Transcript

Sarah Hatcher

Good afternoon, everyone, and welcome to the RECOVER Research Review or R3 seminar. My name is Sarah Hatcher, and I will serve as the moderator of today's seminar. I am a research epidemiologist at RTI International, which serves as the Administrative Coordinating Center for RECOVER. Next slide. The goal of this seminar series is to catalyze a shared understanding of the research within the RECOVER Consortium. Please note that the research presented today is scientific information and should not be considered medical advice. A FAQ document will be posted with the recording of the seminar on recovercovid.org. The FAQ document will include the answers for submitted questions relevant to today's presentation. Answers to broader questions about RECOVER are also available in the FAQs at recovercovid.org. Slides for today's presentations will be available upon request. Next slide.

Our presenters today are Dr. Joel Trinity, Dr. Katelyn Ludwig, and Dr. Naomi Hamburg. Dr. Joel Trinity is an associate professor in the Department of Internal Medicine at the University of Utah and a research health scientist at the Salt Lake City Veteran Affairs Medical Center. Dr. Trinity utilizes an integrative approach that combines in vivo and in vitro techniques to examine the impact of age, disease, and disuse on vascular and skeletal muscle function. Dr. Katelyn Ludwig is the biochemistry director of the Integrative Physiology of Aging Lab led by Dr. Douglas Seals at the University of Colorado Boulder. She earned a PhD in biochemistry at the University of Notre Dame where she focused on the development of capillary electrophoresis and ultra-performance liquid chromatography, mass spectrometry based phospho proteomic techniques to analyze molecular changes in colorectal cancer. Dr. Ludwig then joined the National Cancer Institute's Genetics branch as a post-doctoral fellow in Dr. Natasha Kaplan's lab where she used CRISPR-Cas9 and small molecule screening to study the modifications and stability of the oncogenic transcription factor EWS/FLI1, which drives the pediatric cancer Ewing Sarcoma.

Dr. Naomi Hamburg is the Joseph a Vita professor of Medicine, chief of the Vascular Biology section, and interim director of the Whitaker Cardiovascular Institute at Boston University School of Medicine, and an attending in the cardiovascular medicine section at the Boston Medical Center. Dr. Hamburg is a recognized expert in the field of vascular medicine and in the clinical translation of vascular biology. She has held multiple leadership positions in the field of vascular medicine. Her research seeks to understand approaches to restore vascular health in patients with cardiometabolic disorders and peripheral artery disease, and to evaluate the impact of novel tobacco products and long COVID.

Dr. Janet Mullington will serve as our discussant today. Dr. Mullington is a professor of neurology at the Beth Israel Deaconess Medical Center and Harvard Medical School. Dr. Mullington is also vice-chair for research in the Department of Neurology at the Beth Israel Deaconess Medical Center and serves as the co-chair of the RECOVER Integrative Physiology Committee. Her area of expertise is sleep deficiency and its associated

pathobiological consequences for the inflammatory, autonomic, state-related neurophysiology and cognitive systems and for subjective fatigue and mood. Her research team uses highly controlled approaches to induce experimental sleep deficiency of different durations to study effects of deficiency buildup in healthy sleepers as well as the recovery process when sleep resumes.

In addition, her group's translational work tests the efficacy of manipulating the timing and duration of sleep to affect health outcome indicators. Next slide. The topic of today's seminar is the Vascular Pathophysiology of PASC. Today's speakers will share information about our current understanding of the vascular pathophysiology of PASC, the gaps in our knowledge about this subject, and how RECOVER will contribute to filling these knowledge gaps. And with that, I will hand it over to Dr. Trinity for our first presentation.

Dr. Joel Trinity

Thank you for the introduction. Can everyone see my slides? Great, thank you. So, here's a brief outline of my talk. So, we were talking about vascular dysfunction in long COVID and why should we care about this? We'll talk about assessments of vascular function just to get everyone on the same page in terms of what is being done in terms of assessing vascular function. What do we currently know? So, I'll present some preliminary data on vascular function from our laboratory and then a couple studies that have assessed vascular function in the acute phase as well as in the long COVID phase. We'll discuss some potential mechanisms. This will primarily be the topic for talks two and three, and then I'll have a free slide on where should we go and how can RECOVER be involved in endothelial function and vascular dysfunction in long COVID?

So, the circulatory system is often presented simplistically with distinct and separate vascular systems. So, the heart provides the pump, and the conduit arteries are the macrovascular that deliver oxygen and nutrient-rich blood to the microvasculature where exchange occurs. This overly simplistic depiction provides the basics of the circulatory system, but it's easy to forget just how complex the circulatory system is. In fact, the circulatory system is very extensive. Because the micro circulation is pervasive and present in every tissue in the body, it has the unique ability to influence the local environment in the majority of tissues and organs. Furthermore, damage to the microvasculature disrupts oxygen and nutrient delivery while damage to the microvasculature impairs the exchange of oxygen and essential nutrients. The endothelium is a single layer of cells that line each blood vessel throughout the body, and due to location of the endothelium, this layer of cells is ideally situated to respond to changes in hemodynamic forces and blood signals.

Classic risk factors for impaired vascular function include diabetes, hypertension, hyperlipidemia, aging, smoking, and obesity. When these risk factors are present, inflammation and oxidative stress result leading to systemic endothelial dysfunction with cardiac, cerebral vascular, renal, ophthalmic and thrombotic manifestations. So vascular dysfunction is linked to the initial stages of progression of cardiovascular disease, atherosclerosis and hypertension. It typically occurs before overt disease occurs. The assessment of vascular function provides

independent and predictive information for future cardiovascular events beyond traditional risk factors. And recent evidence indicates that acute COVID-19 is a risk factor for endothelial dysfunction and by the end of the seminar series, I hope that we will provide compelling evidence that long COVID is also a risk factor for endothelial dysfunction.

Early viewpoints and research investigations surrounding acute COVID provided important insight into how the endothelium is involved in the progression of disease from infection to widespread symptoms. So, under normal conditions, the endothelium is critically involved in several processes promoting healthy vascular function and contributing to the anticoagulant, profibrinolytic, anti-inflammatory, vasodilatory and antioxidant properties. However, chronic acute SARS-CoV-2 infection, the endothelium is directly impacted, and it loses its cardioprotective properties and now contributes to the procoagulant, pro-inflammatory, vasoconstriction and pro-oxidant properties. Together, the activated endothelium contributes to tissue and organ damage such as myocarditis, ischemic stroke, acute kidney injury, and thrombosis.

Moving from acute to long COVID. I want to highlight two important studies that evaluated long-term outcomes following COVID-19 in veterans. So, I'm presenting these papers out of order based on their publication date to first highlight the long-term cardiovascular outcomes of COVID-19, and then I'll focus on the widespread symptoms and complications of long COVID. So, in this study, the authors used the US Department of Veteran Affairs database to build a cohort of over 150,000 US veterans who survived the first 30 days of COVID-19. They also included two control groups, a contemporary cohort consisting of approximately 5.6 million users of the VA system that had no evidence of COVID infection, and then a historical control of 5.9 veterans that had never been exposed to it since they were evaluated in 2017. So based on this analysis, COVID-19 increases the risk in 12-month burden of incident cardiovascular diseases including cerebrovascular disorders, dysrhythmias, inflammatory heart disease, ischemic heart disease, thromboembolic disease, and other cardiac disorders including heart failure, cardiac arrest, and cardiomyopathy. Importantly, these risks were evident regardless of age, race, sex, and other cardiovascular risk factors including hypertension, obesity, diabetes, and chronic kidney disease and hyperlipidemia.

This group of researchers also presented compelling evidence that the impact of COVID-19 is not limited to just one organ or organ system. The effects are widespread and nearly every organ system appears to be impacted. So here the same group of authors again use the VA database to identify six-month incidents including diagnosis, medication use, and laboratory abnormalities in patients with COVID-19 who survived the first 30 days after diagnosis. So, this cohort is a little bit smaller using 70,000 users of the VA system and compare that to nearly 5 million users that have never had COVID. So, the results suggest that beyond the first 30 days of illness, people with COVID-19 are at a higher risk of death and are more likely to use healthcare resources and exhibit a broad array of incident pulmonary and extra pulmonary clinical manifestations including nervous system and

neurocognitive disorders, mental health disorders, metabolic disorders, cardiovascular disorders, and GI disorders. They also have general poor wellbeing including malaise, fatigue, musculoskeletal pain.

So why do we care? I think it's pretty obvious that vascular dysfunction is directly related to cardiovascular disease and long COVID is also an important risk factor for vascular dysfunction. Importantly, long COVID is not only associated with cardiovascular disease through the vascular dysfunction but extends well beyond the cardiovascular system. It includes the muscular cell system, pulmonary system, neurocognitive system, and the endocrine systems, just to name a few. So, moving on to assessments of endothelial function. Endothelial function can be assessed peripherally as well as in the brain. We can also assess coronary microvascular function or coronary vascular function. Today we're going to focus on the flow media dilation technique or FMD, finger plethysmography or EndoPAT, I'm bringing this up because this is being discussed as a possible tier three test for the RECOVER initiative, and then passive leg movement or PLM, which is a test that our group has developed over the last 10 to 15 years.

So FMD is a non-invasive test to assess vascular function. This test is typically done in the brachial artery with doppler ultrasound. Baseline measurements are made for up to one minute. A cuff is placed around the forearm. That cuff has been inflated for five minutes causing the occlusion in the distal part of the hand and the lower forearm. That cuff is released. This causes a dramatic increase in blood flow, which increases sheer stress. The sheer stress then acts on the endothelium to release vasodilators and nitric oxide and under healthy situation, that artery dilates. However, when the endothelium is dysfunctional, the dilation is abolished. You can see here in the figure this is just an image of the brachial artery followed by the time course of the response in the brachial artery diameter showing the baseline, the release followed by the dilation. FMD has been used for nearly 30 years in the research realm, however, we do not currently have a clinically approved or clinically recognized test for vascular function.

I would argue that FMD is probably the closest thing we have, and it's gaining momentum and has gained momentum over the last 20, 30 years based on studies, some classic studies that I'll present here. In this first study, FMD was looked at in corresponding risk factors. So as the number of risk factors increases, your FMD goes down indicating reduced vascular function and increased risk of cardiovascular disease. Another study that really propelled FMD into the research spotlight was a study by Anderson in 1995 that looked at brachial artery diameter and coronary artery diameter. So, this study assessed the two, the relationship between the peripheral measurement and the central measurement in the heart and show that there's a positive relationship. We recently used the current guidelines for performing FMD and improved upon this relationship again showing that coronary artery diameter and brachial artery diameter are highly related.

So, moving on to the EndoPAT or finger plethysmography. So, the EndoPAT technique uses a similar setup to the FMD. However, now the measurement of vascular function is restricted to the microvasculature and is measured in a finger cuff. So, you can see from this picture here, the individual sits quietly in a seat, there's a cuff

placed around the forearm on one arm and then the two finger cuffs on the arm that's going to be occluded and then the control arm. The cuff is inflated for five minutes, then it's released. There's a hyperemic response, and that hyperemic response can then be our evaluation of endothelial function or endothelial dysfunction.

A test that our group has pioneered over the last 10 to 15 years is passive leg movement. So instead of using upper body, we use the lower body. We measure blood flow to the leg in the common femoral artery using doppler ultrasound. This technique requires one individual to scan the leg and another individual to move the leg passively through 90 degrees range of motion. We can move this leg passively continuously for a minute or we can do a single movement. I'll present some preliminary data on both. You can see here in the doppler ultrasound there's a baseline period start of movement and then there's an increase in the pulse wave velocity showing an increase in blood flow. And if we look at the overall response in the leg blood flow response, we can see that there's a robust hyperemic response that's transient in response to that movement.

We have also looked at mechanisms as what's causing this increase in blood flow. So, this first study from 2012 from our group looked at the role of nitric oxide in the hyperemic response and by infusing LMMMA, which is a drug that inhibits nitric oxide synthase, we're able to diminish the hyperemic response by approximately 80% in young healthy individuals showing that the PLM response is approximately 80% nitric oxide mediated. We've also published data looking at how activity and aging impact the hyperemic response. So, this kind of agrees with the FMD and the risk factor scores. Although we have not specifically looked at risk factors, we can see that with young active adults, they have a very robust hyperemic response and it's progressively declining as activity level and age increase until our final point with heart failure where the hyperemic response is very, very low.

So based on these tests, there are several ways we can go with measuring vascular function. The three tests that I talked about here are all non-invasive. They're relatively simple. However, there is not one test that's clinically acceptable at this point to measure vascular function. So, moving on to vascular dysfunction in long COVID. These are preliminary data that we've collected over the last year. So, on average, these patients were approximately six months post COVID. This test here on the left is our passive leg movement where we did a single passive leg movement test in approximately 10 healthy controls and 15 individuals with long COVID. And we can see that there's about a 50 to 60% reduction in that PLM response. And we can also see in the FMD response again about a 50% reduction in the FMD. Together, both of these results indicate a substantial reduction in vascular function that may be nitric oxide.

Moving on to another study. This study looked at COVID participants that were at least four weeks post-infection. So more in terms of not exactly long COVID, but I do like this study because they do show a breakdown of individuals that were asymptomatic following COVID. There is no difference between the control and the asymptomatic individuals. However, when you look at those with symptoms still after four weeks of infection, there was a significant reduction in FMD, again indicating a reduction in vascular function.

A more recent study that just came out a couple months ago in hypertension looked at vascular dysfunction in long COVID and they also looked at autonomic dysfunction. So, this study here is looking at the micro, or excuse me, the MSNA or muscle sympathetic nervous system activity. On the top is a tracing from a control subject. And then on the right is a COVID-19 survivor. I should mention that these were quite severe COVID-19 survivors that were hospitalized anywhere between one to six months, and these measurements were taken approximately three months after they were discharged from the hospital.

So, you can see MSNA measured in bursts per minute was elevated and when it's measured as bursts per 100 heart beats was also elevated indicating a possible increase in sympathetic nervous system activity. This study also evaluated vascular dysfunction looking at pulse wave velocity being elevated. So, a higher pulse wave velocity is indicated of arterial stiffness, increased arterial stiffness, and then panel or figure B here is the FMD, again showing a reduction in FMD of about 50%. And when this FMD is normalized for the sheer rate response, it is still diminished by about 50%. One final study looking at FMD is a meta-analysis. So, this study included 12 individual studies and over 600 participants. 600 long COVID participants and 662 controls. You can see here in this figure here there's a significant reduction in vascular function when measured by FMD.

One other study is a study that used EndoPAT. This is an interesting study. The relatively large number of participants where they had 72 control groups or individuals in group three measured to an acute COVID-19. So, this is approximately 10 days post COVID infection, and then again, after COVID, group two was approximately three and a half months after COVID. You can see a significant reduction in endothelial function. This is specifically microvascular dysfunction in these individuals that were long COVID. What's interesting here is there's also a group, a smaller cohort of 14 individuals where they were measured, they had repeat measurements, and you can see after the acute infection in the long-term follow-up, there's a substantial reduction in those individuals indicating microvascular dysfunction.

So, providing a brief summary here, vascular dysfunction and long COVID. So, there's increased risk of adverse cardiovascular outcomes one year post COVID. Endothelial dysfunction appears to be present up to one year post COVID infection. We still don't really have any data that I'm aware of looking at past one year. Both micro and macrovascular dysfunction are apparent. There seems to be a lack of consensus on how COVID severity and comorbidities contribute to vascular dysfunction. In the larger epidemiological studies, the rate of hospitalization and use of healthcare facilities did not seem to be dependent upon age or sex or severity. However, there are other investigations that do point to the severity being an important factor. So, these are things that we need to figure out moving forward. And it's unclear what may be causing the reductions in vascular function. So, the usual suspects are oxidative stress leading to decreases in nitric oxide bioavailability. There's some evidence that increased sympathetic activity, which will vasoconstrict the periphery, could be causing this vascular dysfunction.

We also know that fatigue, muscle weakness and physical activity are common in long COVID. And this brings me to one of my final slides looking at a topic, a very integrative topic of cardiovasomobility. So, this topic really talks about how the cardiovascular system, the skeletal muscle system, are very integrated. And this figure here on the right, you can see when people are physically active, this is the preferred cycle. A cardiovascular system is properly functioning, and skeletal muscle function is good, and this leads to active aging and increased health span. However, we know that increased fatigability and muscle weakness are rampant in long COVID. So it could be, excuse me, that the physical inactivity coming from fatigability and muscle weakness might be also contributing to the vascular dysfunction that we're seeing. Thank you for your time. Just wanted to acknowledge the laboratory that I'm part of, the Utah Vascular Research Lab, and the current funding that I have. Thank you.

Sarah Hatcher

Thank you, Dr. Trinity. Now I'll turn it over to Dr. Ludwig for our next presentation.

Dr. Katelyn Ludwig

Are you able to see my slides okay?

Sarah Hatcher

Yes.

Dr. Katelyn Ludwig

Great. Okay. So today I am going to be talking about some of the work that I've been doing in Dr. Doug Seals' lab here at the University of Colorado Boulder, where we have been looking at circulating factors in COVID-19 plasma and how those can cause endothelial cell dysfunction and NAD depletion with prevention by Nicotinamide Riboside. So, SARS-CoV-2 is known to be linked to cardiovascular diseases as Dr. Trinity was just explaining. And he actually highlighted the same study that I have here where they were looking at the Veterans Affairs data. And actually, I wanted to highlight a slightly different part of the figure than Dr. Trinity was looking at. And that is down here where these researchers were showing that these populations who had COVID-19, at one year after infection, they were associated with 45 incidents of any cardiovascular outcome per 1,000 people.

And they were also associated with 23 incidents per 1,000 people of major adverse cardiovascular outcomes including myocardial infarction, stroke, and all cause mortality. So, this study was really important in establishing a link between COVID-19 and cardiovascular diseases although the exact mechanisms linking those remain unknown. In our lab, we're really focused on vascular dysfunction. And that's because vascular dysfunction is a known antecedent to cardiovascular diseases. And as Dr. Trinity was discussing, numerous studies have shown

that COVID-19 can lead to vascular dysfunction, both acutely and post-infection, indicating the vascular dysfunction may be one of the primary mechanisms linking COVID-19 and cardiovascular diseases.

And one of the mechanisms that drive vascular dysfunction is endothelial dysfunction. As we have explained previously, the endothelium is the inner single cell layer lining vessels that has a number of important physiological functions. And these range from maintenance of barrier integrity, regulation of vascular tone and regulation of hemostasis among others. And one of the key molecules responsible for endothelial maintenance is the vasodilator nitric oxide. Studies have shown that the vasculature and in particular the endothelium can be infected with SARS-CoV-2 and that endothelial dysfunction is likely a driving factor of COVID-19. So endothelial dysfunction is generally defined as decreased nitric oxide bioavailability and increases in vasoconstrictor substances.

The decrease of nitric oxide bioavailability occurs partially because of a decrease in nitric oxide production due to oxidative stress. Oxidative stress is a term for the imbalance of the production and mitigation of reactive oxygen species within the cell. And specifically, nitric oxide can react with the reactive oxygen species superoxide to form peroxynitrite. So not only does this deplete nitric oxide, it creates a reactive molecule peroxynitrite that can go on to cause oxidative damage within the cell. Both oxidative stress and inflammation contribute to a feed forward loop that further drive both of these processes. And they've both been found to play roles in COVID-19 pathogenesis and maybe one of the driving mechanisms or two of the driving mechanisms of endothelial dysfunction during COVID-19.

So, it has been shown that endothelial cells can be directly infected with SARS-CoV-2, and this is one of the first papers that was highlighting that in 2020. So primary infection can lead to an increase in reactive oxygen species by the virus directly entering the cells through ACE-2 inhibitors and activating NADPH oxidases. So direct effects of the virus can lead to endothelial dysfunction. However, endothelial dysfunction has been shown in the literature to persist even after the virus has been cleared from the system. So, this indicates that there are other mechanisms that can drive endothelial dysfunction after infection. And it's not really clear whether this endothelialitis as they're calling it is a result of direct infection or indirect changes in circulating molecules that are perpetuating long COVID or perhaps both.

So, what I want to focus on a lot today is talking about circulating factors in COVID-19. And circulating factors include cells and biomolecules that interact with vascular cells by circulating through the blood and throughout the body, and they can affect the function of vascular cells and specifically endothelial cells. So, I'm going to highlight one study here where they were looking at circulating factors in COVID-19 plasma. In this study, they took plasma samples from 156 healthcare workers and specifically these were healthcare workers that had non-severe COVID.

And they looked at the plasma proteom content from these healthcare workers and how it changed over time. And what they found was that for at least six weeks post-infection, the circulating proteins in these

healthcare workers were perturbed from their baseline levels, and specifically their differential abundant proteins were mostly involved in lipid and cholesterol metabolism as well as complement and coagulation cascades. So, this is one of the earlier studies or one of the studies indicating that protein circulating factors are changing with COVID infection, and not just with severe COVID, also with non-severe COVID, and that these proteins might have the potential to affect endothelial function in the peripheral vasculature.

The second study I wanted to highlight is shown here. In this study, they were assessing circulating factor effects on endothelial cells after COVID-19, and they weren't just limited to non-severe COVID. In this study, they studied more severe patients. They found that the cytokine storm present in COVID-19 patients induced massive cell activation with production of tissue factor mainly by platelets, granulocytes and microvesicles. And they also found that there was impaired nitric oxide biosynthesis in these patients as well as platelet activation and coagulopathies. And I couldn't show it here, but what was interesting is that when they examined the circulating factor content by dissecting out using an inhibitor of the interleukin 6 receptor, they showed that interleukin 6 was playing a role in driving the endothelial dysfunction that was induced by the circulating factors in these COVID-19 positive patients. So, this is really giving precedent to the idea that COVID-19 circulating factors may be having an effect and that studying these circulating factor changes may provide insight into potential therapies that are different than just treating the actual virus.

So, the last aspect of circulating factors that I want to touch on in my introduction is regarding Nicotinamide adenine dinucleotide. The structure of NAD as I'm going to call it is shown here on the left, and NAD is a co-factor that's required in many cellular reactions that enzymes will use as a co-factor. NAD can be depleted during active viral infection, presumably by upregulation of the enzymes that consume it. And this has been shown in viruses like HIV but has not yet been established in COVID. And as I said, one source of circulating factor changes could be the cytokine storm, particularly in severe COVID. And the cytokine storm is going to increase cytokines in circulation like interleukin 6 and interleukin 10. These increases in cytokines can activate interferon beta systemically, and this interferon beta is going to activate interferon stimulated genes.

Importantly, some of these interferon stimulated genes are poly ADP ribo polymerases or PARPs. These are enzymes that are involved in DNA repair, and they're also involved in antiviral mechanisms. And importantly, the PARP enzymes require NAD as a co-factor and upregulation of these enzymes can deplete cellular stores of NAD. When you deplete cellular stores of NAD, you can actually result in decreased nitric oxide bioavailability. And that is because depletion of NAD is going to prevent enzymes like the cert enzymes from using NAD as a co-factor. And those enzymes are important for regulating nitric oxide synthase which produces nitric oxide. So, all of this in summary to say that circulating factors may be playing a role in reducing NAD bioavailability, and this may be a mechanism of endothelial dysfunction.

So here in the Seals lab we're really interested in the effects of circulating factors on endothelial function. And given the known effects of COVID-19 on endothelial function and its effects on NAD bioavailability, we sought

to determine whether circulating factors in COVID-19 are one of the mediators of endothelial dysfunction. To do this, we facilitated a collaboration with the University of Colorado Anschutz Medical Campus, and they provided us 47 plasma samples from COVID-19 positive hospital patients or COVID-19 negative hospital controls. So, these are patients who were all in the hospital who either tested positive for COVID by PCR or tested negative. And we think that these hospital controls are a good control because they can help control for increases in circulating inflammation that could be due to the stress of hospitalization rather than just healthy controls.

And when we use these samples, we use them in our ex vivo model of endothelial cell function. This model is part of a larger sequence that we utilize to determine the effects of circulating factors on the vascular endothelium. We begin by obtaining blood samples from human subjects. Typically, we perform in vivo measures of endothelial function such as flow mediated dilation that Dr. Trinity just described. However, in this case, we don't have those in vivo measures and we're just using our ex vivo model. We then expose endothelial cells in culture to the patient direct plasma, and we measure our markers of endothelial function, which in this case are nitric oxide bioavailability and reactive oxygen species levels.

So specifically in this set of experiments, we exposed ourselves to the COVID positive plasma and the COVID negative hospital control plasma in our cultured aortic endothelial cells and we used fluorescence microscopy to measure nitric oxide and reactive oxygen species levels. When we measure nitric oxide, we actually measure it pre acetylcholine addition and post acetylcholine addition to get the full change of nitric oxide that is bioavailable in the cells upon stimulation by acetylcholine. We measure ROS as basal levels after exposure to the plasma and we also measured NAD levels in cells after exposed to the plasma using a luminescent assay.

So, these are some of our results from our ex vivo assay. To begin, I have the nitric oxide production as a full change relative from our positive to our negative group. And you can see that after exposure to the COVID-19 positive plasma, there was approximately a 20% decrease in nitric oxide bioavailability as assessed by fluorescence microscopy. We also analyze reactive oxygen species production after exposure to the COVID-19 positive plasma and the negative controls, and we found that there was approximately 1.5 times as much reactive oxygen species in the cells that were exposed to the COVID-19 positive plasma. So, using this model, we established that circulating factors from COVID-19 positive patients resulted in endothelial dysfunction as measured by nitric oxide bioavailability and reactive oxygen species levels in the cells.

We also assessed NAD function or NAD content as I stated in the beginning. And when we exposed our cells to COVID-19 positive plasma, we found that there was a decrease in NAD content in these cells normalized to our cell viability. And we also found that there was a decreased NAD to NADH ratio in the cells indicating that exposure to the circulating factors from COVID-19 is depleting these cells of their NAD content. So, we wanted to determine exactly if the NAD content was really driving this mechanism.

And so, we tested the effect of using supplementation of an NAD boosting compound, nicotinamide riboside as shown here. Nicotinamide riboside is a molecule that can be converted to NMN and then it can be used

in the NAD salvage pathway within the cells because NMN can be converted into NAD to boost the bioavailability of NAD in the cells. We chose to use nicotinamide riboside or NR because we performed a pilot trial in humans looking at NR supplementation. And we're currently running a larger trial to determine its effects on vascular function. So, we wanted to use this as a translatable molecule in our ex vivo model.

We once again expose the cells to the COVID-19 negative and positive plasma. But this time we also supplemented our COVID-19 positive plasma with nicotinamide riboside, and we again assayed for nitric oxide production ex vivo. And you can see that when we supplemented with nicotinamide riboside, the decrease in nitric oxide bioavailability that we previously observed was mitigated and brought to the same levels as our COVID-19 negative controls. We also found that the increase in reactive oxygen species production was mitigated, and it was decreased to levels similar to our controls, indicating that increasing NAD bioavailability was able to restore endothelial function in this ex vivo model as measured by nitric oxide and reactive oxygen species. So, it's possible that nitric oxide is mitigating the reactive oxygen species production or affecting enzyme function that restores nitric oxide bioavailability in these cells, perhaps by affecting nitric oxide synthase where it could be affecting both of these processes.

So, like I said, this ex vivo model is part of a larger workflow that we use to identify and analyze circulating factors. So, I just described the first two modules that we usually perform in this workflow, and the next steps would be to identify the circulating factors that are linked to the phenotypes that we're seeing. And once we do that, we can actually modulate in our ex vivo model, which is one of the great advantages of it, to be able to see direct effects of these circulating factors. So, we can supplement with circulating factors that have decreased, we can try to remove the effects of circulating factors that may be causing endothelial dysfunction, or we can treat with small molecules to inhibit or activate biological processes to get a better understanding of what's going on in these cells after exposure to the COVID-19 plasma.

So overall, our findings suggest that circulating factors promote COVID-19 related endothelial cell dysfunction by increasing ROS bioactivity and decreasing nitric oxide bioavailability. Supplementation with NAD boosting compounds like nicotinamide riboside may be an effective strategy to prevent endothelial dysfunction or mitigate it and reduce the risk of cardiovascular diseases with COVID-19. So, like I said, some of the next steps that we're interested in are looking more specifically at the circulating factors and how NAD might be mitigating them, but also as part of the RECOVER consortium trials, we are trying to expand this into more long COVID work. So, we're working with some groups at the University of Texas Health Center where we're going to use this assay to look at longitudinal samples from patients with COVID-19 so we can track how their endothelial function changes over time, because currently this is just acute COVID data that we have. So, with that, I would like to thank our group here at CU Boulder as well as our funding. And I look forward to questions in the Q&A.

Sarah Hatcher

Thank you, Dr. Ludwig. Now I'll turn it over to Dr. Hamburg for our final presentation.

Dr. Naomi Hamburg

Thank you so much. Great to be here with you today. So, I'm going to talk about endothelial health in the clinical expression of long COVID or PASC with a specific focus on cardio metabolic disease, vascular aging inflammation and thrombosis. On disclosures, none of which are relevant directly to this talk. So, I thought I'd start with a case which I know there was a lot of questions about how all of this relates to the clinical disease. So, this is a patient I've been following for a number of years. He's a gentleman in his 50s. I had seen him historically for super ventricular tachycardia and he has now a chest pain and dizziness. He has a job as a bus driver and he experienced COVID-19 initially in the initial wave of disease in April 2020. He had upper respiratory symptoms, but he did not require hospitalization.

But since then he's had persistent chest pain, near syncope and slow thinking and dizziness. He had an extensive cardiovascular workup that was largely normal with an echocardiogram, a pharmacologic nuclear stress test, a loop recorder for his SVT, and really nothing was identified. He recently had a metabolic stress test, and it showed a tremendously reduced ability VO2, which is a measure of your ability to exercise at 12.6. And this is really similar to someone who would have advanced heart failure. And so, I think this raises a lot of questions. It shows you that there's something abnormal, we're not picking it up on other tests, and what are really the mechanisms of this cardiovascular expression of long COVID? What is the relationship of endothelial dysfunction that we've been talking about to these symptoms? And even in patients who don't have symptoms, is there evidence that there's accelerated vascular aging due to prior COVID infection? And how do risk factors modulate these events?

So, we've been talking about the cardiovascular manifestations of long COVID with a focus here on what's happening at the level of the arterial wall with inflammation, potentially with thrombosis. And really I think the question is sort of the way that this figure is laid out. We have each of these elements kind of on their own, but the question is, what are the intersections? What is the crosstalk between what's happening in the vasculature, the heart, with the symptoms that people are having chest pain and with autonomic symptoms like POTS? So, we've seen some different data about that there's effects in the cardiovascular system from prior COVID infection.

Here's some additional data focused on the thrombotic complications. So, this is a cohort study that happened in the UK of already over 48 million adults and they looked at both incidents of arterial and venous thrombosis since the time course after COVID infection. And the black line here is the cumulative incidence. And you can see that most of this incidence of arterial and venous thrombosis is happening in the early phase, but it continues, it persists. The slope continues to rise in the time after infection. And the question is, what is going on that leaves you with this persistent vascular abnormality?

So, we've seen some data, but I'm looking at flow dilation. I thought I'd show you a little bit of data with another measure of vascular function. This is a measure of arterial stiffness. So, we measure arterial stiffness looking at the way that the blood flow pulse moves through the vascular system. And the concept here is that a more compliant system, the pulse flow is going to move more slowly, but as the central arterial system gets stiffer, that you have a faster propagation of the blood flow through the vasculature, of this pulse wave through the vasculature. And this is a study that came out of Europe and they had a cohort of individuals with a history of COVID and then they followed them over time. And you can see that this baseline is the time of acute COVID compared to controls in black.

They clearly have a heightened arterial stiffness, but then even in follow up, you see some improvement from the time of acute COVID, but it is still worse than the matched control cohort suggesting that perhaps there's this persistent effect in terms of arterial stiffening with prior COVID. And so, this is a model that we proposed in an editorial to this, which is that perhaps there's accelerated vascular aging that happens after COVID-19 infection. So, we have this acute infection and I'm going to talk more about some of the events that might happen with acute infection that damage the vasculature with inflammation, coagulopathy and microvascular damage, that this maybe then interact with underlying risk factors like age and cardiometabolic risk to heighten the stiffness of the arteries to kind of give you this premature aging phenotype. And that in some people there's persistent injury over time and that this may be related potentially to long COVID and cardiovascular risk. But then in other people perhaps you get some vascular healing and maybe we can accelerate that with therapies to restore cardiovascular health.

So, to move to some of these upstream events in a little bit more detail or just complementing what some of my other speakers talked about in this session, this was a study that came out of Alex Schmaiers. This is a group at the Beth Israel Deaconess Hospital here in Boston. And they looked, again taking plasma from patients who had COVID varying severities, and they treated cultured human endothelial cells and they showed activation of pathways involving angiopoietin that really led to increased thrombo inflammatory gene expression. And you can see that with increased thrombin generation, largely in the individuals, the red bars of the individuals who had severe COVID compared to more mild disease and controls. And that they were able to block this pathway by blocking Tie2 activation through this pathway suggesting that potentially this might be a modulatory way that we could protect the vasculature from the impact of COVID related inflammatory and thrombotic activation.

This is another study that comes out of Yogen Kanthi's group at the NIH, and they've really been interested in this concept that there's an inflammatory activation and particularly activation of autoimmunity that may be related to what happens in the endothelium. So again, they also took blood samples, here serum from individuals who had had COVID, been hospitalized for COVID, compared to controls and treated cultured endothelial cells. And they looked at the expression of adhesion molecules that are important in activating the endothelium and promoting both thrombosis and inflammation ICAM, VCAM, E-selectin. And you can see that the

individuals who had a COVID infection had more of activation of these adhesion molecules. And they went on to look at whether or not these individuals had expression of a set of auto antibodies called antiphospholipid antibodies. And more than 45% of the subjects who had COVID infection had at least one of these auto antibodies. And when they depleted the IgG auto antibodies from the serum, they saw less of this endothelial activation suggesting that perhaps one of the pathways to injuring the endothelium is through this activation of autoimmunity and inflammation.

This is one study that came out of Europe and I thought this study was really nice because it puts together this concept of what's happening in the endothelium and flow meter dilation with the microvasculature and then what's happening in the heart. So, they had 70 individuals who were, actually, now this is a post COVID study. They were four months after having had COVID and they used two control groups, 70 individuals who are age and sex matched but who had a cardiovascular risk factor. They had hypertension. And then 70 healthy individuals without cardiovascular risk factors. And what they found was that even at four months you can see this persistent abnormality and flow media dilation compared to the healthy controls.

It was actually similar to those with hypertension. Again, this idea that there's perhaps this accelerated aging and cardiovascular phenotype. Now, this is with prior COVID. They did an interesting measure looking at microvascular function where you actually image the microvascular in the mouth in the sublingual circulation. And you can see similarly in a similar pattern where it's abnormal compared to the control individuals at four months after COVID. And then looking at the heart, they saw impairment in coronary flow reserve. And there was some association of circulating biomarkers of oxidative stress, von Willebrand factor and thrombomodulin with the abnormalities and endothelial function, coronary flow reserve and in the microcirculation.

So, I think all of this putting together is again this concept that perhaps there's intersections between what's happening with inflammation, with underlying risk factors, and then in the heart, and that's really the basis for our ongoing study that I'm leading along with Jennifer Ho, who's at BIDMC. This is, we call it the CLEO study and it's looking at the long-term endothelial effects of COVID-19 with a focus on obesity. And we're measuring a set of phenotypes, we're measuring the endothelial phenotype, we're measuring vascular function, looking at brachial artery flow, media dilation and circulating biomarkers of endothelial health. And then we're looking in detail at isolated endothelial cells from the individuals along with looking at coronary microvascular dysfunction using cardiac PET of the heart and then looking at whether some of these cardiometabolic risk factors like obesity might intersect with these pathways of vascular function both in the conduit, the endothelial cell level, and in the coronary microvasculature.

So, a little bit more detail about background for this. So, we know that there's an intersection of obesity with outcomes in COVID and this was data from a study that Jen Ho led at Mass General at the time of close to 800 individuals admitted at Mass General Hospital with COVID-19 and close to half of them had obesity. And you can see that those individuals with obesity, marked in black compared to the gray, have higher levels of circulating

markers of inflammation including CRP, Sed rate, IL-6 and D-dimer. And that obesity was related to multiple adverse outcomes in these hospitalized patients that you can see in the hazard ratios of outcomes. So, we think at least in the acute setting that obesity may modulate risk. They also looked at plasma proteomics and identified endothelial pathways as well as aging pathways like senescence-associated secretory phenotype as being important in the individuals with COVID-19 compared to controls doing a comprehensive proteomic paneling.

And part of this and highlighted was ADAMST13, which is a secreted protein that's related to cleaving von Willebrand factor that is a marker of microvascular endothelialitis, and so lower levels relate to abnormalities in the endothelium and they were related to the severity of COVID-19. In terms of our work specifically with endothelial cells, so I'm showing you some work that we've done with isolated endothelial cells now in diabetes. And the idea is really to try to look at this in patients who've had prior COVID infection. So, we are able to isolate endothelial cells from human beings and this is using a minimally invasive methodology to insert a wire into a forearm vein and collect some endothelial cells.

And when we treat individuals who are healthy with insulin, we can improve the production of nitric oxide in part through improvement in the phosphorylation of the enzyme that catalyzes nitric oxide production. But this is absent in individuals with diabetes and we're interested in then seeing how this may happen in patients who've had prior COVID. We're also able to isolate the RNA from the endothelial cells, and in individuals with diabetes, we've shown that there are activation of pathways that relate to oxidative stress, to mitochondria in the ER, and we're interested in now looking at doing comprehensive RNA sequencing in these endothelial cells from individuals with prior COVID-19 infection.

And so, this is the overall CLEO study design. So, we're recruiting individuals who are cases with prior COVID infection and controls and they're undergoing comprehensive vascular function testing including brachial artery inflammated dilation, as well as coronary flow reserve using PET scans. And as part of that we'll be able to look at phenotyping their adipose depots with the CT scan. We're also looking at the endothelial cell RNA sequencing, looking at specific pathways like nitric oxide production and endothelial cells and doing a proteomic profiling to look at circulating factors. And then we're planning to follow these individuals at six months to look at the longitudinal change that will help us really see what the intersections are between underlying risk factors and the prior history of COVID. And this includes both individuals who have persistent symptoms and those without persistent symptoms.

I'm going to shift a little bit to talking about the treatment. So back to our patient. I talked about this 56-year-old gentleman with largely normal cardiovascular testing but markedly abnormal abnormalities in this VO2 max suggesting moderate to severe limitations in the activities of daily living. And so, the question is, what are some of these pathways that people have talked about in terms of treating the cardiovascular symptoms, how might they be targeted at the endothelium? And so, I took online from looking at what has been proposed and what's ongoing in terms of different types of therapies that people are trying for long COVID. And you can see

some of these are sort of cardiovascular targeted, these antithrombotic therapies. We've also talked a lot about how the inflammation might affect the endothelium and there's a large number of them that are targeting inflammation, and other dietary supplements we heard about with nicotinamide riboside and and what of these might help affect their exercise intolerance?

So, what about antithrombotic therapy in post COVID-19? And so, there are a lot of questions and I know some questions about microvascular clotting. So, could teeny blood clots cause long COVID's puzzling symptoms? So, we know that in hospitalized patients there's some evidence that both prophylactic and full dose antithrombotic therapy can be beneficial in reducing adverse events in individuals hospitalized with COVID. But there have been a number of studies that have looked at what about continuing this post-hospitalization or just treating outpatients with asymptomatic COVID-19 with anticoagulants, both antiplatelet directed therapies or anticoagulant therapies that have largely shown no benefit. And there are some ongoing studies that look at whether this might have a benefit in long COVID, but there's no current data to support the use of antithrombotic therapy in individuals with long COVID. So, I think we really have to be cautious about people taking on these therapies without any proven benefits.

Similarly, what about targeting vascular inflammation? Will we all encourage individuals to get vaccinated to prevent recurrent infection? That likely has adverse impacts. There's some ongoing studies looking at steroids or antihistamines, the thought being mass cell targeted inflammation or dietary supplements, but there's no proven therapies. And again, I just caution people against taking on these therapies that don't have any proven benefit in terms of reducing symptoms currently. But I do think that part of what we're thinking about here is maybe some of these endothelial markers could be useful as secondary endpoints when we think about how we're going to design our trials to really understand whether these potential therapies have any benefit. And lastly, a little bit about this exercise intolerance. So, it's really interesting, there's multiple different levels that you could have the exercise intolerance, whether it's related to pulmonary effects, cardiovascular or peripheral vascular effects.

And there's, I think, a lot of interest in exercise rehabilitation and this I do suggest to all the patients that I see, a gradual reintroduction of exercise if they have difficulty with standing trying to use non standing approaches like recumbent bicycle and gradually building up their exercise tolerance. And there's some evidence that this has benefit with the cardiac rehabilitation to reduce self-reported fatigue. And we know that exercise interventions have been shown to have benefits on inflammation, mitochondrial function, endothelial health and other conditions that have both with risk factors in cardiovascular disease. So, I think that this is a safe and known therapy that's reasonable to suggest to patients. So, I'll finish with some take home points. There are diverse long-term impacts of COVID-19 infection on the vasculature. And there's this concept that perhaps the accelerated vascular aging may be important in inducing this profound functional limitation that we see in patients.

And it really is likely an intersection of your underlying risk along with acute processes that include inflammation, activation, thrombosis and endothelial injury. And there are ongoing studies including our own that

investigate the connection via vascular dysfunction with both circulating biomarkers and coronary vascular function. And we really need, now that we have this RECOVER platform that's helping us identify the physiology of long COVID, to understand what are the strategies that help protect and restore endothelial health after COVID-19 infection? And with that, I'll just thank many collaborators who are both involved in my laboratory, the COVID-19 work and our diabetes work. And I will finish there and looking forward to the discussion.

Sarah Hatcher

Thank you, Dr. Hamburg. Our discussant, Dr. Janet Mullington, will now provide a brief synopsis of the research presented today.

Dr. Janet Mullington

Well, thank you very, very much to our speakers for wonderful coverage of the endothelial aspects of COVID and long COVID. We heard from Dr. Trinity a wonderful overview on vascular physiology and methods of measurement as well as data, present presentation of data looking at changes in velocity and stiffness. And then we heard from Dr. Ludwig some interesting new findings, preliminary findings, and looking at the in vitro, sorry, in vivo, excuse me, in vitro, the dish studies that she showed looking at the regulation of NO production, ROS production and NAD content. And I think that these ex vivo models will certainly be important to watch as we move forward. And then we heard from Dr. Hamburg on more clinical aspects, vascular arterial stiffness and vascular changes through acute COVID and looking at the different model of accelerated aging discussion of the currently ongoing CLEO study looking at obesity phenotypes and biomarkers of endothelial function.

And the discussion then went to the microvascular clotting antithrombotic therapies. Dr. Hamburg reviewed that currently there is need for more investigation. The antithrombotic methods interventions have not come up with prudent therapies and there are some strategies regarding exercise, gradual exercise for improvement of endothelial function. I would just like to maybe open up the discussion by asking our speakers if they would like to discuss some of the host defense mechanisms. There's an overreaction with the sympathetic and vasoconstriction factors leading to endothelial dysfunction and the age and sex factors are important, I think, to consider, sex in particular, because long COVID has been shown to be more prevalent in female sex of course. And wondering if our speakers would like to comment on sex differences, potentially age differences and social disparities. Anybody would like to respond to that question? You could start there. Dr. Hamburg.

Dr. Naomi Hamburg

Sure, I'll start with that. I think it's a really important question. We know that there have been disparities in terms of the impact of acute COVID across our different communities. And the question I think is really, and

then all I think there in terms of the long COVID, we have been seeing differential impact I think in women as compared to men. I think part of this that we need to know better and hopefully we'll learn better from RECOVER is really to me, there's two pieces of this. Do we think that there are biological impacts?

And that may be some of these underlying risk factors. Are there underlying differences in how this inflammation, thrombosis, the endothelial activation? Are there biological differences that happen between men and women based on underlying risk factors, based on some of the social determinants of health that may lead to differences in incidents of long COVID? And then the second question is, who are we seeing in our clinics versus who's all getting long COVID? And I think that's going to be really important to look at too and hopefully recover with the prospective design is going to give us really a better sense of, is what we're seeing that, in terms of who comes to our long COVID clinics, that I do tend to see more women. Is this really true or who's coming? And that will, I think, help us understand that better.

Dr. Janet Mullington

Great. Thank you. Dr. Trinity or Dr. Ludwig, would you like to comment on that? The potential sex or?

Dr. Katelyn Ludwig

Yeah, I don't have too much to add because we unfortunately couldn't tease out the differences in age or sex based on our data just because we didn't have a big enough sample size. It wasn't dispersed enough amongst those groups. So, we're hoping in our RECOVER project where we have a much larger sample size that's going to be more evenly dispersed with men and women of different ages. But that's something we can look at in terms of circulating factors. It's possible that we might see a difference, especially between young and old where we would expect to see those differences. So, it'll be interesting to see if we're able to find that. But at this time, I just don't have any data on it.

Dr. Janet Mullington

Thank you very much. And Dr. Trinity, would you like to? Or if you feel everything has been said, I will throw out another question. So please go ahead if you want or I'll-

Dr. Joel Trinity

Yeah, we can move on to the next question.

Dr. Janet Mullington

Okay. Well, maybe actually you might have a perspective on this one. With regards to the exercise, I think it's a very interesting and delicate aspect to navigate in long COVID, because as with MECFS, we see that too much can really be potentially long and harming and exacerbate symptoms for a period of time. And so, I think there's real opportunity to look at who may benefit and who may be at risk in terms of the different population or kind of symptom clusters. And I'd be interested in hearing everybody's perspective on how to move carefully forward with that. Sort of related to that is this sort of sympathetic and parasympathetic balance. And I wonder also about the restorativeness of activities like relaxation, sort of biofeedback relaxation, but also sleep, where we see increased parasympathetic and lower blood pressure. And just wondering if our panelists would like to comment on any of those. Go ahead, Joel.

Dr. Joel Trinity

I think the idea of exercise rehabilitation is, like you said Janet, it's very tricky with the long COVID patients, right? I'm just noticing in the Q&A that one of the people, their lived experience is any type of exercise, rehabilitation, even if it's very low intensity, seems to exacerbate and make things worse. So, thinking physiologically about what's going on at the basic level of exercise, it's an acute stress that the body has to deal with. I think we really don't know, especially with aging or with long COVID, what are the cellular and molecular signals that are dysfunctional or dysregulated in response to that acute stress? So, we know that there's increases in oxidative stress, increases in inflammation that occur. And in the healthy individual, those lead to adaptation that's beneficial. So, you can stress the system, keep stressing it, those acute bouts then lead to positive adaptation.

But in long COVID, it could be very similar to the MECFS cohort or patients that anything that happens, it's just such a stress that throws everything out of whack and we don't know how to deal with that. So honestly, I don't know how to best address that. Being a exercise physiologist by training, and we like to say exercise cures everything, and for most things it seems to do a great job. But this is a very interesting situation where it could make things worse. So, I'm really interested in, maybe not exercise per se, but physical activity. Getting up, moving around, standing, just getting your muscles active, increasing blood flow. Very simple things that really aren't exerting yourself but might be increasing blood flow. And I think that'd be an interesting first step to that realm.

Dr. Janet Mullington

Thank you. Dr. Hamburg? Unmute yourself.

Dr. Naomi Hamburg

I think that's important qualification. I think that a lot of it is targeting to the patients, and I think I tried to highlight this patient where I think it's helpful to have objective evidence that goes along with people's experience of how difficult it is to move. So, the reason it's difficult to move is there's real physiologic changes that are making it truly difficult to do any activity. And so, I think that it's not simply telling people to ignore it and move. That's not the recommendation. But I do think that thinking about how we're going to design interventions that help people restore their function is really what's important because this is extremely debilitating condition and we want to try to help think about how to effectively, and maybe that is other approaches that help induce the physiologic benefits of exercise with other safe approaches for individuals. And we need trials of all of these pieces specifically with these patient groups.

Dr. Janet Mullington

And I'm wondering what Dr. Ludwig thinks about whether it might be possible to look at plasma or do some of your dish studies with samples from exercise versus in some of these affected patients as well?

Dr. Katelyn Ludwig

Yeah, we certainly could do that. We haven't done any of those studies directly on exercise patients before, but we are interested in our lab in kind of some exercise mimetic interventions that I think could be really interesting in the context of long COVID. So specifically, we look at a few things like heat therapy where you're immersed in hot water for long periods of time. We also look at inspiratory muscle strength training. So that is a breathing device where you kind of breathe against resistance to also act as an exercise medic. So, we've done a little bit of that in the cell culture model, but it would be interesting to look at all of those in the context of COVID if it was possible.

Dr. Janet Mullington

Very interesting on the temperature as well, thermal use. Okay. I think that we are ready to turn it over to our moderator to go into questions.

Sarah Hatcher

Thank you, Dr. Mullington. So, we've had, I'm pulling questions from both pre-submitted, pre-webinar submitted questions as well as the Q&A, and we have several questions about treatment. I know we've talked a bit recently in the discussion about exercise as a treatment, but would any of the panelists like to share any additional information about therapies that are being considered for treating vascular dysfunction after COVID?

Dr. Joel Trinity

So, we have a trial that's starting soon within the veteran population looking at a mitochondrial targeted therapy to hopefully improve the vascular function but then also to potentially improve the ability to perform an exercise rehabilitation. So, I think the mitochondrial dysfunction is definitely associated with vascular dysfunction and I think that could be an interesting avenue to look at.

Dr. Naomi Hamburg

I think there are also ongoing studies in the UK looking at this anti-thrombotic therapy question about whether there are effects in long COVID. But again, I just want to make sure that we preface any discussion about therapies to say that there are, as of yet, no results of any trials of therapies. But there are a lot of people treating people out there with individual therapies. So just to understand that there's currently no proven therapies.

Dr. Katelyn Ludwig

And I know there are some trials looking at NAD boosting compounds in COVID, but again, like Dr. Hamburg said, there's no results on them. So, I don't know how they're doing in the clinic.

Sarah Hatcher

Thank you. So, another question we have is, since arterial stiffness is also associated with menopause vasomotor symptoms, which are generally treated with estrogen and sometimes SSRIs, are there any studies being done with hormones and PASC related arterial stiffness?

Dr. Naomi Hamburg

Maybe that's a two-part question. I mean, I think part of it is about the hormones and then the other question I think is maybe about the integrative physiology question. That is to say, so I don't know of any that are specifically estrogen related and one may have the concern that there's some risk of unopposed, of estrogen therapy with thrombosis. So, I would want to think carefully about how those studies would be designed. But I think the other question is how did the endothelial piece integrate? We all kind of alluded in different ways about the integration with other physiology, with the autonomic nervous system or with the neurologic.

And I'm interested in other people's thoughts on this too, but I think it would be really interesting to see whether things that may target the nervous system, like you suggested SSRIs, but that's only one of many therapies that might impact that, would have an effect on the vasculature. So, making sure that in the studies that

we design, we kind of broadly look at the physiologic impact of therapies for long COVID. I don't know, Joel, if you have other thoughts about that from your looking at the autonomic nervous system piece.

Dr. Joel Trinity

Yeah, I think there's substantial evidence that there is this autonomia and I think that could definitely be contributing to the vascular dysfunction. So, we have this balance of the basal constrictors from the sympathetic nervous system activity versus the vasodilators. So, I definitely think that is another avenue that we could approach. Trying to turn down the sympathetic activity could benefit the vascular function but then also some of the other autonomic disorders that we're seeing.

Dr. Naomi Hamburg

I think there's also a real question that I know was raised before about whether the other way. We don't really fully know which, it could be bidirectional that the microvascular dysfunction or vascular dysfunction, all of the nerves are vascularized as well. Is there some impact of the microvasculature on the autonomic nervous system as well? And certainly it's a little bit challenging. I would also just caution about anecdotal reports, but I've certainly had anecdotes where patients are on some of these therapies targeted towards autonomic and their other symptoms like say chest pain or other pieces get better. So, there may be pieces that go together.

Sarah Hatcher

Any other comments on this question or topic? Okay, I'll shift gears away from treatment for a minute. We have a question about vasospasms. Do vasospasms have a role in pathophysiology of the disease? And would any vasospastic, mitochondrial, immunological, micro clots, et cetera pathways be potentially involved in worsening of symptoms and maybe further vascular damage?

Dr. Naomi Hamburg

Well, I mean, certainly I've seen people who have manifestations of acute COVID that seemed like vasospastic disorders. Probably a component of, we talked about the COVID toes early on. I haven't seen patients with that since the early days of COVID to be honest. So, I don't know if it's the incidence is going down or with milder disease. And I think it's people who have those types of symptoms have tried some of the treatments that we use for vasospastic disorders. But again, I think it's a reasonable hypothesis about which there's not a lot more evidence. I do wonder in the data that was shown with EndoPAT and why it would be nice maybe to have in the RECOVER study is that in the acute setting, perhaps some of the differences that we saw might be that there's vasoconstriction and less pulse amplitude at baseline and then that was sort of actually showing paradoxically

more percent dilation and that over time that was what got worse. So, it'd be interesting to look at what the baseline pulse amplitude, because certainly in the acute setting, you might have more vasospasm than in the long-term setting.

Sarah Hatcher

Any other comments on that? Okay. So now onto a question we have live, we probably have time for this one question before closing out. Is there a theory as to why the vascular dysfunction in long COVID patients isn't showing up in the acute phase but does show up weeks or months later?

Dr. Joel Trinity

I think it could be due to the tests that are being performed to assess vascular function. The vasculature is highly responsive to multiple signals. So, it could just be, in the acute phase, there's so many things going on. It could even be an overactivated immune response that's causing increases in blood flow, something like that, that you're not then seeing decreases until that resolves. And then long term there's something else going on. So, I think it's a combination of the physiology of what's going on, controlling blood flow regulation and vascular tone. And then, we still, like I said earlier, we don't have a clinically relevant test to measure vascular function. So, I think Dr. Hamburg and Ludwig did a great job of looking at endothelial cells in these downstream more mechanistic things, but we still don't know what our best approach should be for measuring vascular function, which to me is important because we have to have that before we can start guiding therapies that will treat and improve vascular function.

Dr. Naomi Hamburg

I mean, I do think it goes along with this really interesting question about a lot of what CI showed in the acute setting or others are showing in the acute setting is that the people with more severe disease have more endothelial damage. But in terms of, I think there's some questions in the comments about this paradox, like the patient I discussed and many other patients I see in the office, they didn't necessarily have severe COVID but prior to severe long COVID. And I think the question is maybe getting at that a little bit and just trying to speculate it. It may be that whatever is helping you fight off from having more severe infection has some lingering effects in terms of these other long-term symptoms. And I think it's going to be really interesting to see from a cover, how did the acute immune markers relate to the incidents of long COVID and maybe they're not the same as who ended up with more severe illness.

Sarah Hatcher

Thank you. Well, we're almost out of time, so I want to thank our presenters for sharing your time and important work with us and thank our audience for attending and engaging with this Q&A. As a reminder, a recording of today's seminar will be available on recovercovid.org within a few weeks, and we will be posting a Q&A document that has responses to the many questions we received today, including those we did not have time to address. Shane, would you like to put up the final slide? This is our last R3 seminar of 2022, and this slide lists the topics for future sessions. R3 seminars are held on the second and fourth Tuesday of the month from 12 to 1:30 PM Eastern Time.

The RECOVER team is finalizing the rest of the 2023 schedule and will post more information to recovercovid.org when it is available. The RECOVER team has developed a short three question survey that you're seeing on your screen now to learn about how we can improve the R3 seminars. Please fill out this brief survey if you have time before you leave the webinar. Thank you. Happy Holidays, happy New Year, and have a great day.

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