Responses to Participants' Questions

The overarching goal of the RECOVER RESEARCH REVIEW (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 seminar participants related to the following presentations at the R3 Seminar ***Persistent SARS-CoV-2 Antigens and Correlation with Long COVID Symptoms: Findings from a Multi-Cohort Study*** held on December 10, 2024:

- ***Current Understanding of Biomarkers for Long COVID***
  David Walt, PhD
- ***Background of the Assay, Brief Overview of Cohorts***
  Zoe Swank, PhD
- ***Study Findings, Detail about Cohorts, Symptom Clusters***
  Elizabeth Karlson, MD, MS
- **Discussant: David Walt, PhD**

* Responses may have been edited for clarity.

All Presenters: Questions and Responses

Q. Have similar studies been conducted for other viruses, CMV or EBV, showing viral persistence in tissues? If yes, do other viruses show similar persistence? If no, is it possible this persistence is not unique to COVID virus?

Responses:

Dr. Walt: There definitely have been other studies that have postulated viral persistence. We have not applied our methodology to any of those other persistent syndromes. It's obviously something that's worth doing because of the ultra-sensitivity of our assay, but I think that there have been a number of studies, including influenza, where folks have postulated and actually measured some viral persistence in individuals who have post-viral infections syndromes.

Dr. Karlson: Just for mononucleosis. I think for Epstein-Barr virus that there have been studies that have shown that Epstein-Barr virus can reactivate and produce more symptoms but not with your technology. So it's a different

technology. They look for very specific antibodies and sometimes they look for circulating virus but not looking for the antigen particles yet.

Dr. Swank: I would also add that Ebola and Zika viruses are similar ribonucleic acid (RNA)-type viruses that function in a similar way. Although they are less well studied, there's also evidence that they persist for longer and people also have symptoms for longer. There's been some animal studies that show that the virus can persist in tissue as well.

Q. Can you infer anything meaningful based on the highest antigen detection being four to seven months after infection or do you attribute this to a measurement artifact?

Response:

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

Q. Techniques like intravenous immunoglobulin (IVIG) are being studied to combat persistent reservoirs, early results are mixed. Could the location of the reservoir impact the effectiveness of IVIG?

Response:

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

But as far as I know, there aren't any controlled trials trying to treat the antigen level in the tissue yet. The RECOVER-VITAL is the first one that I know of that's using the antigen assay in the blood to look for response.

Q. Were pregnant individuals represented in your study?

Response:

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

Q. What significance, if any, do rapid tests have in Long COVID? For instance, I continued to test positive on several brands of rapid tests for almost two years, negative on PCR.

Response:

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

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

Response:

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

Q. I thought Paxlovid was already disregarded as a possibility to treat Long COVID or was this another study and are you studying it in another way?

Responses:

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

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

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

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

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

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

Response:

Dr. Walt: We're not. Being able to obtain tissues is a challenge, so I'm not sure it would be particularly worthwhile. We obviously can test stool in these individuals, but we think that our marker is sensitive enough to pick up antigen in blood. So that's been our go-to sample.

Q. Is there a path to making these assays, whether analog or digital, more widely available for researchers and clinicians?

Responses:

Dr. Swank: For the RECOVER-VITAL trial we did validate this assay in a clinically certified lab. So now the clinical Translational Biomarker Core Lab at Brigham and Women's Hospital is running those samples according to clinical guidelines for the RECOVER trial. In terms of adapting this assay to make it more widely available, we're also trying to translate it to another platform that uses flow cytometry as a readout. So, you would not need the instrumentation that's associated with the single molecule array (SIMOA) assays. You can do everything by hand and then read out with flow cytometry. And this also has the benefit that it can be more sensitive, as well. It was developed in our lab and there's a few of us working on trying to translate this assay, which could then be used more readily by everyone because flow cytometers are more widely available, I would say.

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

Q. For the RECOVER cohort, when reporting the percentage of the cohort with symptoms or with antigen, what denominator was used? Was it the full cohort or recognizing your 64 patients with no symptoms but antigen were those 64 patients removed from the denominator?

Response:

Dr. Karlson: We use the total number of participants in each cohort as the denominator. For example, RECOVER had 392 individuals, so our percentages reported in those heat map figures are out of the 392. We didn't remove the asymptomatic people. So, it's percent positive among 392. And then regarding the 64 RECOVER participants with no symptoms—the table in the paper and that I showed on the screen (Table S5) of the asymptomatic participants—we use the total number in each cohort so that we have the same denominators across all of the figures and that table.

Q. What do you make of patients with SARS-CoV-2 spike antibody levels that keep rising after infection? Can anyone address an autoantibody theory? Do any results greater than 2,000 units per mL have any significance?

Response:

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

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

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

Responses:

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

Dr. Swank: it's hard to understand if the rates are different due to the variant itself or due to the vaccination because vaccination also became more readily available during the Omicron variant era. We also relied on participants to report if they had been infected before or if they were reinfected. But a number of times in our laboratory, we also saw people who were supposedly uninfected but had antibodies against nucleocapsid. So even

if they were vaccinated, in which case they would have spike antibodies, we saw also nucleocapsid antibodies indicating an infection. So I think it's quite difficult as the pandemic moves on to actually find good controls who were never infected.

Dr. Karlson: The best control would be people who were never vaccinated and never infected—since up to a third of people have asymptomatic infections, about a third of even people who think they've never been infected have been infected. So it's really hard, as Dr. Swank said, to really answer that question.

Dr. Walt: Dr. Swank has tested individuals who have claimed they've never been infected. We've determined that 99.4% of all samples have nucleocapsid antibodies. So pretty much everybody's been infected whether they know it or not.

Q. There were no post-exertional malaise (PEM) symptoms in the Long-term Impact of Infection with Novel Coronavirus (LIINC) study group. How can that be explained?

Response:

Dr. Karlson: LIINC did not include questions about PEM, so we do not have the ability to study antigen rates in the PEM symptom group in LIINC.

Q. Which part of S1, or of the full spike, is detected precisely?

Response:

Dr. Swank: The epitopes of the antibodies against S1 are proprietary, but somewhere within the receptor binding domain (RBD). The epitope against the S2 subunit targets amino acid residues 1029-1192, containing the heptad repeat domain 2 and it was shown to be able to bind this epitope even if spike is incorporated in a membrane.

Q. What is the origin of the full spike? Spike does not get secreted by infected cells, right? Would it be debris of infected cells or virions?

Response:

Dr. Swank: We have been trying to understand this ever since we detected the full spike protein in Multisystem Inflammatory Syndrome in Children (MIS-C) patients. It may be that the RNA is being transcribed to produce full spike and somehow spike is getting into the bloodstream. So it could be that it isn't coming from full virus, which you'd expect would produce only S1. We have also detected a spike in exosomes from some patients but not all. It could be aberrant protein processing that doesn't enable spike to be cleaved into its subunits—still a puzzle.

Q. How were reinfections diagnosed/excluded? Was serial serology for nucleocapsid antibodies performed to exclude boosting due to non-diagnosed reinfections?

Response:

Dr. Walt: We ran assays for nucleocapsid antibodies but not on every sample, so we did not test for reinfection. In some cohorts, some, but not all, participants self-reported reinfections. Some of our positive detections could be due to active infection but we tried to eliminate these samples.

Q. Could the SIMOA assay be used to study other diseases or illnesses with a potentially similar mechanism, such as HERV/EBV antigens?

Response:

Dr. Walt: Yes, we use these assays for neurodegenerative diseases but have not looked at other infectious diseases with persistent symptoms for viral remnants.

Q. Could persistent presence of the spike protein be coming from the mRNA vaccines?

Response:

Dr. Walt: We measure S1, not full spike in vaccinated individuals but it goes away in 3–5 days.

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

Response:

Dr. Walt: No, we will not.

Q. Does the finding that four-fifths of folks did NOT have antigen detected suggest that most Long COVID patients don't have circulating antigen, or that it just wasn't detected?

Response:

Dr. Walt: It could be either. It's likely that most do not have antigen because it isn't the cause of their symptoms.

Q. For the RECOVER cohort, when reporting percentage of the cohort with symptoms or with antigen, what denominator was used? Was it the full cohort or, recognizing your 64 patients with no symptoms but antigen, were those 64 patients removed from the denominator (i.e., is the denominator for the heatmap only symptomatic patients)?

Response:

Dr. Karlson: In all cases, the denominator used to calculate percent values is the total number of participants in each cohort: LIINC (n = 171), MGB (n = 63), Allen (n = 80), and RECOVER (n = 392).

Q. Do you think that an anti-spike antibody would be helpful to treat Long COVID patients who have a positive SIMOA signal?

Response:

Dr. Walt: Many of the individuals who have positive spike have good immune responses and have robust levels of anti-spike antibodies. So, I'm not sure if an antibody treatment would be able to get to the reservoir.

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