

## Responses to Participants' Questions

The overarching goal of the R3 Seminars is to catalyze a shared understanding of the research being conducted by the scientific stakeholder community within the RECOVER Consortium. The R3 Seminars and the Q&As typically feature highly scientific material intended for researchers and clinicians. For other audiences interested in these topics, a link to the National Library of Medicine's MedlinePlus medical dictionary is provided at the end of the Q&As as a resource to help in understanding the scientific terminology.

This document provides responses\* to questions raised by webinar participants related to the following presentations at the R3 Seminar *Mechanistic Pathways of PASC: Session 1 — Overview of Mechanistic Pathways* held on January 10, 2023:

- **Presentation 1: *Immunology of Long COVID***  
Akiko Iwasaki, PhD
- **Presentation 2: *Pathology of SARS-CoV-2 Infection: Implications for the Biologic Mechanisms Underlying PASC***  
Jim Stone, MD, PhD
- **Presentation 3: *An Overview of SARS-CoV-2 Reservoir in Long COVID/PASC***  
Amy Proal, PhD
- **Discussant: Marrah Lachowicz-Scroggins, PhD**

\* Responses may have been edited for clarity.

## All Presenters: Questions and Responses

**Q. How are we translating the information that was presented today into treatment of and care for 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, 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?**

**Response:**

**Dr. Iwasaki:** 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's why I emphasize the importance of trying to understand the endotypes of Long COVID. Let's say, for example, if the viral persistence was the main driver of Long COVID in a subset of patients and if we can find biomarkers to subset those patients,

then we can start providing them with appropriate medicine, such as antivirals or monoclonal antibodies, or something to get rid of the viral reservoir. If it's just RNA alone, it might be 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 of patients is suffering from autoimmunity, antivirals are not going to work. So, we might have to use anti-inflammatories or antibodies against cytokines or Janus kinase inhibitors to tamp down the autoimmune components. And again, understanding what biomarkers might indicate which people are suffering from autoimmunity as compared with viral persistence would be very helpful in categorizing patients for treatment options. And if latent reactivation of Epstein-Barr virus (EBV) is contributing to the symptoms, there are antivirals that are potentially useful in that area as well against EBV and other DNA viruses.

Understanding which type is present in which patients is a key first understanding to even suggesting appropriate treatments. At the same time, this is a very urgent issue. People are very desperate for therapy and we should go ahead and do well-designed randomized clinical trials to see which patients are benefiting before and after and have biomarkers that correlate with that response. That way, without knowing the molecular mechanism, we can start to understand through experiments and surrogate measurements of these patients who might benefit and how that can inform future therapy options. So, I'm giving you two possible ways to approach this, but they should be done at the same time.

### **Q. How are the tissue pathology studies translating into potential clinical interventions?**

**Response:**

**Dr. Stone:** It's unfortunately a long course. It takes a while to develop therapies. The tissue pathology studies are supportive of the fact that for patients who are suffering there is indeed something going on in the tissues. We're seeing viral persistence and we're seeing other changes in the tissue, such as inflammation, but it's going to be difficult to sort out what the proper target is and whether it's inflammation or whether it's virus without inflammation that's the issue. I know people really want us to be launching into therapies right away, but it takes time to figure out the right therapies so that we do more good than harm as we try to launch new therapeutic trials.

### **Q. What potential biomarkers seem most interesting and most valuable for us to translate into the clinic?**

**Response:**

**Dr. Proal:** One of the top potential biomarkers would be studies in which antigen was found in plasma. These studies need to be replicated in other cohorts, but we have yet to see that. For example, the Harvard team that used the very sensitive Samoa assay to find spike in plasma, I believe they did work on a different cohort that had

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. Yet, being able to identify antigen and plasma would be huge because plasma is regularly most easily collected from patients, but it's a big challenge when SARS-CoV-2 has been shown capable of infecting nearly every human tissue type throughout the body and brain. It follows that SARS-CoV-2 may persist in tissue reservoirs in Long COVID patients, where viral protein may not necessarily "leak" into the blood where it can be easily measured. So, the virus may be in someone's nerve or in someone's tissue sample, but of course getting a biopsy from patients can be difficult.


That being said, our Long COVID research consortium is working on some tissue types that can be biopsied more easily at routine appointments than others. For example, there are punch biopsies that happen with teams we regularly work with at Harvard Massachusetts General Hospital and we're working those samples up in case there's signal in those tissue samples that are regularly collected at appointments from patients. Also, lymph node aspirate can sometimes be collected with a fairly routine procedure.

We're making sure we analyze all these different types of samples to best understand what can be found where. Also, it's key that in the research studies we're doing that 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 can somewhat reflect what might also be happening in tissue. That's the most hopeful biomarker.

Also, I believe there are one or two Paxlovid clinical trials that will be using spike protein as one of the outcome markers. Dr. Iwasaki could speak to what she's been measuring and being used as outcome measures in clinical trials. So that is moving forward, and while more research is needed, there are some leads especially on what can be measured in blood from what she is measuring and the spike in the antigen.

### **Q. What do we know about the role mitochondrial dysfunction plays in Long COVID?**

#### **Response:**

**Dr. Proal:** I wrote a paper with my close colleague Mike VanElzakker that walked through the mechanisms by which viral, bacterial, and fungal pathogens hijack the metabolism of the cells they infect ([Pathogens Hijack Host Cell Metabolism](#) ). One thing to understand about viruses is that they're obligate intracellular pathogens. When they replicate, they need to create another copy of their backbone and they must pull from substrates produced by the host cell mitochondria 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 and viral replication in the metabolic profile of the cell. It's a completely connected topic and 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 as two separate things and say, "Oh, this team's going to work on the virus and this team's going to work on

metabolism.” The field of immunometabolism is combining these topics now. And researchers in this field—for example, our articles published in the journal *Immunometabolism*—are combining these areas of research and it’s going to be important to pull this into the PASC field.

**Q. Several epidemiological studies are now pointing to an increased incidence of PASC in women. What do we know about mechanisms for this sex difference?**

**Response:**

**Dr. Iwasaki:** That’s a great question. We’re preparing a paper on that as we speak. The MY-Long COVID study that I described to you today was not sex disaggregated, but we’re now doing a study on that subject to see whether there are any sex differences in immunity and symptoms. There’s 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 particularly interesting thing we’re seeing is that the EBV reactivation and the IL-6/IL-4 double positive CD4 T-cell link is particularly dominant in women as compared with men. We’ll be learning a lot about that, but it’s a great question.

**Q. Are microthrombi in the lungs an acute infection the same as microclots detectable in Long COVID?**

**Response:**

**Dr. Stone:** That’s 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. Even within deaths in the acute phase, thrombi can either be entirely fibrin thrombi or they can be platelet microthrombi. The type of thrombi is often determined by how the patient was anticoagulated prior to death. There 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 these patients and to compare them. So at this point, we don’t know if there’s a systematic difference or not.

**Q. 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?**

**Response:**

**Dr. Iwasaki:** This is an excellent question. As I mentioned, cortisol is regulated in diurnal fashion during the day. We’re now collaborating with David Putrino’s lab to collect diurnal levels of saliva cortisol from some of his patient participants to see if there are any changes in their levels during the day. As I mentioned, so far we’ve only collected a one time-point sample from these participants. We want to know during the day if there’s any difference in the pattern. And if so, if there’s any difference in the ACTH (adrenocorticotrophic hormone) level and

other hypothalamic controls. For that, we may need to do more MRI studies and others, which are also ongoing with David Putrino's group.

**Q. Were you able to segment immunoprofiling for just brain fog sufferers? And was there any pattern of interleukin elevations of interest?**

**Response:**

**Dr. Iwasaki:** That's a great question. I mentioned the study that we published with Michelle Monje's group at Stanford (<https://pubmed.ncbi.nlm.nih.gov/35768006/>) where we saw elevated CCL11, which is known as Eotaxin-1 as well, in circulation that correlated with the brain fog reported by the same Mount Sinai participants that we're studying. And that tended to be elevated more in male than female patients. So that's interesting, that specific chemokine is correlating with brain fog, but more dominantly in males. There's a lot to study there.

**Q. How do you define/differentiate the "healthy, uninfected controls" (HC) cohort (i.e., how do you know they were never infected)?**

**Response:**

**Dr. Iwasaki:** Healthy uninfected controls were recruited based on the participant reporting of having never had COVID. To rule out asymptomatic infection, we also tested their antibody levels to the SARS-CoV-2 nucleocapsid, which were negative. However, it's not possible to completely rule out having been exposed to the virus.

**Q. Why is there no biomarker included in the lab studies that measures a very important aspect of COVID-19 and of the subsequent PASC/Long COVID: the redox imbalance? Serum concentrations of free thiols (µM) significantly discriminated between patients with COVID-19 and healthy controls and showed slightly higher discriminatory power compared with CRP concentration. Since free thiols are a reliable marker of oxidative stress, their quantification in COVID-19 could be a promising and noninvasive strategy.**

**Response:**

**Dr. Iwasaki:** Thank you for the insightful suggestion. We'll look into this in future studies.

**Q. HSV-1 and HSV-2 looked reduced in Long COVID patients; do you think these may serve a protective role?**

**Response:**

**Dr. Iwasaki:** We believe the reduced IgG to HSV antigens reflects reduced seroprevalence for HSV-1 and HSV-2 in our Long COVID cohort compared with CVC (convalescent SARS-CoV-2 individuals without persistent symptoms) or HC (healthy controls with no prior SARS-CoV-2 infection). Determining whether people who have never been infected with HSV are at reduced risk of Long COVID would require a much larger study because of multiple confounding factors.

**Q. Is there any evidence or testing available to evaluate reactivation of HSV type 1, 2, or 6?**

**Response:**

**Dr. Iwasaki:** Thus far, our study did not indicate evidence of reactivation by HSV-1, HSV-2, or HSV-6. However, this would require a larger study to better understand the role of these viruses.

**Q. Are there any cardiac biopsy specimens from MIS-C/MIS-A patients?**

**Response:**

**Dr. Stone:** There have been a few small studies and case reports looking at cardiac biopsies in patients with MIS-C and MIS-A. In general, the findings from these cardiac biopsies are similar to the findings from heart evaluations from autopsies on patients dying from acute phase COVID-19, but they're more limited because of the much smaller amount of tissue available for analysis.

**Dr. Iwasaki:** We do not have access to these biopsies, but I'm sure other groups do.

**Q. Are researchers considering a link between the low cortisol levels and the overreactive sympathetic response that has been discussed in other RECOVER talks? Have individuals' cortisol levels been retested over time? It seems plausible that they would be elevated during early stages and then become depleted and lead to chronic fatigue and post-exertion malaise.**

**Response:**

**Dr. Iwasaki:** This is such an important point. Our MY-LC study is only cross sectional. We'll need a longitudinal study to better understand the changes in cortisol over the disease course.


**Q. Is there a level of virus in biopsied thyroid nodules in Long COVID patients?**

**Response:**

**Dr. Stone:** The virus can be detected within the thyroid gland and thus may be present in biopsies of thyroid nodules.

**Q. Dr. William Haseltine has outlined how 18 SARS-CoV-2 viral proteins suppress type I interferon and innate immunity. Do you think this virus is capable of “hiding” in plain sight? If so, would the activation of innate immunity via vaccination explain the phenomenon of development of PASC symptoms post vaccination?**

**Response:**

**Dr. Iwasaki:** We don't yet know how vaccination results in PASC-like syndrome. We're currently recruiting participants in our Yale LISTEN study to investigate the immune phenotypes of post-vaccine long haulers (<https://medicine.yale.edu/ycci/listen-study/> )

**Q. What can be said about the state of the innate immune system in patients with PASC?**

**Response:**

**Dr. Iwasaki:** Our MY-LC study reveals activation of certain innate immune cells and factors in Long COVID. However, the exact players identified appear to depend on the timing from disease onset to analysis. There's also evidence of microclot formation and platelet activation, suggesting there's endothelial inflammation in PASC.

**Q. How are you considering the phenotype of the individual (multiple comorbidities) that appears to correlate with the degree of severity of PASC (independent of the severity of acute infection)?**

**Response:**

**Dr. Stone:** In general, studies with human patients are often complicated because of comorbidities. Large studies like RECOVER will allow for the assessment of the impact of comorbidities on the symptoms being reported by patients with PASC.

**Dr. Iwasaki:** Our MY-LC study focused on individuals with limited comorbidities. However, others have found various comorbidities to be risk factors associated with the development of Long COVID:

(<https://www.nature.com/articles/s41591-022-01909-w/tables/2> )

**Q. Is S1 persistence in vaccine-adverse-effect patients being looked at as a form of “reservoir”?**

**Response:**

**Dr. Proal:** Currently, no research team I work with is performing a study of S1 protein in post-vaccine patients. Overall, when I use the word “reservoir” I'm referring to the persistence of the SARS-CoV-2 virus itself. If SARS-CoV-2 protein (including spike from vaccines) can persist on its own for long periods in the absence of SARS-CoV-2, we need to better understand the mechanisms by which that would be possible and use a term other than “reservoir” to describe the phenomenon. For current data on S1 or spike duration in plasma after vaccination, see this article by David Walt's team at Harvard

(<https://academic.oup.com/cid/article/74/4/715/6279075?login=true> )

**Q. Based on previous research of endothelial damage in PASC, would medication focusing on immune modulating and endothelial health, such as statins, be worth trying for treatment or prevention of PASC?**

**Response:**

**Dr. Stone:** Aberrant endothelial activation may play a role in PASC. Further research is needed to understand the role of the endothelium in PASC and the relationship of endothelial activation to viral persistence and tissue inflammation in these patients.

**Q. Is there value to repurposing multiple sclerosis (MS) drugs for those suffering Long COVID mechanistic/mobility muscle, nerve, and connective tissue issues (most likely caused by endothelial damage and microclots) to help ease symptoms?**

**Response:**

**Dr. Proal:** It's worth considering that MS was recently linked closely to the activity of Epstein-Barr virus (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9382663/> and <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3179777/>). Reactivation of Epstein-Barr virus has also been documented in some patients with Long COVID. So, it may be that therapeutics aimed at controlling herpesvirus activity will prove to be most useful in helping both MS and Long COVID patients.


**Q. Please discuss the role of chronic autonomic dysfunction/autonomic nerve damage in driving inflammation, autoimmunity, and coagulation.**

**Response:**

**Dr. Proal:** The order of events might in fact be reversed for how coagulation and inflammation might contribute to chronic autonomic dysfunction/peripheral neuropathy in Long COVID. Patients with Long COVID have been shown to harbor fibrin/amyloid microclots in blood. It's possible that these microclots accumulate in the capillaries, preventing blood and oxygen from reaching peripheral nerves, which could exacerbate problems with autonomic signaling. We have a study focused on determining if that might be occurring in Long COVID.

**Q. What role, if any, do you suspect GPCR AABs are playing in the pathomechanism?**


**Response:**

**Dr. Proal:** If G protein coupled receptor autoantibodies (GPCR AABs) play a role in the Long COVID disease process—more data are needed—it's worth considering that certain herpesviruses encode proteins that are very similar in size/shape to human GPCR receptor proteins (<https://www.annualreviews.org/doi/pdf/10.1146/annurev-virology-100220-113942> ). Herpesvirus reactivation has been documented in some patients with Long COVID. Consequently, molecular mimicry between herpesvirus proteins (called viral GPRCs) and human GPCR receptors may lie at the root of why the immune system might target GPRCs in certain individuals with Long COVID.


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