ME/CFS Research Roadmap Webinar Series – Circulation
Open Session
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Vicky Whittemore: Okay. I think we'll get started. Good morning, everyone. I'm Vicky Whittemore. I'm a program director at the National Institute of Neurological Disorders and Stroke at NIH, where I oversee grants on ME/CFS. And I've been helping to coordinate this webinar series.

So, I'd first like to recognize -- excuse me -- all of the members of the ME/CFS Research Roadmap Working Group of Council who have participated in planning and working through all of the eight webinars as part of this series. And especially recognize Cindy Bateman and Maureen Hanson, the two co-chairs of this Working Group of Council.

I'd also like to recognize the specific individuals who organized this webinar that you'll hear today on circulation. So, the chair is David Systrom, who is a physician at Brigham & Women's Hospital, and also an assistant professor at Harvard Medical School. And you can see the rest of the individuals here who have been part of this particular webinar planning group. So, thank you to all of you. I'd also like to recognize my team at NINDS, who've been really instrumental in helping us to coordinate and put all of these webinars in place.

And a special recognition since this is our very last of these -- of the series, to our colleagues at RLA, who have really done a tremendous job working with us, especially Holly, Damon, Michelle, and then the writing team, Nancy, Maya, Cooper, and Martin. So, thank you all very much. It's been really my pleasure working with all of you.

So, for more information about this roadmap webinar series -- and if you would like to go and view or read the transcripts from past webinars, you can go to this link that you see here. And all of the videos are there for the past webinars, including the transcripts.

For guidance for this particular webinar, the goal of these webinars is really to identify research priorities for research. And in this particular webinar, on circulation issues in ME/CFS. So, what do we know? What don't we know? What do we need to know to accelerate research? And how will it better, how will it help us to have a better understanding of the circulation issues in ME/CFS to really lead to new targets for treatments and acceleration of research toward treatments and clinical trials?

So, after each presentation, if there's time at the end of each presentation, we will take some of the questions, either from the panelists, who you're welcome to raise your hand and ask a question or from the Q and A from individuals who are online watching the webinar via Zoom.

Please note, we're not able to answer questions related to your individual health issues. And questions should really focus on clarifying points from the presentations and be directly related to the topic of this webinar.

If you have other questions about NIH, ME/CFS research, research funding, et cetera, you should send those questions to nindspublicinquiries@ninds.nih.gov. So, all of the research priorities for the webinars will be used to form a report that will be delivered and presented at the May 2024 NINDS Advisory Council and NINDS leadership meeting. This will be done during the open session of the council meeting. And we will circulate the link to that -- for that meeting when it becomes available for anyone who would like to see the delivery and presentation of that report.

For additional feedback, you are always welcome to send emails to this mecfsresearchroadmap@ninds.nih.gov email address. And the best way to receive announcements and updates for all ME/CFS research activities is to sign up for the ME/CFS Listserv at this URL, simply www.nih.gov/mecfs. We will be soliciting information and feedback from the community on the research priorities identified during the webinars using a crowdsourcing platform.

And we're hoping to get that launched next week. We're working hard to get that up so that we can get additional feedback from everyone in the community. So, with that, I would like to introduce David Systrom, the chair of this webinar. As I said, he's a physician at Brigham & Women's, well-known in the ME/CFS community now for his research, and an assistant professor at Harvard Medical School. So, I will turn it over to you, David. Welcome.

David Systrom: Thank you so much, Dr. Whittemore. And thank you for the wonderful introduction, for organizing the webinars, and this one in particular. And a special call out to Drs. Bateman and Hanson, whom you mentioned earlier. It's my pleasure to introduce our first speaker of the day, who is a patient with lived -- or a person with lived experience, Gwynn Dujardin.

I should tell you I know a bit about her. Don't know her perfectly well, but remotely, we've connected. She's had ME for almost a decade and is incredibly well-versed in both really the science and the experience of having ME. And she's going to open things up and tell us a little about her experience as a person with a lived experience. Gwynn.

Gwynn Dujardin: Good morning, everyone. Welcome patients, caregivers, researchers, and members of the public to the final research roadmap webinar on the topic of circulation. I would especially like to thank the patients and caregivers committing time and energy to this session on this cold morning in January.

My name is Gwynn Dujardin. And I'll be opening today's session by describing the patient experience of circulation. And also presenting some history on early science to provide context for the historical moment we find ourselves now, compiling research priorities to advance ME research toward necessary clinical trials.

I'd like to reverse the standard order of operations to begin by acknowledging the insight and generosity of people in my patient communities. And other organizations and individuals who helped prepare me for this presentation. Thank you. I want to note though that while I'll be quoting from individuals with diverse experiences of circulation, I claim full responsibility for the assertions in this presentation. Also, any treatments I mention are not recommended per se, but presented to provide researchers with a holistic context of how we manage our illness.

Two things bring me here. First, until this past fall, any significant improvement I made seemed to be related to circulation. I traced my onset to a trigger in late 2015 that without medical support from my reported symptoms, kept me working and teaching in the university classroom in Canada for another year until I became homebound and bedbound from repeated ongoing cognitive and physiological exertion. I had to medically retire from my position and return to the U.S. and begin treatment here.

In the early dark days, I improved modestly by learning to regulate water and salt and taking Florinef and learning how to pace my exertion. After my POTS and overall circulation condition worsened after the COVID vaccine, a prescription for midodrine, a blood pressure medication, reversed that decline and restored some functioning. But the biggest improvement came after my September 2022 COVID infection when I went on the Nattokinase anti-clotting protocol used by people with long COVID.

Within six months, including over winter, I had reduced PEM and had a larger energy envelope. I also had vastly improved condition and a sense that blood was reaching and saturating extremities where it hadn't been for years. When I nominated myself to be a person with lived experience, I aspired to represent the community with this experience and hopefully, help people. But wait.

Unfortunately, in the great game of ME Chutes and Ladders, I became infected again for a second time. And found the same practices and protocols I used the first time of no help in preventing a significant relapse and pronounced decline. I'm currently experiencing rolling PEM

and back to home-bound bed life. That saturated, refreshed feeling I had was gone and replaced with overall weakness and stagnation.

My cognition is dull and impaired, and especially affecting my speech and ability to write. I have constant breathlessness. And my heart is my constant companion, with palpitations and fluttering, which are much worse. My doctor has found a heart murmur and I'll be getting an echocardiogram later this month. So, instead of representing improvement, I am instead here as evidence that some treatments work sometimes and not others. And I look forward to our researchers' perspective on that question.

I'm also here because once I felt some improvement, I committed to using whatever skills and knowledge I had remaining from my privileged prior career to help other patients and researchers and the general public understand the place we've come to and the culture with ME. I used to study how the English language became a prestige language of knowledge and learning in the 16th and 17th centuries. Up until that time, the principle languages of learning and knowledge were Latin and Greek.

At that time, works of ancient Greek and Roman authors were translated into national mother tongues, like the English language, and circulated through the medium of print. This became known as the age of the vernacular. In 1605, however, Sir Francis Bacon proposes a new form of learning that isn't book learning, but is interested in matter or observations of the natural world. He petitions King James to effectively fund or advance a new form of knowledge in a treatise he calls the "Advancement of Learning."

Interestingly, deliberately countering the trend of publishing in English, Bacon resolves to translate the "Advancement of Learning" back into Latin, looking to reach other men of learning on the continent who spoke no English. If you look at the title in Latin, "De Augmentis Scientiarum", you can see the name for this new knowledge from the Latin scientia: science.

Patients who have felt the language of medicine is forbidding inaccessible can thank Bacon for gatekeeping knowledge through education and language. But Bacon wasn't just against English, but against any elaboration of language, considering it a distemper of learning that, "Men began to hunt more after words than matter." By which he means phenomena in the natural world. Still looking to classical authorities, Bacon turns to Aristotle's theory of empiricism or the study of the natural world through the senses, particularly observation.

Bacon is known as the father of modern science for defining and promoting empiricism, the scientific method of experimentation. I'm interested in several of Francis Bacon's legacies, which bring us here, present a challenge specifically for ME patients. The first step when Bacon promotes the new science, he's concerned to diminish and limit the influence of all language and

culture on scientific study as having a corrupting, subjective influence. But how do we communicate our symptoms except through language?

The next legacy is that a contemporary and colleague of Francis Bacon called William Harvey applies his scientific method to -- and I quote, "Discover circulation", or specifically, learn that the heart pumps blood throughout the body instead of the liver, which was previously imagined. This revelation is seen as a victory for empirical experimental science, what brings us here today. And also, ushers in the age of anatomy in the study of physiology as discreet systems, the very silos researchers lament in trying to study a heterogeneous condition like ME.

A third cut legacy concerns Bacon's proposals, which led to the founding of the royal societies, or state-sponsored organizations for scientific study, which in the U.S. takes the form of the NIH, which brings us here. My objective as a language scholar and person with ME is to buck 400 years of Baconianism to promote patient testimony, not only as credible and authoritative, but also useful to clinicians and researchers in their work.

On the left, I've listed the topics of circulation that our group of scientific researchers will be presenting on today for causal agents of our disease. On the right, I've listed just a sample of symptoms and conditions related to circulation from head to toe as they manifest throughout the body that people with ME commonly experience.

On the far right, I've also marked orthostatic intolerance and postural orthostatic tachycardia syndrome, or POTS which admittedly, is properly a neurological condition of dysautonomia -- speaking of silos -- and it was covered in the first webinar of the roadmap series. But people with ME predominantly experience through the circulatory system. People with ME describe these symptoms as ranging from unbearable to terrifying and subjective language that Bacon would lament but reflects the intensity of our experience.

A note to everyone in the public and in our research that the intensity of our language is a reflection of the intent and the extremity of our experience, which is alien to the able population in both quality and depth of suffering. I believe that when you collect testimony across the patient community and pay attention, do some close reading, signals emerge through the noise, even as everyone's experience of ME may differ.

In my conversations with my community, symptoms of circulation were first present at ME onset, could be constant and intermittent, a dominant part of illness, specifically contribute to the dynamic fluctuating experience of ME, affects appetite and digestion, is subject to seasonal variation, interacts with other comorbidities, is impacted by additional trauma and infection, can worsen over time without diagnosis and treatment, and worsens with cardiac age. Indeed, at age 56, with a family history of heart conditions and EDS, my echocardiogram for my heart murmur

is long overdue.

What we discover by looking throughout patient testimony is that while circulation is part of the autonomic system, colloquially, supposed to happen in the background. For people with ME, symptoms related to circulation, related to the heart, blood, and lymph, form a dominant and daily part of illness worth researching. The lived experience of ME related to circulation also encompasses our encounters with the medical system.

Our symptoms in the language we use to describe them lead not only to misdiagnosis of anxiety or deconditioning, but also to prescriptions or counter-indicated measures like CBT and GET. In everyday clinical settings, the reliance on the routine blood test and the privileging of quantified visualized data, following Bacon's directive to limit language, dispose medical professionals to dismiss serious illness and rule out further investigation.

An ME diagnosis can also inhibit physicians from taking cardiac symptoms seriously. My own doctor dismissed my concerns for years saying it was just part of my esoteric condition. Patients also have a bad experience in emergency care with circulatory symptoms, often being admitted by triage when they believe it could be a heart attack but dispensing with care when that's ruled out. I've had a horrible experience in that regard. Encounters within the medical system in urgent care are especially fraught for people with ME and the BIPOC population and other at-risk communities.

I'd like to note for our researchers that -- to remind everybody that circulatory measures such as the basic pulse Ox may not work on all populations, and once again, lead to mistaken conditions. Thus far, I presented the predominantly negative aspects of circulation for people with ME. I would be remiss not to mention ways that patients manage their blood flow for relief and symptom improvement. The most common and accessible intervention is to manage posture, to regulate blood flow, especially to the brain.

And I can't emphasize enough that what appears as rest or inactivity to the outside world often represents strenuous effort to manage heart rate and blood flow. Most patients wear some form of compression clothing. Many report benefits from massage and specifically lymph massage to relieve the constant poisoned feeling we have. I'll let the physicians present possible medications but note that the other intervention that often helps improve our circulatory system are saline IVs, and if you're lucky to get it, of course, IVIG.

The other positive aspect of the patient's experience of circulation involves new wearables that provide people with ME the ability to track and measure circulatory conditions, both as evidence of symptoms and dysfunction for their care providers. And as a means to pace and manage their energy envelope and avoid overexertion. I've seen many patients volunteer to share their data if

that will help researchers. I'd like to see a plausible goal for research to make these devices available with accommodations to benefit communities who most need them but don't have access to them, which would also expand research samples.

As we proceed from the webinar series to compile and whittle down research priorities for ME, I make the following humble suggestions on the basis of what I've gathered. In the first group, I hope that we can combine resources with other groups in the working group project to ensure that patient priorities, such as POTS and lymph advance in research their dominant conditions of illness. I was also delighted to have Beth Pollack's presentation last week and hope researchers will take her up on her call to record stages of menses in ME patients under study.

In the second group, I hope we will find methods and accommodations to expand our patient base to include patients with severe ME. Those beyond words and who can't come to an office appointment for a CPET, BIPOC and at-risk communities, people at different stages of illness, including long-term with cardiac impact. As part of that, I recommend we maintain steady communication throughout all our work with our PWLEs.

And our last -- in the last group, I gesture to the problems of COVID. I'm now doubly infected with COVID. Where do I stand as a patient with ME? How does that factor in research? Please ensure COVID safe meetings and research practices in whatever you formulate. And finally, like Bacon, who pursues his own advancement, let's fund ME.

As I finish, I acknowledge I've spoken a great deal about words and language. I'd like to conclude with this Instagram image by ME artist Lia Pas, whose embroiders of ME symptoms bring another dimension of meaning and knowledge to this illness that translates without words. Indeed, I'd like to conclude by dedicating this presentation to our fellow severe people with ME and to those we are missing in the community this year due to suffering and lack of treatment. Let's restore the heart and blood of our ME communities and make some new science, some new learning this year. Time can't wait. Thank you all.

David Systrom: Thank you so much, Gwynn. An incredibly moving and eloquent description of what you've experienced. I too in the clinic have heard from patients, time and time again, that they actually sense circulatory abnormalities, amongst other symptoms. I think you've pointed out correctly in a wonderful way that at all times, investigators have to remember the patient. The patient has symptoms.

The symptoms have organic pathophysiology underlying them, which we're investigating. But at the end of the day, we have to close the loop, determine what ails an individual with ME and long COVID. And then circle back and determine whether we have made the individual feel better. Do we do questions now? I'm asking the organizers.

Vicky Whittemore: No. Move on to the next speaker, please. Thank you.

David Systrom: Okay. Great. So, it is my distinct pleasure to introduce our next speaker, who is Professor Jane Mitchell. Who's going to talk to us about the first of several different hypotheses about what might underlie circulatory abnormalities in the systemic circulation of individuals with ME. Professor Mitchell has an amazing pedigree. She is the head of the cardiovascular division at the National Heart and Lung Institute in London. She's based at the Imperial College in London. I could go on and on. I heard her speak at the long COVID symposium, the Keystone meeting in Santa Fe, was absolutely wowed. And I'm so pleased that she's able to share her findings with us here today. Professor Mitchell.

Jane Mitchell: Thank you very much. Thank you, David. And as you mentioned, you very kindly asked me to present at this webinar after my presentation at the Long COVID Keystone meeting. So, the presentation is geared through a COVID lens, but obviously, with direct relevance to ME and CFS. So, I'm hoping that's okay. And I want to thank Gwynn for a fantastic presentation. Very, very difficult to follow. So, I'll do my best.

Endotheliitis is a relatively new word to me. Actually, it was given to me by the keystone organizers. But I'm using it a lot, it seems, since then. So, we'll first work out what that means. And to understand that primarily, we need to remind ourselves of what endothelial cells are. And I just want to point out, from Gwynn's talk, I actually did my PhD studies at the William Harvey Research Institute in London. So, that was really interesting that she mentioned him in her presentation.

So, endothelial cells are a very delicate lining cell that covers the entire internal surface of the vasculature. And you can see in the panel on the left is an image of the vascular innovation in man. And I put this in just to remind me and remind us how extensive the vascular system is and that it extends to every region of the body, every organ, every tissue requires a blood supply. And because the endothelium lines the inner surface of every blood vessel, it's instrumental in homeostasis, in health, and in any kind of disease. It's a very important tissue. But obviously, the direct study of it is -- falls generally within a cardiovascular framework.

So, the endothelium lines, the blood vessels, and here on the right-hand side of the slide, you can see an image from some cells grown in my lab. And these are endothelial cells grown from a human aorta. So, the main artery in the body. And you can see they have this cobblestone morphology. They're very beautiful cells. And they grow in a single mono layer as they do on the surface of blood vessels.

And they have a very interesting, unique property in that they'll align in the direction of flow. And as blood flows through blood vessels, where the vessel is straight, the direction of flow is linear. And in those areas, endothelial cells all line up like little soldiers. Areas of branch points, where the flow is turbulent, the endothelial cells are all completely rounded and don't align. And that's actually important for cardiovascular function. But I won't discuss that today. But it's a very important, interesting feature of the cells.

So, the endothelium and blood vessels and the marriage of those two terms in science has been studied for decades and decades. But a fundamental change in how we view blood vessels and endothelium, and the function came after this paper, highlighted on this slide here by Robert Furchgott, "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine."

And this paper, together with some other observations in the field, rewarded Robert Furchgott, together with Louis Ignarro and Ferid Murad, with the Nobel Prize in 1998 for the discovery really of this endothelial factor, as he called it. And you can see in the bottom of the slide, the exponential rise in publications. This is just a PubMed search for endothelial function and cardiovascular disease. It really took off and many clinical discoveries have been made on the back of this.

So, I just want to show you what that paper actually did because it's an old paper, 1980. But it's a beautiful scientific observation. What Robert Furchgott did was he took a ring of blood vessel. You can see in the bottom left how the ring is clamped together and fixed in an apparatus where you can measure the ring contracting and relaxing. And that kind of models what would happen for blood flow, where the vessel contracts and blood is forced down one route or relaxes and so on.

And the middle of the slide, you can see what are vascular traces -- the trace of the vessel contracting and relaxing. And you can see that the vessel is given a drug to make it contract. So, the line goes up. And then it's given acetylcholine, ACh, and the vessel the line comes down because the vessel is relaxed. And that only happens in either the ring or an equivalent strip -- a vessel. That only happens if the endothelial layer is present.

You can remove the endothelial layer very easy by gentle rubbing because it's so sensitive. And then this response completely disappears. It's a fundamental observation. So, what we now know is that the endothelium is very thin, single-cell layer, contains three really important hormone pathways that together regulate vasoconstriction and vasodilation. And so, really, are very powerful regulators of blood flow.

So, the first on my list here is nitric oxide. And nitric oxide is the mediator that Robert Furchgott found as his factor. It's a vasodilated gas. It's formed from the amino acid, L-arginine by an enzyme called endothelial nitric oxide synthase or eNOS. It causes vasodilation. And it's antithrombotic and it's antiproliferative through a second mediator called cyclic GMP.

Another pathway packed into the endothelium is another vasodilator -- another cardioprotective hormone called prostacyclin. And prostacyclin, in this case, is a lipid mediator formed from arachidonic acid by the enzyme, cyclooxygenase, abbreviated here as COX. And here, prostacyclin has many of the features of nitric oxide, but it works through the second messenger, cyclic AMP.

And then thirdly, but not lastly, is endothelin-1. This is a very interesting mediator. It's made almost exclusively by the endothelium. And it's a very potent vasoconstrictor peptide. So, here, we have a gas, a lipid, and a peptide, two dilators, and one extremely potent constrictor. And

together, these hormones made by the endothelium, work in a kind of balance to impact on blood vessel responses to need and cause the vessels to contract or relax. And also, importantly, for nitric oxide and prostacyclin has very potent effects on platelets and thrombosis.

So, I'm going to gear my talk around these three mediators, nitric oxide, endothelium, and prostacyclin, and the vasomotor responses of blood vessels. But I just want to acknowledge that endothelial function is not just the endothelium controlling the underlying smooth muscle and the platelets. It's also -- endothelial function is also central for permeability. Things have to pass through the endothelium. It's central for cell growth -- angiogenesis. It's central for thrombosis and for cell migration. So, it has an endothelial function. And endothelial cells can be many things to many people. And with these properties shown on this slide, it also means that the endothelium is a very important card-carrying immune cell. And, of course, that has relevance to vascularitis and to disease.

So, what's vascular endotheliitis? So, it's regarded as a host immune response of the endothelium, which we now know forms the inner surface of blood vessels, in association with a -- as a direct consequence of pathogen invasion and resulting in endothelial dysfunction. This is a pretty established definition of the term. So, I just want to take a little bit of time to explore further endothelial dysfunction and what we mean by it in this setting.

So, how is endothelial function measured in this kind of contraction and relaxation blood flow lens? So, there are three main ways you can get an angle on endothelial function in this kind of classic endothelial function definition. The first is in vitro. So, take a blood vessel, very similar. So, the information on the left-hand side of the slide similar to the experiments that Robert Furchgott did that got him the Nobel Prize. And you can take a piece of blood vessel from a patient with a disease or from an animal model, put it in this kind of apparatus, study the endothelial function and its change related to disease, et cetera.

In the middle panel is a very important way to measure endothelial function, which is used -- can be used clinically. But here, it's measuring endothelial function in vivo in people. And there are a number of flavors of apparatus to do this, but they tend to all work in a similar way. They're non-invasive. And blood flow is measured maybe by a light sensor or by changes in pressure or size of digits, et cetera. And it's -- it kind of works by generally occluding a limb, occluding the arm, releasing the occlusion. And the rush of blood that goes back to the arm causes a hyper activation of the endothelium. And you can then get a marker of endothelial function in vivo.

And the end panel there is a very straightforward way to measure markers of anything you're interested in in vivo. And that's an ex vivo approach where we can measure those three elements that I mentioned. So, we can measure nitric oxide, although that's very difficult to do in plasma. But we can measure -- I forgot to mention, actually. I apologize. The endogenous compound

called dimethylarginine, or ADMA which is a natural inhibitor of eNOS because it competes with arginine. So, we can measure ADMA and arginine. And they give us an indication of how the endothelium is likely to be working on the nitric oxide pathway. We can measure endothelin-1 easily and prostacyclin easily by a standard enzyme colorimetric based assay.

So, the infectious pathogen invasion part of the definition of endotheliitis is there's a lot of literature that addresses this in terms of bacterial infection and sepsis. There's a huge body of work on how bacterial infection really catastrophically affects endothelial function. And now, pertinently, and more recently, is respiratory viruses as the infective pathogen. And, of course, SARS-CoV-2, which was the presentation that David heard at Keystone from me.

And that's where I'm going to go on to spend a little bit of time now about SARS-CoV-2, COVID-19, and the cardiovascular system. And that is pertinent, obviously, to ME and CFS because, as David published recently, there are commonalities in symptoms of long COVID and parallels for us to understand and work through. Also, in ME and CFS patients, there are studies showing that endothelial function in people in vivo, again, this flow mediated dilatation is reduced in patients.

And the bottom half of the slide shows levels of endothelin-1. So, that's our ex vivo. One of our ex vivo measures of endothelial function is also increased in the serum of patients with ME and ME/CFS compared to healthy controls. So, just quickly to remind -- not that we need reminding -- about the cardiovascular alignments with severe COVID and morbidity and mortality with COVID-19.

So, from the very early first information coming out of Wuhan about patient characteristics of individuals who got COVID-19 and succumbed to the disease, then individuals' age was obviously a huge indicator. But cardiovascular -- pre-established cardiovascular conditions increase the likelihood of severe disease and death in patients with COVID-19. And this has been repeated many times now since the pandemic has ensued.

Also, post-COVID is associated with an increased risk of cardiovascular events. So, this is data from a really excellent study. Looking at -- the paper looks at a number of outcomes. But on this slide is cardiovascular outcome, the bottom part of the panel, any cardiovascular outcome -- if I just draw your attention to that. That's a little bit easier to see.

There's an increased excess number of individuals or risk of individuals having an increased risk of individuals having an event post-COVID. And that's proportional to the level of hospital care that individuals require. So, the highest risk is in those patients that were -- ended up in ICU, hospitalized. But there is an increased risk even in individuals who got COVID-19 and were not hospitalized.

And I think, just swinging back to endothelial function, again, measured noninvasively, is flow-mediated dilatation. There's a number of studies now, quite a number of studies, all -- more or less, all universally showing that endothelial function is reduced post-COVID. And in this particular paper, you can see endothelial function is reduced up to six months after COVID-19. What's a little bit more worrying or very worrying is that endothelial function is reduced after COVID-19 or SARS-CoV-2 infection in very healthy, very young adults.

Here, in this study, this was -- around about 20-year-olds was the average age. And this was three to four weeks after they've had SARS-CoV-2 infection. And these volunteers had minor or negligible symptoms. And we don't know, obviously, how long this dysfunction would continue. So, beyond endothelial function and flow-mediated sanitation, there's a huge body of work appreciating the role of the endothelium in COVID-19.

And the bigger area of the endothelium in that immune-type response with what happens to levels of cytokines and other inflammatory mediators in various cohorts. And I'm -- just alert you to have a look at this review I've put on this slide if you want to know more about that side. But for me and my expertise is at the level of vascular reactivity and these three mediators that I've explained: nitric oxide, endothelin-1, and prostacyclin.

So, in trying to work things out in this area, an obvious question is, what's the mechanism for how these mediators and how endothelial dysfunction might happen in COVID-19? And in understanding that, we might understand how it happens in other respiratory viral diseases. And this is a paper that we published when the pandemic was -- just started in 2020, and the labs were closed. And a very talented, early career PI in my group, Blerina Ahmetaj-Shala performed this study where she interrogated transcriptomic repositories including various human organs and human cells. And we looked for key entrance genes -- SARS-CoV-2 entrance genes to see how they were distributed across organ systems and cells.

And what we found in this dry lab project focusing on ACE2 and TMPRSS2. So, the last two bar charts, top and bottom on both squares, and focusing on those two because it turns out that those two are the key entrance pathways for SARS-CoV-2. What we found was that cardiovascular tissues expressed a respectable amount of ACE2, but not TMPRSS2. And that was also true for isolated cells.

This panel on the right-hand side, if, again, you look towards the end of that, on the top row, ACE2 is present in endothelial cells. That's the red dots, even if it's lower than nasal epithelium, which is the natural infective cell type for SARS-CoV-2. But TMPRSS2 is extremely low in those cells. And when the labs opened, we tested what we thought we knew from the transcriptomic analysis by infecting endothelial cells from three main sources: grown from the

human aorta, from the lung microcirculation, and from circulating stem cells.

And we infected them with live SARS-CoV-2 virus. And while the virus very clearly and happily infected airway epithelial cells, as you can see in the top middle line graph, it did not infect at all endothelial cells from three separate donors. Either from the aorta, from the lungs, or from the circulation. And this was true whether the endothelial cells were in a resting state or whether they were stimulated with IL-1β.

I'm sorry. I didn't realize I was being so late in time. But I will skip along quickly to say that what we think is happening then the endothelial cells are not permissive to SARS-CoV-2. But the increase in TNF and interferon that occurs in SARS-CoV-2 infection is driving a reduction in endothelial nitric oxide synthase and an increase in endotheli-1. I'm sorry, I've let myself run on. So, I'm going to skip through the other slides, which just show data that confirms that statement. And finish up with the summary of where we are now in our understanding of COVID-19 and the circulation.

Definitely, we have cardiovascular risk factors will predispose to severe or fatal disease. Cytokines, particularly TNF and interferon, are really important, we think, in this endothelial dysfunction. We think they're responsible for reducing eNOS and increasing endothelin-1. And there's plenty left to do in terms of integrating this information. And what we're planning on is to run a full study comparing those readouts and mediators with an -- a parallel influenza study.

And I'm just going to finish with a time. This was my future work from the Keystone meeting. But I'm also hoping perhaps that if I can be of any help to your community, perhaps I can contribute in some way to studying endothelial function in ME and CFS. Thank you. Hope I didn't overrun too much.

Vicky Whittemore: Thank you so much, Professor Mitchell. That was excellent overview. So, I'm going to address some of the questions that came in to the Q&A. And also, as I said, if any of the panelists would like to raise their hands and ask a question, that's fine as well. So, the first question is, the circulation treatment called enhanced external counterpulsation, EECP, was found to improve the endothelium. Has this been considered as a treatment for ME/CFS and long COVID? Are you aware of that?

Jane Mitchell: I'm so sorry. I've -- I'm really not aware of that. But I can -- if someone can pop it in the chat or send me the terms, I'll happily look it up. And send a response when I've had the chance to look.

Vicky Whittemore: All right. No problem.

David Systrom: Maybe I could quickly just address that.

Vicky Whittemore: Yeah. Sure.

David Systrom: EECP has been used in ME/CSF somewhat sporadically. We've gotten some interesting anecdotal reports back from patients. That after a treatment protocol that lasts for several weeks, it basically facilitates in a cardiac gated fashion venous blood flow and return to the heart. It's been used in heart failure. And in ME patients, it appears there's a subset who may benefit and have a lasting benefit for it. Maybe not forever, but more work needs to be done there and in long COVID, I think.

Vicky Whittemore: Great. Thank you. Thank you very much. Another question is the technology for measuring endothelial function that you described is from the 70s and 80s. Do you have a wish list or are there newer measurement technologies that could be innovated by engineers?

Jane Mitchell: Is that -- that's to me, Vicky?

Vicky Whittemore: Yeah.

Jane Mitchell: Yeah. Well, they were a golden age, the 70s and 80s, weren't they? But yes, there's -- there is a particular piece of kit called an endo-PAT apparatus, which David might be familiar with that clinicians often are there. And it's not super new, but it's an improvement in some way. It's more portable. It's a little bit more user friendly. And for the patient or the volunteer, the participant having their endothelial function measured -- and I've had mine done. It's not very onerous. It's not too tricky. But like all measures of endothelial function in vivo in people, they're all limited.

Because the endothelium is very complex. It's very heterogeneous. You can't really do a snapshot as such. But using endo-PAT or other similar approaches, older ones, there is a very convincing body of work that they're useful in as a biomarker or predicting response to drugs. I think there is a lot of potential to get a better -- to get better wearables that could measure endothelial function. I mean, what you get on your Fitbit now, I don't think it's that far away. Probably a little knowledge gap, but I think it's not beyond the realm.

Vicky Whittemore: Fantastic. Thank you. We have to move on in our agenda. But if you're able to answer some of the questions in the Q&A, that would be appreciated. Or we may come back to them at a later discussion. So, thank you very much. And, David, back to you to introduce the next speaker. Thank you.

David Systrom: Thanks, Vicky. So, it's my pleasure to introduce our next speaker, Resia Pretorius, who is a distinguished professor and the head of the Department of Physiologic Sciences and the Faculty of Science at the Stellenbosch University in South Africa.

Like Dr. Mitchell's talk, I heard Resia speak at the Keystone meeting last fall and was absolutely wowed by her work on micro clots in long COVID. And she's here to tell us about that and any potential overlap with ME/CFS. It is a fascinating area that, hopefully, will drive therapy one day. Dr. Pretorius.

Resia Pretorius: Thank you very much. And thank you very much, everyone, for the opportunity to talk to you. I just want to check, everyone can see my screen? I'm sure you can see my full screen. Okay. Perfect. So, hi, everyone. I'm going to talk this afternoon in South Africa. I know it's your morning in the U.S. -- on thrombotic endothelialitis and microclots in long COVID. And I am going to -- although focusing on acute COVID and long COVID, I will relate it to ME/CFS.

This is just a list of my very close collaborators and some of my students as well. Doug Kell, Amy Proal from Polybio Research Foundation in Harvard, Martin Kraeter from the Max Planck Institute, who kindly donated some of the instrumentation that they made specifically for blood flow and tests for microfluidics to us. And then Asad Khan, like many of you might know, and some of our clinical collaborators. And then a whole team of my postgraduate students.

So, my lab and our collaborators from various labs all over the world have been identifying inflammatory molecules and circulation that might be involved in and drive pathological clotting. And we have also focused our research endeavors on studying the effect of increased circulating inflammatory molecules. And how they interact with cells of the hematological system.

We focus in particular on platelets and red blood cells, as well as on the clotting protein, fibrinogen. We are also interested in identifying novel inflammatory molecules that might play a role in the persistent symptoms of long COVID and also play a role in ME/CFS. I'm particularly interested in platelet signaling and the role in abnormal clotting. Platelets in circulation play a critical role in healthy blood clotting, as we all know.

However, they can become overstimulated and can drive pathological blood clotting if there are inflammatory molecules in circulation. This can also happen in the presence of viral infections where platelets can act as important signaling entities. There is a complex relationship between the receptors on platelets and endothelial cells where circulating biomarkers may bind to.

As we now know, damaged endothelial cells, and also, so eloquently presented by Jane in the previous presentation, endothelial cells, as well as platelet hyperactivation are central in the pathologies in acute COVID and long COVID, and perhaps also in ME/CFS.

Our research group has shown it in acute COVID in various published papers. And if you just look at these two diagrams, you will see there are a plethora of places where inflammatory molecules can interact on platelets. But also, where platelets can then interact after these binding has taken place onto endothelial cells, which might drive damage.

In the context of COVID-19 and post-viral infections, platelets are therefore central in immune

activation and general coagulation pathology. And they also can form various complexes and obviously, also with themselves. This is then known as platelet clumping. We're also interested in pathological blood clotting involving the main clotting protein, fibrinogen, which we all know is a soluble protein in circulation if you are healthy.

If you focus your attention to the three cartoons shown here, on the left, cartoon A is a healthy protein structure. This is the biochemical structure of a protein. And a typical healthy protein structure will have many alpha coils and few beta sheets. However, in the presence of inflammation, oxidator stress, circulating biomarkers, including viral and bacterial molecules, inflammogens, these -- the structure will change. Where the inflammatory molecules, also, the spike protein, may bind to the fibrinogen as shown in cartoon B.

Such direct protein-protein interactions may cause the alpha coils to untwist into beta sheets as seen C -- in the cartoon on the far right. We, therefore, suggest that fibrinogen molecules then have a fibrinaloid structure -- fibrin amyloid structure. And we have shown this with various amyloid protein markers, including those that bind to other general amyloid-like proteins, like beta amyloid.

But please, do not -- don't get confused. Are we not saying that fibrinaloids are beta amyloid? It's a protein, but it's not the same thing. And the markers that we have used include thioflavin T as well as amytrackers. And we have been calling these fibrinaloid plasma proteins, microclots. Our research, therefore, focuses on the pathology of platelets and endothelial cell markers and micro clots. And ultimately, point to widespread vascular endothalmitis due to the interactions of the platelets and the microclots with the damaged endothelial layers.

As the focus of this talk is ME/CFS, but long COVID is very pertinent here, here's just some very worrying thoughts about estimates. We've been looking at Professor Daniel Altmann's, a collaborator of ours, data. And the worrying thing is that perhaps we have a conservative estimate of 5 percent suffering from long COVID.

And my specific concerns are that some of these patients may progress into ME/CFS. I'm also sure that most of you have seen the fantastic work of the Wüst group that's recently published in the recent Nature Communications paper. They found amyloid deposits in muscles using our methodologies. It's the green little dots that you see in the micrographs. And that they are called amyloid deposits.

If we agree that ME/CFS is also post-viral disease, perhaps there are lessons to be learned from acute and long COVID. And our research will be looking at both acute and long COVID, but also, at ME/CFS. So, I'll now just give a bit of background on our research endeavors since the start of the acute COVID pandemic. We have looked at blood clotting in acute COVID using the

scanning electron microscope. And this is what you see here, some of our micrographs.

We have found that platelets are damaged and clumped together and attached to red blood cells. The clotting proteins that are supposed to be soluble as previously mentioned, form spontaneous microclots. In the micrographs on the far left, you can see platelets that are hyper activated. And it seems as if they simply exploded. Note the yellow arrows.

In the middle are micrographs where platelets are attached to red blood cells. Note the white arrows. On the far right, you will see micrographs are spontaneously formed microclots that even glue together red blood cells. Note the blue arrows. And if you look at those red blood cells, they will definitely not be able to give over oxygen and transported properly to the tissues where it's needed.

Now, from the ultra-structural pathology, and with this in mind, we looked at platelet poor plasma and exposed it to our fluorescent marker, thioflavin T that I briefly mentioned earlier. Now, ThT binds to open hydrophobic areas in proteins that are damaged. And this marker was first used, as I briefly mentioned, to detect amyloid protein in brains of Alzheimer's disease patients. Although in 2014 already, our research group discovered that ThT can also bind to misfolded fibrinogen, which is also a protein.

And we can show this binding in pathological blood clotting situations. Here you can see the differences in platelet-poor plasma structure in acute COVID where we compare plasma smears from the acute patients in 2020, to healthy and also diabetic patients. And you can see microclot formation demarcated is the green signal from thioflavin T, our marker for misfolded protein.

We found small areas of nearly abnormal detected clotlets that are present in plasma of healthy individuals. And this can be seen in the micrographs on the left. Please note that you will always find some sort of misfolding in healthy individuals. We all have a level of inflammation, even if it's only due to aging.

So, you will always see that. And depending on the level of inflammatory markers, the level of gut dysbiosis, getting inflammatory molecules into your system, even in a healthy individual, there will be levels of microclot detection. But the abnormal microclots that we see in diabetes, we have found numerous times previously. But it was quite significant to us to see the extent of the abnormal misfolded proteins in acute COVID, as can be seen in the far right. And also, note the scale bar to look at the amount of clots and the size of the clots in the micrographs.

We also needed to quantify our platelets and microclots as viewed under a fluorescent microscope. Because as we know, unfortunately, microscopy is not a quantifiable -- easily quantifiable technology. So, it's more quantitative. So -- qualitative. Apologies. In our

experiments, we looked at hematocrit samples that we exposed to fluorescent platelet markers, CD62P, which is the pinkish signal.

And that is a marker for P-selectin. And P-selectin is an interesting molecule. It can be inside platelets when they are healthy. Or they can be on the membrane. And then there, they act as a molecule of attachment as a receptor. They act as a receptor. And it can also be soluble, where it can be in the plasma where one can -- we measure it using ELISAs. And then we use PAC-1, which is the greenish signal that identifies platelets through the marking of glycoprotein IIb/IIIa, which are only found on platelet membranes.

So, as I mentioned, as microscopy results are quite difficult to quantify, we suggested a microclot and platelet grading system that we published a few years ago. And here are examples of our platelet grading system. We look at spreading and clumping, where one and two will be found in healthy individuals. And obviously, in clumping -- in a clumping grading scale, you will not expect to find any clumps in a healthy individual that would not have platelet pathology.

And then it progresses to a four, where you find significant platelet clumps, and clumping as well as spreading. And here is just our microclot grading system, one and two demarcates probably healthy individuals progressing into COVID and acute, and as well as long COVID. And you can also see just a -- in the micrograph is just no fluorescent signal on the sample. Where you can actually also see, with just normal lights function, microclots in the sample. And then the overlay, you could see in C.

Now, platelets in long COVID as I mentioned, is, we progressed to that. We looked at that. The samples that I showed previously were from the acute COVID samples. And probably, not to our surprise we were -- we thought we were going to see that. We also saw significant platelet hyperactivation and platelet clumping. And also, note the scale. And here's just an example of a really bad long COVID patient, where the whole slide platelets were just absolutely hyperactivated, clumping together, and significantly diseased platelets noted.

We also, again, turned our attention to our scanning electron microscope to look at the ultra-structure of long COVID. The previous ones were acute COVID. It's just an example of how a typical platelet would look like. And then on the right, a few platelets just clumping together and just being activated and hyperactivated. And you can imagine that these platelets can just go and plug onto damaged endothelial cells.

Here are examples of our red blood cells in a healthy individual. Nice red blood cell that can carry oxygen well. And in long COVID, we found these microclots gluing together the red blood cells. And we can imagine that those red blood cells won't be able to flow properly. They won't be able to deform properly. And they will most definitely not be able to carry oxygen into

the the tissue via the endothelial layers. And here's just the close up.

And here, you can even see the little breakage in the actual membrane of the red blood cell. And note the scale bar as well with these numerous microclots just forming these courses and course layers over the red blood cell membrane. And this is an example of a microclot inside a whole blood sample of a long COVID patient.

Now, the question that's -- that I'm always asked is, so what? Do we see microclots in other diseases? And the answer is, yes, we do. We see it. We have personally seen it and published on it in lupus samples, rheumatoid arthritis, and Alzheimer's disease. But what is very important to note is that not necessarily the presence or the absence, or even the size or the numbers of microclots, but the content. So, that's, I think, the most important thing for us to remember.

And interestingly, we have looked at spike protein. And we've actually taken the spike protein, the S1, and we added it to healthy blood samples. And we could induce fibrinolytic resistant microclots as well as platelet hyperactivation. And we published that a few years ago -- two years ago. Where the spike protein actually interacts -- direct protein-protein interactions with the fibrinogen causing the microclot formation as well as interacting with platelets causing them to hyperactivate.

We also used scanning electron microscopy where the addition of spike protein caused numerous clot formations. And if you look at the micrograph on the right, you could see our healthy sample, a COVID sample, and the healthy sample at the top. And where we expose it to spike protein, where we found numerous clots in a microfluidics system. We are going to explore this more with the Max Planck's system that is being delivered to us last year from Martin Kraeter's group.

So, we also looked at, are there differences between Omicron and the beta and alpha variants? Yes, there are. We also know that the Omicron variant was not so significantly damaging to patients. However, still microclot formation. And interestingly, in patients having Omicron going into long COVID, we still find numerous microclots that we -- that's very much comparable to beta and alpha. Lots of platelet hyperactivation in Omicron as well. Now, with all the morphology behind us, we turn our attention to proteomics. So, we planned an experiment. We looked at proteomics of a healthy -- of healthy plasma, type 2 diabetes, acute and long COVID. And we also added, obviously, diabetes because we know people suffering from diabetes, they're prone to severe, acute COVID.

So, for our proteomics analysis, we prepared platelet poor plasma from citrated blood, and we followed standard procedures. We have -- we trypsinized -- to break up -- trypsin is the enzyme. It breaks up proteins and that's what you use for mass spectrometry. To our surprise, we found a

visible deposit still in the acute COVID samples and the long COVID, but not in the diabetes samples and also not in the controlled samples. Plasma protein is, therefore, were fully digested in controls and diabetes suggesting that the trypsin could degrade all of those plasma proteins, but not those found in acute and long COVID.

And this is just a simple diagram to show we took this first trypsin digestion samples and we rushed to our lab to look at the clot structure. No clots were present in plasma for controls and the diabetes, but still all the clots were still present in acute COVID and long COVID. Then we developed another methodology together with our biochemist collaborator. We did a second trypsin digestion step where they could digest all the samples.

And then we ran our mass spectrometry for proteomics, and we detected very inflammatory molecules. That was substantially increased inside the digestive microclots of the acute and the long COVID patients versus the equivalent volume of fully digested field of the controlled samples. A particular interest was alpha2-antiplasmin and the various fibrinogen chain, which you would expect because it's clots and fibrin. And also, lots of molecules that you would see as an inflammatory molecule, von Willebrand Factor. We found numerous C-reactive protein molecules. We found complements, plasminogen, really a mix of inflammatory molecules entrapped inside the microclots.

Now, here is just a basic diagram of the clotting cascade. I'm not going to go into detail. But just to show you where alpha2-antiplasmin and why we were so happy to find that molecule in our analysis. So, this is the clotting cascade, the intrinsic and the extrinsic pathway. But the clots might break down. It needs to break down as well. However, if alpha2-antiplasmin is involved and present, it means that it blocks the process of lytic enzyme formation and breakdown of clots.

So, that gave us a good reason to suspect that there is a failed fibrolytic system in long COVID, as well as in acute COVIDs that we looked at. We repeated our study in 2022. That was published in 2022. It's a much larger sample size. And we found exactly the same molecules that we found in the first study, but we also found a little bit more.

Now, the question is, what can we do about it for patients? These that I've just mentioned are all research type methodologies. So, are there a place for flow cytometry methods? And we think there is. Last year, we were very lucky to get imaging flow cytometer funded by Balvi Research Foundation, Polybio Research Foundation and many, many patients, and ME/CFS patients as well. It had -- we had a crowd funding initiative via KERNLS, and we've got our instrument in our lab. And we did find significant microclot differences in controls, numbers and sizes compared to healthy individuals.

And here, you could see some of our results. It was recently published in Heliyon. But this still is just as numbers and size type of analysis. We need to determine what's inside the clots. That's the important thing.

So, just briefly, we looked at this, I must say, is together with -- our clinical collabators are not a research clinician. So, we just looked at could -- is there a place for treatment of microclots and platelet hyperactivation in long COVID patients? And indeed, we found that anticoagulation made a significant difference in the patient's symptoms that they self-reported. And in fact, 80 percent of patients felt that they were much better. And when we looked at these patients -- and I thought that in this PGIC score, we found that if they say they were better, then -- as we didn't find microclots and platelet hyperactivation anymore, if the 20 percent -- in the 20 percent where they said they weren't better, still lots of microclots and hyperactivation of platelets as well.

So, we think what's happening in here is that those patients might be those that have gone into autoimmunity or immune responses, which is very troubling for us, indeed. We also have been playing around with other types of instrumentations. We got a metasystems automated system funded by Balvi Research Foundation where you could just put in your slide under the microscope, and it does all your analysis for you automatically. So, it prevents cherry-picking from a research point of view.

So, just to come to a final conclusion about microclot presence and how it's involved as we see it. We know that these fibrinaloid microclots are widely present in chronic, inflammatory diseases. That's not a problem for us because the issue is not the presence, but the content. And we know that we can stain them with flurogenic dyes such as Thioflavin T. We have seen that they are resistant to normal processes of fibrinolysis. We know it can induce it by adding various inflammatory molecules including spike, but also estrogen, RPS, and lipoteichoic acid from bacteria. And we know that they can exhibit structure and even spectral heterogeneity, reflecting the molecules that are bound to them and are entrapped in them.

And with that, it's not just simply size and numbers. So, there is not an argument to say they are presenting any other disease or inflammatory disease, too. We need to look at the content. We need to look at the activity. We need to look at the biochemical characteristics. Obviously, we can't ignore hyperactivated platelets. These two pathologies go hand in hand, and they definitely drive thrombotic endothelialitis from our point of view.

But actually, back to ME/CFS, we published a paper a year or two ago where -- in 2022 where we showed that, indeed, in ME/CFS, we also have microclot formation. And we also have significant platelet hyperactivation. Platelet hyperactivation might be similar to that in long COVID and microclots less then in long COVID and acute COVID, but that's it. This patient population that were pure ME/CFS patients, they were not exposed to acute COVID or have had

it, or any exposure. So, we were very lucky to have this. The picture will definitely change if ME/CFS patients have been exposed to acute COVID, I think.

So, some of the things that we have been doing, we've been looking at endothelial debris in platelet poor plasma in ME/CFS and long COVID. This is Polybio funded. This is an endothelial marker for E-selectin. And you could see this whole, long string that was put in the two micrographs because one view on the microscope was not enough to capture this long string.

But -- so, it seems to us is as if the endothelial layers are sloughing off, just coming off it from the insides of the vasculature and it's in circulation. And we think many of these endothelial E-selectin, which is a marker, can also be entrapped inside the microclots. And here, you could see, we actually find the brighter bits in a microclot. And this is our next project where we will be publishing some of our data.

So, the next steps at the last slide are commercial endeavors. We need to have imaging flow cytometry. And perhaps, microclot microscopy testing done more widely, and we take that into pathology labs. We need, as researchers, to look at ranges of microclot presence, also for other diseases. But we need to find molecules that are disease specific that we could use as markers in our flow cytometry and perhaps -- not imaging flow cytometry. I'm talking about normal flow cytometry.

We're finishing off another proteomic study with really interesting results. And we are definitely looking for endothelial debris markers and even biofilm markers. Luckily, there are a few trials that we think, hopefully, this year will be the year in the U.S. and the U.K. for anticoagulation, and we proceed with a lot of collaborator grant applications. So, with that, it's an open invitation for anyone that wants to join us. Please get into contact. Thank you very much.

David Systrom: Thank you so much, Resia. Vicky, we're doing questions?

Vicky Whittemore: Yes, we are. Sure. So, there's quite a number of really excellent questions for you. Let me go to the questions. So, while I'm pulling those up, just a question, have you evaluated in long COVID or ME/CFS the microclotting and seen differences between male and female? You mentioned estrogen. And so, I was interested is -- if you see differences based on sex?

Resia Pretorius: We have not done that by ourselves. But I was just in a meeting a few days ago where one of our collaborators in the U.K. actually is looking at that and she's been finding differences, so more microclots in females. So, it seems as if estrogen is playing a role. And we know estrogen is a significant player -- role player in long COVID. Some people complain about very increased blood flow during menstruation and all sorts of things associated with

estrogen.

So, definitely, I think these are -- there is, definitely, something to look at. And we do know that estrogen can cause abnormal clotting. And looking at people taking the pill, and in hormone replacement treatments in older females. We know some of them are more prone to stroke due to taking of these medications, so definitely something to look into.

Vicky Whittemore: Sort of a follow-up question is, have you looked at changes over time, so with length of disease? You know, particularly in long COVID or, you know, in your ME/CFS study, how long have those individuals been ill with -- or have -- since their diagnosis of ME/CFS?

Resia Pretorius: So, we haven't -- in ME/CFS, we haven't looked over time because it's such a new endeavor for us. But we have been following a few long COVID patients that have developed long COVID, or we actually have got from the acute phase, we -- and into long COVID and then over the progression of the disease. So, we're actually following one of our patients for over three years now. And he's been on various treatment regimens. When he's more ill, the microclots and the platelet hyperactivation are more prevalent.

If they -- when we followed patients during treatment with clinician-initiated coagulation treatments, if they feel better, the clots disappear. If they don't feel better, the clots and the platelets are still there. And we followed some of them over a nine-month period. We haven't published that yet.

Vicky Whittemore: Yeah. That really ties into one of the questions about the functional consequences of the clots -- of the microclots in terms of symptomatology and how that correlates with feelings of wellness. And I was really intrigued by the finding of -- when treated with anticoagulants that there was a significant improvement in people's fatigue or feeling of fatigue that this slide you showed. That's very interesting.

Resia Pretorius: Absolutely. And I think something to take note is the Wüst paper that just was published last week in Nature Communications. They actually showed with a little bit of exercise, they found microclots or amyloid deposits using our ThT method inside the muscle. And that might be indicative of why you get pain and the muscle not being able to function properly. And perhaps, also tell us a little bit more about the endothelial permeability, which is adding a very important thing that we must look at. Because everything that is in circulation if the endothelial layers are so permeable, it can just travel into organ systems. And that's worrying.

Vicky Whittemore: Yeah.

David Systrom: Vicky, may I ask a very quick follow-up, please?

Vicky Whittemore: Yeah, sure. Yeah.

David Systrom: So, Resia, intriguing notion about the mechanical presence of the microclots and vascular dysfunction -- systemic vascular dysfunction that you just mentioned, and then the packets of proinflammatory cytokines that are buried, and you need to really use detergent to get them out. So, one could actually envision clinically using an anticoagulant, breaking up the clots and having a period of time of cytokine storm and release from the clots, but that's not what you're observing. Would that speak more to the mechanical presence rather than the cytokine storm?

Resia Pretorius: So, I can just mention what patients have been telling me anecdotally. When they go onto anticoagulants and some even go on to Nattokinase and Serrapeptase, which are enzymes, which are over-the-counter enzymes that break down clots, many of them use the term that they feel toxic for a few days. So that they actually feel as if they're back into acute phase COVID.

So, I have not seen anything like a cytokine storm reaction. But I've definitely seen patients really feeling ill before they're suddenly feeling better. And I think the important thing with the anticoagulation regimes together with the antiplatelets is if you could just get the platelets to calm down and if you can get the microclots to not cause havoc and entrap so many molecules -- inflammatory molecules, then you can have your endothelial layers, get them respond to the rest.

And I think the important thing is they need to -- the endothelial layers need to become healthy again. Then that's the point when the patient will be starting to feel better. It's not necessary that the microclots or the platelets that, I think, are the main culprits in their symptoms. It's the damaged endothelial cells. So, I think the anticoagulation regimes are just one of the things that one needs to address possibly in a subpopulation, perhaps, of patients.

Another thing is, definitely, the work of Akiko Iwasaki is to -- that they looked at the immune and autoimmunity. And we definitely need to look at the possibility that they might be a viral persistence or even RNA persistence. Some people have been thinking that is not to say virus that goes persistent, but RNA persistence. That's a great question to answer.

David Systrom: Incredible.

Vicky Whittemore: Yeah. Thank you very much. So, we'll come back to more of the questions later. But at this point, we'll take a break, and we'll reconvene at 12:35. Thank you.

Resia Pretorius: Thank you so much.

David Systrom: Thank you.

Vicky Whittemore: Okay, welcome back from the break. And over here, David, to introduce the next speaker.

David Systrom: Excellent. I see Linda on the screen, and I know Frans is in the vicinity. I have on my order Frans first. Is that not the case?

Linda van Campen: Well, the blood volume is first, and I will do that [laughs].

Vicky Whittemore: Linda is going first.

Linda van Campen: Yeah.

David Systrom: Okay. Linda is going first with cerebral blood flow?

Vicky Whittemore: Yeah, yeah.

David Systrom: Okay. Excellent.

Linda van Campen: No, with hypovolemia.

Vicky Whittemore: No, with hypovolemia.

David Systrom: Oh, okay. Let's go with hypovolemia.

Linda van Campen: Yeah. So, we tossed the coin, and I got this one.

David Systrom: No worries at all. So, I should say as a pulmonologist, it's doubly intimidating to have two cardiologists talking to us about this, but it is wonderful. And we thank you for staying up late in the Netherlands to talk to us. So, Linda van Campen is a cardiologist and a director at an independent treatment facility, the Stichting Cardiozorg in Hoofddorp, Netherlands. She and Dr. Visser, who will follow, have done phenomenal work in the area of hypovolemia and cerebral blood flow, utilizing such methods as the upright tilt table test and measurements of cerebral blood flow over the years.

It's fascinating to me, at least in my part of the United States, cardiology is not particularly

invested in these things. It's usually in the neurology camp. So, it's wonderful to have the subspecialty represented. I should also say I reached out to Peter Rowe in organizing this webinar. And it was a slam dunk. He sent me in your direction and Dr. Visser's. So, welcome, Dr. Van Campen, and we are looking forward to hearing about hypovolemia in ME.

Linda van Campen: Well, thank you, Dave, for the invite. We're very honored to have a part of this meeting. Let me see if I -- yeah. So, the outline I have is some requirements of standing. I will not talk a lot about orthostatic intolerance because that will be part of the presentation of Frans. I will also not, for the sake of time, talk about the regulations that come with blood volume, which impasses a lot of sympathetic, parasympathetic, tubular -- glomerular tubular feedback hormones, raw system, erythropoietin. It's too much for this presentation. I will shortly touch on the methods that have been used for blood volume measurements. And then I will give kind of a literature overview on what has been done in ME/CFS patients with blood volume.

So, standing successfully requires the interaction of a lot of factors compensating for gravitation. One of them is blood volume. There are a lot of physical factors involved, like musculature. There are a lot of neurological factors involved, autonomic nervous system neuropathy. Humoral factors involved plasma renin, angiotensin, aldosterone, erythropoietin, antidiuretic hormone, vascular factors, vascular tone, venous valve competence. Probably also endothelial have a lot of things to do with it.

And studies in this patient group have shown some conflicting results in blood volume changes. So, first, some methodologies, the gold standard, obviously, has been a double labeling of red cell mass and plasma volume. In Netherlands, it's not practiced anymore since, well, a decade, I think. There has been a comparison with the Daxor Corporation, Volumex, where they have one label and use the red cell mass with hematocrit measurements. There have been comments on the reliability of those measurements, which is not that great. But the comparison in 2007 between the two measurements was pretty good.

Some groups have used Evans blue. Indocyanine Green has been used. Carbon monoxide blood volume measurements have been used and cardiovascular MRI with ferumoxytol. How do those methods compare to each other? There's not been a great study on that. So, I don't know really.

What is important is that blood volume measurements in isolation can be difficult to interpret because what number say something. Clinicians don't want to know about the absolute number, but whether the result is abnormally high or abnormally low. As blood volume changes with size, lean versus fat body mass and gender, one number can be abnormally low in one patient and abnormally high in the other. So, also the relation between height and mass and blood volume is not linear, but curvilinear related.

So, that's why even in 1977, a method was made up for normal ranges, kind of quantifying what is normal. And they used the increments of eight persons as that was the standard error of blood volumes. So, within eight persons of predicted, it's normal. Minus 8 to minus 16, it's mild hypovolemia. Minus 16 to minus 24, moderate hypovolemia. Minus 24 to minus 32, severe

hypovolemia, and more than 32, extreme hypovolemia.

The question you can probably have with that is what -- how does that relate and translate to the clinical picture of the patient? Does it relate to complaints of orthostatic intolerance, and does it relate to exercise intolerance?

So, now, I have compiled the studies that have been done. The first one is from 1998 in -- from Streeten. They included CFS patients with NMH and delayed orthostatic hypotension. They found excessive blood pooling in lower limbs and subnormal venous constriction. They found a reduction of the red blood cell mass. They included through Fukuda criteria and they included a severity criteria where they had less than five hours being upright activities included. They used the gold standard, double isotopes, and they included 19 patients, five -- 15 females and four males.

So, they found that red blood cell mass was below the normal range in 14 or 15 female patients and two or four males. Plasma volume was less variable below and only below the normal range in 10. Abnormalities in blood volume were very common. In 16 of 19, there was a reduction in red blood cell mass. Eleven had low plasma volumes, and totally -- total circulating blood volume was subnormal in 12 of 17. Actually, the conclusion of this article was that, especially abnormalities were there in the red blood cell mass and that there were not very significant differences in blood volume and plasma volume.

The next one I'm addressing is from Jacob from 1997, a study exploring orthostatic intolerance patients and checking especially the RAAS system. They included chronic orthostatic intolerance patient and high norepinephrine levels, assuming that these chronic orthostatic intolerance patients probably will be chronic fatigue patients because of the long duration and because of the high percentage of post-viral illness. The study included 14 females and two males and had two matched controls. They used the Evans blue dye method for blood volume.

So, they followed a standard diet with sodium and potassium for a couple of days, and the balance in that diet was reached after three days. In this table, you see the difference in heart rate and blood pressures and what you see in the patients that they will have a POTS response going from 77 to almost 170, whereas in the normal subjects, that was not the case.

Obviously, there was a difference in norepinephrine as the inclusion was already high in norepinephrine level. So, nine patients in this group were hypovolemic, seven were normovolemic. The range was from minus 23 to plus 5 as you can -- especially, you see here that the plasma reading level was kind of different when there was a hypovolemic patient or a normovolemic patient. Wherein, the hypovolemic patient, the plasma renin level was extremely lower than in the normovolemic patients.

Next one is from 2000, also from Streeten. In this one, we included the same type of patients, but -- and we used 15 patients and 15 healthy controls and had a head tilt test withstanding up to 90 minutes, which in our opinion, is extremely long duration, again using dual isotope volume.

And in this study, he described and studied the effect of lower body compression with MAST, the military antishock trousers on hemodynamics and symptoms. This is the first one actually addressing symptoms. So, with the MAST inflation, six patients have had the symptoms completely disappearing and seven-day improved. And only one of this group had unchanged symptoms. When the MAST was deflated, all patients had, again, orthostatic symptoms. And obviously, none of the healthy volunteers had orthostatic symptoms.

Farquhar in 2002 tried to relate blood volume, stroke volume, and peak oxygen consumption as part of exercising tolerance in chronic fatigue syndrome. And his hypothesis was that volume status had a strong physiological correlation of exercise intolerance. Included with 17 patients and 17 controls, he used Evans blue dye with hematocrit measurements. And he used for cardiac output measurements the Fick methods.

In this figure to the left side, you see the peak oxygen consumption, which was significantly lower in patients than in controls. On the right, the blood volume with also a lower but broader range, and therefore, not completely significant, but maybe this blood volume was not corrected to the norm. So, blood volumes and plasma volumes are not significantly different in this group, although there was a significant trend. Peak oxygen consumption was significantly lower in the CFS group. And the fundamental relationship between blood volume and peak oxygen consumption was confirmed and extended not only in healthy controls, but also in CFS patients as is shown in this figure.

Raj in 2005 described a group of POTS patients. And in our opinion, a lot of POTS patients probably are CFS patients. He compared them to healthy controls and especially looked into the plasma renin and the aldosterone in plasma volume regulation, 15 patients, 14 healthy controls. Again, the same diet that was described earlier. This group used this Daxor Corporation blood volume measurements and they corrected it for the ideal weight.

To the left, you see a difference -- the response in heart rate, which obviously with standing is having way higher increase in the POTS group. Also, for the norepinephrine, which is kind of part of the study design. Aldosterone also increased in both, but in the POTS patients more. In renin, both increased, but there was no difference found.

So, when he looked into the differences in plasma blood volume and red cell volume, there was a highly significant decrease in all those three measurements. And this table is for your reference

where all the measurements and the percentages and the ideal volumes are there to check.

Hurwitz did a study in 2010 to look into different deficits in cardiac output and blood volume and linked to illness severity and sedentary lifestyle. He had 146 participants and divided them into CFS groups based on symptom severity data where they -- he had 30 severe, 26 non-severe, and two healthy control groups based on physical activity and sedentary group and a non-sedentary group. This group also used the gold standard of double isotopes and did the graded exercise test with aerobic capacity.

So, in this group, you see that only the total blood volume is significantly different, but plasma and renal blood cell volume are not. When you normalize it to the ideal, then all are significantly different. And that's why what -- the point I made in the beginning that you have to normalize it to age, gender because there's differences over time.

He checked cardiac output and he found differences between the four groups with the method he used. But then again, if he normalized those differences, all those cardiac output and index differences disappeared. So, obviously, the differences he saw in this table were there because of the differences in blood volume. When the blood volume was corrected, the differences in cardiac output disappeared.

Newton in 2016 studied cardiac volumes done by MRI, exploring the mechanism, 47 patients, 47 matched controls, used the standard double isotope, and did cardiac MRI for volumes and cardiac mass. In those patients, there was a relation with -- and diastolic wall mass and the -- and plasma volume, red cell volume, and total volume. In our data, by the way, we don't find lower blood -- lower cardiac volumes.

So, in this picture to the right, you see the relationship between plasma volume and the fatigue impact scale that they used. And you see that there is a relation. That the lower the volume, the higher the fatigue score. So, there's obviously a relation between blood volume and symptomatology. This is for your reference, the cardiac MRI figures on cardiac volumes.

So, then we end with our work where we, first of all, try to correlate blood volume in ME/CFS with presence or absence of orthostatic intolerance symptoms. We also did it with the double isotope gold standard. It was normalized to the ideal weight, and we had 12 ME/CFS participants, 11 females, one male. The total group had a mean of 59, plus or minus eight meals per kilo, and the reduction was minus 11.

In this picture, you see that when you divide, we had four patients with no orthostatic intolerance and eight with orthostatic intolerance. You see that there's a difference in the patients who have no and who have orthostatic tolerance that the patient with complaints have a significantly lower

blood volume. The range of the blood volume of the patients without orthostatic intolerance is nearly normal. And the same is here when it's in percentage. There is a difference when there are orthostatic intolerance complaints.

So, the next one, we -- besides orthostatic intolerance symptoms, we also looked at the maximal oxygen consumption on CPET. It's actually the same study group, but we took into account now the CPET information. Here, you can see that blood volume, RBC volume, and plasma volume in percent are similar for all, but there is a part of this group who is above 80, which is considered normal.

Here, you can see the -- actually similar to what you've seen in the last publication, but more subdivided into blood volume, RBC volume, and plasma volume as an absolute value. And then you see no difference in the plasma volume when it's normalized. The difference in plasma volume is there again. In this left-sided picture, you see that there is a correlation between the percent oxygen consumption and the present blood volume where the lower the blood volume, the lower the condition is the lower the oxygen consumption.

So, in conclusion, blood volume, plasma volume, and RBC mass are often decreased in patients with ME/CFS. Measurements are currently hardly ever done but can be informative or diagnostic and therapeutic purposes. Current measurement techniques are expensive, time-consuming, and the gold standard ones come with a high radiation exposure. Decreases in blood and plasma volume seem to be related to orthostatic intolerance symptoms and decreases in exercise capacity. And this or more studies focusing on these relations are actually needed. Thank you.

Vicky Whittemore: Thank you very much, Linda. So, have you or has anyone looked at changes in blood volume over time? So, compared or -- and/or compared to individuals who are, say, three to four years post-diagnosis of ME/CFS versus a longer length of illness?

Linda van Campen: We haven't done it. We were actually cut short doing this because of financial issues with health insurance. But that's a really interesting question. And maybe with retrospect, looking into the studies that have been done, the studies that I've shown, we can figure something out.

It's definitely in our experience that we have some -- and Frans will show one slide on that -- some patients who don't have orthostatic intolerance when they have -- when they come early in the disease will develop orthostatic intolerance when they become more severe. So, I can imagine that the four patients who were not having orthostatic intolerance, probably if we see them now, will have that and will have a decreasing blood volume.

David Systrom: Vicky, a quick follow-up.

Vicky Whittemore: Yeah, yeah. Sure.

David Systrom: Linda, I'm aware of some data that suggests that the perennial confounding variable and the deconditioning can in itself lead to hypovolemia. So, is there a way to sort that one out here? And maybe the other follow-up, the Holy Grail would be what is the cause of the hypovolemia, the root cause?

Resia Pretorius: Well, the first is, for me, a nice one because I like talking about deconditioning, especially kicking it. We did a manuscript -- and I will send it to you, David -- on looking into actually the cerebral blood flow abnormalities that come with orthostatic intolerance and CPET outcomes where Parsaik in neurology, 2012, are, yeah, series coming into. Parsaik from Mayo in 2012 defined deconditioning as below 80 percent of VO2 max. And below 65, it's severe deconditioning.

In the group we did, there are patients who have a normal percent VO2 max as defined by the CPET who do have abnormal cerebral blood flow. And by that account will have abnormal blood volume. So, there's no relation in our opinion or my opinion that deconditioning has something to do with blood volume.

And to the other one -- well, there's -- I first made a complete overview with all the factors playing a role in blood volume things. And it has to do with autonomic function. It has to do with the glomerular tubular feedback with natriuresis probably in ME/CFS, which is a brain disease. It has something to do with the regulatory things going into the brainstem with the hypothalamus, with the autonomic nervous system, something like that, I would say.

David Systrom: So, the root cause may be heterogeneous and therefore, potential treatments beyond volume loading may be targeted potentially in the --

Linda van Campen: Yeah, yeah, yeah.

Vicky Whittemore: Yeah. So, I don't see any other questions. David, do you have any other questions, or should we move on to Frans?

David Systrom: I guess we could move onto Frans. And then potentially, there would be some overlap questions there.

Vicky Whittemore: Yeah. Okay.

David Systrom: All right. Lovely. So, my pleasure also to introduce Dr. Frans Visser, who is a Dutch cardiologist and researcher. He did his cardiology training at the VU University in Amsterdam. And he, too, has focused over the past 15 years and done a phenomenal work at an independent treatment facility, the Stichting Cardiozorg facility in Hoofddorp, Netherlands. He and Linda have put on the map a huge amount of meaningful data that linked systemic vascular changes, including the upright tilt table test to cerebral blood flow. So, Dr. Visser, our pleasure to welcome you.

Frans Visser: Well, thank you. The interesting thing is that the first patients mentioned that she had a lot of orthostatic intolerance problems, and at least in our patient population, it's a very, very common abnormality.

So, I'm going to talk what exactly is orthostatic intolerance, signs and symptoms. And the core thing is the cerebral blood flow measurements. And finally, I'll tell something about the -- also the systemic circulation abnormalities. So, the orthostatic intolerance is a term, which is -- refers to a group of clinical conditions. It's not only ME/CFS, but the core thing is that symptoms worsen with an upright posture and many but not all are improved upon lying down.

For example, fatigue and brain fog can persist long after assuming a recumbent position. I'll come to that later. The symptomatology of orthostatic intolerance is highly variable. The most prominent is, of course, dizziness, lightheadedness, syncope, diminished concentration, headache, blurred vision, fatigue, et cetera. These are all symptoms which are also present in ME/CFS patients addressed that are more pronounced while standing, sitting, or walking, et cetera.

To demonstrate the highly variable symptomatology, we administered the questionnaire to patients -- ME/CFS patients the first minute and the 10 minutes of the tilt test and compared that with 50 or 40 healthy controls. Also, in healthy controls, immediately after the upright position, there is some mainly dizziness, but it disappears over the minutes after they had the upright posture. But on the left side in dark blue, you see the prevalence of the orthostatic symptoms immediately after standing up and after 10 minutes.

And if you compare this symptomatology with the healthy controls, there's a huge difference in in the patient population. Again, indicating or suggesting that the orthostatic intolerance symptom methodology is highly different between patients. There are also -- except for the symptomatology, there are also objective signs of orthostatic intolerance. And the most famous one, I may say, is, of course, pulse in which the heart rate increases substantially during tilt testing. This is a patient with a heart rate of 110 going up to 180. That's an exception, but it occurs.

The other one is the orthostatic hypotension, stable heart rate. The red one is the systolic blood pressure. The black one is the diastolic blood pressure. And this particular patient after initiation of the tilt had a gradual decrease of the systolic blood pressure while the diastolic blood pressure increases, but that's leading -- then we stopped here because we were afraid of syncope. But if you continue this -- to tilt these particular patients, then they may have syncope, which is also very clear saying this is a patient, who during the tilt test, suddenly here developed symptoms, but he has suddenly -- the blood pressure drop was enormous, and she fainted.

These are the well-known objective signs of the orthostatic intolerance. Another one, which is overlooked, and you have to look at the hands and the feet of these patients if they have blue feet. It's still completely an unknown factor why some patient derived blue feet and other not. I really don't know, but it's -- if you -- it's there and then you're sure that they have orthostatic intolerance.

And the cause of the orthostatic intolerance is reflected here. This is a slide from the syncope guidelines 2009. And centrally here is that there is a low blood pressure or a global hypoperfusion. And this is the main business of the orthostatic intolerance. So, reduction of the blood flow to the brain with oxygen and nutrient reduction, they give dizziness, light headedness, headaches, vision problems, memory problems, fatigue, syncope, et cetera. But also, another part of the orthostatic intolerance symptoms is related to the activation of the stress system, palpitations, chest discomfort, diaphoresis, et cetera.

So, it's two parts. It's the cerebral blood flow reduction. That's one, and secondary, the activation of the stress system. So, what is the cause of the reduction of blood flow to the brain? First of all, the most important thing, I think, is gravity. Reduced blood flow may play a role. But the extent of the reduced blood flow is, as far as I'm concerned, not completely clear. And there is also a decreased oxygen extraction.

So, what happens is when you're lying down and the heart -- the circulation contains five liters of blood, which is, of course, circulated by the heart when patients lie down, it's completely normal. Interestingly, up to 700 and even to 1 liter may be pulled by the gravity to the legs, which is, I think, an enormous amount. But then healthy volunteers, this gravity effect is counterbalanced by arteries and veins. But in ME/CFS patients, less blood returns to the heart, less blood is then pumped out to the heart and enters the brain.

The reason why patients with ME/CFS have a less than optimal venous return of clot is not completely understood, but it's a very clear thing. And one of the important things is that we were able to demonstrate that the reduced venous return of the blood to the heart in ME/CFS patients versus healthy controls was largely different. This is here in the bars in white are indicated the changes in cardiac index, which is a normalization of the cardiac output. And it's doubled compared to the reduction in healthy controls.

So, also in healthy controls, there's the reduction of cardiac index while standing. But again, that's completely optimized by the cerebral circulation -- by the cerebral autoregulation. While this is what Linda already told, there is a reduction in blood volume. And mainly in patients with orthostatic intolerance, blood volume, erythrocyte volume, and plasma volume are all increased.

And the interesting and fascinating thing is that also that there are patients in which the oxygen

extraction is less optimal. And this in brown here, on the right side, the right-side column are those patients who have a very high with an elevated cardiac output versus the oxygen consumption, but they have the lowest failure to predict peak VO2. And -- well, I think that we can now -- we -- I did have a question mark. I think that the mitochondrial problems are a clear problem in the ME/CFS population.

Now, how do you measure the orthostatic intolerance? Simply by inducing the gravitational effect. There are three common tests -- is the tilt test. It can be performed at 70 degrees, but also at 20 degrees. You can -- you have passive standing test while you lean against the wall. And in active standing test where you stand without support, heart rate and blood pressure measurements are always available.

But you can also include cardiac output, EEG, transcranial Doppler, extracranial Doppler, CO2 expiration, oxygen saturation, whatever you want if you want to explore that this phenomenon more than you add this sort of a test. This one is the same slide we have seen before. We have patients with POTS with increase in heart rate and patients with orthostatic hypotension with a decrease of the blood pressure, syncope patients. But the most interesting -- intriguing part are those patients with a normal heart rate and normal blood pressure, but with orthostatic intolerance symptoms.

And here -- so, you can't dismiss these sort of patients stating that they have -- there is nothing wrong with them. They had orthostatic intolerance. And here, I think comes the use of -- and the availability of the measurements by either transcranial Doppler on the left or by extracranial Doppler. And the principle is that you measure near the two sides' movement in the blood vessel and -- which translates to the velocity.

The transcranial Doppler can interrogate the internal carotid arteries and they can also look at the virtual -- or the basilar arteries. The major disadvantage of this technique is that you can't measure changes in volume of the vessel, and that changes everything. And that's the major advantage of the extracranial Doppler where you interrogate the carotid arteries, and on the backsides, the vertebral arteries.

And what we do is we measure -- in all four arteries, we measure flow and measure the diameter leading to cerebral blood flow. And this is one example, you have a patient here. He -- on the top are the vessels visible. And on the bottom side is the flow velocity profiles of these patients. Well, you don't have to be a rocket scientist to see that the flow velocity in this particular patient is the cardiac cycle. That decreases substantially in patients while standing. And -- but we measure also the diameters and come to the measurement of cerebral blood flow.

And this was our initial publication in the ME/CFS populations group. On average in healthy

controls, the reduction in cerebral blood flow was 7 percent. So, that's not relatively a mild fever. In old patients, the reduction of cerebral blood flow was 26 percent and there was a slight difference between the patients who had a normal heart rate and blood pressure, patients with POTS and orthostatic hypotension. And these reductions are highly significantly different from the values of the healthy controls.

The prevalence is still an issue, shown in this slide from Peter Rowe. Patients had orthostatic intolerance in, well, approximately 42 percent. And in contrast in our population if we look at the data using a normal blood flow value of 14 percent, 90 percent of the ME/CFS patient have an abnormal cerebral flow during the tilt test. In POTS patients, all patients who had cerebral blood flow abnormalities and in patients with orthostatic hypotension, 80 -- at 98 percent. So, if you have POTS or orthostatic hypotension, then you're sure that there is an abnormal blood flow.

But the interesting thing is that in 82 percent of the patients with a seemingly normal test, a normal heart rate and blood pressure, there was also a significant reduction of cerebral blood flow. We've heard that the study in severe ME/CFS patients and even a 20-degree tilt, which is really a very mild tilt protocol, there is a significant reduction in the cerebral blood flow while being tilted to 20 degrees.

And interestingly enough, also in these particular patients if you do a sitting test, while patients were first -- while they're in supine position measured cerebral blood flow and then were seated for 10 minutes, then you see in -- particularly in the patients with an orthostatic intolerance complaints, they have a massive reduction. So, 20 degrees and even sitting may provoke the orthostatic intolerance in ME/CFS patients.

And this is also a very intriguing slide that if you look at the reduction in cerebral blood flow after they have been tilted and brought back to the supine position that in patients with especially mild and moderate disease, 10 minutes after the lying down, and again, there is still an abnormality of the cerebral blood flow. And the minus seven is in the normal range in patients with mild disease. But in severe disease, there is a consistent and persistent abnormality in cerebral blood flow.

How long it will take for return to normal is there's -- actually unknown. We talked about the cerebral blood flow, and we talked about the orthostatic intolerance. And in our first publication, we also showed that there was a clear relation between the percent CBF reduction and the number of symptoms they have during tilt. So, if you have a minor reduction in cerebral blood flow, symptomatology is less than in patients compared to patients with a severe CBF reduction.

And we were further interested in how does this cerebral blood flow reduction translate into practice. We were able to demonstrate that in patients without and with fibromyalgia after the

tilt test, there is a decrease in the pain threshold indicating that the pain sensation is increased in these patients. And in healthy controls, nothing really is different before and after the tilt. And you can imagine that in patients with fibromyalgia, the pain intensity perception is earlier coming up and is more extensive than a patient without fibromyalgia.

Also, the -- we did look at the memory and concentration problems before and after the tilt test in red, the two back test and in dark red, the three-back test. It's all relying on the fact that you have to memorize two or three images before what -- if that was the same picture presented on the screen. And you can see that even 10 minutes after the tilt, the memory and concentration is decreased more of less correct answers. In -- after the tilt test and the time for patients to give an answer, whether good or bad is also increased.

So, this really demonstrates that you can really measure also the memory and concentration problems. And also, the orthostatic stress test itself may result in the post-exertional malaise as shown here. This was at 10 minutes before the test. And the second one is after the test. And you see there, over time, there is an increase in pain. And for example, in the fatigue after 48 hours, there is a reduction to normal.

But again, if you look at the data of the healthy controls here given in blue, there's a way -- there's a big difference between patients and the healthy controls. And -- yeah, of course, in patients with fibromyalgia, they report a longer and more severe pain after the tilt test and also before the tilt test. To further objectify the relation between the orthostatic intolerance and the cerebral blood flow is shown here in the study in which we get our patients to a short tilt test and perform a stocking off and stocking on procedure.

And here, for example, is shown a patient in which is stocking off, a clear reduction in the cerebral blood flow from 240 to 160 mls. And then if we applied the stockings, then they improved. They do not return to normal, but it's clear that you can do something about the cerebral blood flow. And these patients also show intolerance -- orthostatic intolerance improvement.

And finally, this is a very recent and interesting slide showing that in which patients were extracted from my database who came back. And on the left side are the patients who did not have a change in symptomatology, and on the right side, the patient who had worsening of their ME severity. And in these patients, you'll see that there is an enormous decline in the cerebral blood flow abnormalities compared to the patients who had no signs or symptoms of severity improvement.

The last slides are related to a new phenomenon as far as I'm concerned. And that's what we call orthostatic chronotropic incompetence. Here on the left slides are shown the data of the healthy

controls. And also in healthy controls, there is a relation between the decrease in stroke volume and the increase in heart rate. And the green lines here are the 95 percent competence -- sorry, prediction intervals.

So, it makes sense if the reduction in stroke volume is less than the heart rate increase is also less. And here on the right side are the other patients. And the two green lines -- the blue lines and the green lines are, again, are from the healthy controls. But you can see here that a vast majority, 37 percent of patients had a heart rate increase, which was lower than expected based on the findings with healthy controls. And it's -- and these patients, probably the increase in heart rate is less than of the healthy controls for a given reduction in the stroke volume.

And in these patients, probably you have a different therapeutic approach needed compared to the patients who remained in the normal range. So, that's the reason why we can only speculate why these patients do not increase in heart rate for a given stroke volume. And the last slide -- the last two slides are the presence of a cerebral autoregulation ME/CFS patients. This is a compiled review of five studies in patients in the healthy controls.

And for a 10 percent -- for a 30 percent decrease in cardiac output, there's a 10 percent decrease in the cerebral blood flow. That's the normal situation. But here are the most recent slides. We looked at patients in blue, the healthy in black, the healthy controls in blue. The patients -- 10 percent of the patients, the output -- cardiac output and cerebral blood flow reduction is maintained as -- in the range of the study of Ming, et al.

But in 90 percent of the patients here indicated in red, there is almost 1-to-1 reduction in cardiac output versus reduction in cerebral blood flow. So, in these patients, the cerebral autoregulation is completely disturbed. And the reason for this disturbed mechanism is at this moment, actually, unknown. So, conclusions. Orthostatic intolerance complaints are highly diverse. They are real, that's important, and also important that some of the complaints can be objectified. The orthostatic intolerance complaints and the orthostatic intolerance signs like POTS and hypotension are very prevalent in ME/CFS patients. And importantly, not only a 70-degree tilt or standing but also while sitting, and a mild orthostatic read results in an abnormal decrease of cerebral blood flow.

In patients without POTS or orthostatic hypertension, this abnormal cerebral blood flow decrease was also present in 82 percent of the patients, and there is a relation between the complaints and the cerebral blood flow decrease. And finally, as we recently have found out, there are signs of abnormal cerebral autoregulation. Thank you for your attention.

Vicky Whittemore: Thank you very much for the excellent presentation. So, I have a quick question about the slides you showed with the test one, test two, with people who you retested, or

I guess -- so, what was the length of time between the two tests?

Frans Visser: Two years.

Vicky Whittemore: Two years?

Frans Visser: Yeah.

Vicky Whittemore: Very interesting.

Frans Visser: So, these patients came back. And if a patient comes back and says that he had more complaints, then we do this retest. And the other group of patients were patients who were restudied, mainly because of conflicts with the authorities, and they want to demonstrate that the abnormality is real, et cetera. So, it's a selection of patients, but I think the message is clear.

Vicky Whittemore: No. Absolutely. Yeah. So, Gwynn, I know you had a question.

Gwynn Dujardin: Thank you, Vicky. Can you hear me?

Vicky Whittemore: Yes, we can.

Frans Visser: Yeah.

Gwynn Dujardin: Thank you for this wonderfully validating presentation. I have a question from the patient's concerns. What do we know about the long-term impact of reduced cerebral blood flow on general health and condition and or the progress of disease? Thank you.

Frans Visser: Yeah. That's a very important question. We do show -- a patient shows cerebral blood flow abnormalities. Some patients decrease, but that's a subgroup. We really don't know if you have a real continuous result of repeated cerebral blood flow reduction in daily life, which happens, of course. If that in the long term will result in a further deterioration of the clinical condition. We think so, but it remains to be proven.

Linda van Campen: I like to think that when the brain gets into trouble too much, it will make you crash and protect the brain by doing that. And only allowing you to go out being upright again when there's some reparation of the brain.

David Systrom: Vicky, a quick one?

Vicky Whittemore: Yeah. No. Yeah. Go ahead.

David Systrom: Frans, I think you've made a case, maybe, to paraphrase loosely here, that the final common pathway for orthostatic intolerance is really this reduction in cerebral blood flow, irrespective of the original pathophysiology. I'm aware that some neurologists are using specific calcium channel blockers to mitigate cerebral vasospasm, things like amlodipine. I'm wondering if you've had any experience with the direct treatment of cerebral vasospasm.

Frans Visser: No, we don't have it because the pessimist usually occurs in the setting of bleeding. And we didn't see any facial spasms while lying down.

Linda van Campen: Well, maybe in migraines, but maybe we have some patients on amlodipine but that doesn't do much for the orthostatic intolerance.

David Systrom: So, I think one school of thought is that the hypercapnia that sometimes seems to be associated with the upright condition and orthostatic intolerance might be treated with a calcium channel blocker.

Frans Visser: Well, of course, CO2 is an important issue in this final analysis. There is some role for the entire CO2, with a low CO2 leading to a further reduction of cerebral blood flow. But in the end, I think the contribution of cardiac output, so, the heart is more important than the CO2 changes. And it also depends on the CO2 literature. There's a division of abnormal being below 30mm of mercury and above is normal. But if you have a patient who is going from 31 to 29 is an abnormal reaction and the CO2 doesn't change. So, we're now looking at the magnitude of the CO2 changes, how that correlates with the cerebral blood flow reductions.

Linda van Campen: Actually, the hypercapnia is a functional reaction. Well, it doesn't help in the end, but the idea is that the flow through the arteries is lower. And I like to explain to patients that the vessels don't like that and they like to speed up the velocity of the blood flow. And for that, you need vasoconstriction, and for that, you need hypercapnia. So, it's kind of a functional tryout of the ME brain to fix the problem. But in the end, it doesn't fix it at all.

Vicky Whittemore: Quick question before we go to break. Have you -- in your studies, have you looked at any correlation between cerebral blood flow changes and sleep disturbance in individuals with ME/CFS?

Frans Visser: That's a good question. No, we didn't look at it until now, but that's a good one.

Vicky Whittemore: Yeah, okay. Thank you.

Frans Visser: The point is, of course, that when patients lie down and go to sleep, the cerebral

blood flow is presumed to be normal. Maybe it isn't but presumed to be normal. And I think that -- but it's a good thing to look at.

Vicky Whittemore: Interesting question. All right. Thank you. Thank you both very much for excellent talks. We'll take a 10-minute break and come back at 1:45 for the next talk. Thank you. Okay. Welcome back from the break. And over to you, David, to introduce the next speaker.

David Systrom: All right. Thank you very much, Vicky. So, the penultimate speaker is Jiandi Wan, who's an associate professor of chemical engineering at UC Davis. I have been so impressed by his bibliography. He does it all. He studies dynamics of multiphase flow, but he does so in material, he does it in tissue engineering. And then perhaps closest to what we're discussing today, he does so also in the vascular circulation. And he's described some incredibly interesting findings that might beget further study and treatment trials about the interaction of the red blood cell and the vascular endothelium in terms of shear viscosity and interactions with ATP release. So, I am so delighted to introduce Dr. Wan today and to hear what he has to tell us.

Jiandi Wan: Thank you, David. So, my talk today was actually trying to introduce what we recently found out a new blood test for red cells. The new approach we developed is trying to see red blood cells' capillary velocity and how that change with local oxygen tension. And let me explain why we thought that's interesting and important for ME/CFS. First of all, we published a paper in 2016 and provided a new finding showing where functional hyperemia initiate. Functional hyperemia meaning blood flow will increase when there's local metabolic activity starts to increase. So, that's called functional hyperemia.

And what we did is the figure A shown here, we used a mouse, and we open the cranium window and we study the blood flow in the brain. And when we stimulate the feed of the mouse, and we can start to measure the blood flow change in the brain. And figure B was showing the active area we identified when the brain was activated and then the curve showing how the blood flow starts increase upon stimulation. And we can find out the blood flow increase starts from a capillary and followed by these arterioles. So, why that's interesting because normally people believe arterial blood flow starts to increase first when there's a local metabolic change. And because they have smooth muscle cells, they can change the diameter of the blood flow.

But capillaries, they do not have muscle cells over there. So, they cannot change the diameter and therefore they cannot change the blood flow. But this finding was interesting, showing that blood flow increase actually starts in the capillary instead of these arterioles. So, the overall workflow, you know, we presented in the paper was when neurons get activated upon the stimulation and there's a local oxygen tension drop. Because neurons start to consume oxygen, right, and that drop of oxygen triggers the blood flow increase in the capillary. And after this, followed by this blood flow increase in arterioles and arteries. So, this is an interesting phenomenon, but we wonder, you know, why the local dropout PO2 can start to increase blood flow in the catheter. You know, what are the mechanisms?

And following that study, we spent a couple of years to investigate the mechanisms. And we found out, actually red blood cells play a critical role in this process. Basically, when red cells, actually they experience decreased PO2, for example from the tissue and they can become more deformable. And that change of deformability upon change of local PO2, making them move faster in the capillary given a constant pressure drop across the capillary network. So, this is a paper we published 2019 explaining the mechanisms, how change of local PO2 can regulate RBCs flow in the capillary.

And we actually developed this ex-vivo microfluidics approaches. And to mimic the local oxygen tension change in the brain and see how fast red cells can go through this microfluidics capillary. So, this is an artificial capillary we made in the lab. And there's just PDMs, which is a polymer, and there's no endothelial cells, no other cells at all. Just a pure engineering device with only the size of this channel which mimic the size of a capillary. And we inject -- red blood

cells go through this will control the local PO2 levels and see how the change of red cell velocity as a function of oxygen level change. So, this is the video -- oh, I cannot control the video, actually.

Here we go. You can see that we took an image how a single red cell goes through this artificial capillary. We can measure the velocity at given PO2 levels. And the figure to the left showing the oxygen level. We can manipulate and measure the red cell velocity as a y-axis. And we can show when the PO2 level starts to decrease from right to left and the red cell velocity in the capillary actually start to increase, which is consistent with the in-vivo finding that we demonstrated a couple of years ago.

And why that's related to ME/CFS and why, you know, I'm excited about these findings here? That's because impaired cerebral blood flow has been observed, right? In the previous section, Dr. Hamilton talked about this cerebral blood flow decrease in ME/CFS. And also, the compromised functional hyperemia, meaning the initiation of the increase of CSF upon neural activation was not as good as healthy controls. And that will contribute to our cognitive decline in neurodegenerative conditions and also ME/CFS. And more importantly, we find out this reduced red cell deformability in ME/CFS, which is published in 2019.

So, all combined these preliminary previous literature work with our findings, and we thought, you know, the PO2-regulated RBC capillary velocity may be compromised in ME/CFS. And it maybe can be used to measure the progress of ME/CFS or even for diagnostic. So, that's why we developed microfluidic approach to measure RBC velocity to test RBCs from ME/CFS patients and compare to healthy controls, and to see if that's the case or not.

And the other results I will talk about here is not published yet. We are preparing a manuscript and hopefully can submit soon, but these results are not published yet until today. So, what we did in the work is like I showed you before, we fabricated these microfluidic devices. And put the device inside a sulfite sink which can control the PO2 level around the microfluidic channel, the capillary. And then we can inject RBCs to this channel and use high speed camera to see the RBC velocity.

So, this imaging shows healthy control cells and ME/CFS cells, how they go through this microfluidic channel. And we can record this imaging and doing that analysis later on to calculate the velocity. And there are the overall results we got so far. We test healthy control 23 patients and ME/CFS 35 patients so far in the past two years. And we plotted the RBC velocity, as I mentioned before, in the capillary as a function of oxygen level in the sulfite sink. And we can see for a healthy control when you -- for both, actually, when you decrease PO2 level, the velocity will increase. But you can see healthy control have a higher magnitude of velocity compared to ME/CFS.

And also, most importantly, at least to us, the slope was significantly different. So, which is showing here. The slope meaning when you drop PO2, how fast or how the magnitude changes, how those changes with PO2 level. Meaning if this has to control a small change, PO2 level will introduce a large velocity change. But for ME/CFS, a same magnitude change of PO2 level, but the velocity change was not that much at all. Meaning they are not sensitive to local PO2 change anymore, ME/CFS patient.

And then we compared the general effect, and we take on male and female for the healthy control and male and female from ME/CFS. We didn't find any significant difference in terms of the calculus slope, meaning the sensitivity of red cells to PO2 changes between genders. But definitely, you can see there's more data point from female participants compared to the male participants in the study for both healthy control and ME/CFS.

I think this animation here didn't show up somehow, I don't know. It's not here. It should be one slide underneath this one. But anyway, so, this image is showing -- figure is showing how the effect of age on sensitivity to PO2 changes. And this is ME/CFS, and the healthy control, and there's no significant dependence on age. You can see the ME/CFS is the data is all over the places, and a slight, you know, dependence in healthy controls, but it's not that dramatic. So, the age doesn't play a role in this case in terms of the slope.

So, then we thought maybe we can find out what's the best parameter on top of the slope we can use for ME/CFS diagnosis. And so, what we did here is we have all the possible parameters we can have. For example, number one with the slope range means 25 percent, 75 percent range of the maximum velocity. We use that as a parameter or the slope I showed previously as a parameter for sub slopes one, two, three. That's meaning every two, for example, these two adjacent PO2 two levels will have one slope, and this another slope, and this have another slope, right?

This is a sub slope. R square meaning how the linearity of this fitting slope square oxygen tension one, two, three have different oxygen tensions here. One, two, three, four have four: age, gender, slope-old, meaning when the slope -- each data point will have a lower end of this slope we can get and maximum sub slope. And we have all these parameters, and we extract from this curve as input to this machine learning algorithms. And we calculate which one can provide us the higher accuracy, and we can use that for diagnostic ME/CFS.

So, we do this correlation map first and identify one, two, three, four high correlation parameters with ME/CFS. And the slope actually have a pretty good one. It's like 0.968. And we have other 11 left in the correlation, we didn't show up here. These are the most correlated parameters when we run the correlation. And we run the algorithms and we find out as I mentioned before,

one is the slope range, two is the slope, and three and also one is what you see here. But this meaning -- two, 10, 14, 15 meaning we combine all these parameters together as an input and using these different algorithms to calculate the accuracy.

And we found out this slope will combine slope with other parameters was pretty high and used a KNN algorithm. We find this highest is around 78 -- about 80 percent. It's not that high. It's, you know, 98, 99 percent yet. One of the reasons we are thinking is probably we use image analysis method for doing this and we didn't take like a thousand cells for this testing. We only take like a couple hundred, 200, 300. So, there's variation, you know, age or change in ratios. So, that's why the accuracy was not that high yet. And now we're working on different approaches to include, you know, high number of red cells for testing and hope we can increase the accuracy.

But accuracy is not bad. You know, 80 percent was not bad at all. So, we're trying to improve it right now. Since we identify the slope, and the combined slope with other parameters may be a good approach for diagnostic, we are thinking probably we can use the same approach to see longitudinal monitoring assessment of ME/CFS to see if it's working or not. And one experiment we did is we used same patient, you know, zero month, meaning we take the sample, tested the red cell velocity versus PO2 changes, and this is 26 months is the table here, not two months.

Twenty-six months later, we tested again and we can see the improvement of the patient's red cells after 26 months. And during these 26 months, the patient didn't take that much medicine. This is normal medicine is taking. And apparently, you know, the steeper, meaning the lower the value of the slope, meaning the better, right? Because the red cells start to respond to local PO2 changes. So, the significant decrease of this slope meaning is getting better. I like to say we are not targeting treatment or therapeutic approach to do this. The only purpose we're doing this is we're trying to see if the approach we develop, meaning the RBC capital velocity as a function of PO2 can be used to monitor the progress of ME/CFS. So, anything the patient was taking we're doing -- can test it to see if that's any effect on the red cells response to PO2 or not.

So, the second patient was quite interesting study too, and we take the first blood test when the patient was taking these two drugs. And then after this testing, the patient started taking more drugs for two months. And then we take that testing again at the end of the two months, and then they change the drug dose. You can see from here these reduce all these doses for these drugs. And then we take the drug testing again three months later. So, basically, time zero is only these two drugs we test this RBC velocity. And then after those two months for this drug, we take the RBC velocity at the end of two months. And then when they change to reduce the dose, we take another drug -- the blood testing after three months.

And what we find out is the first drug test was this curve, the circle one. And after the second test, meaning they take a whole list of drugs, you can see the increase of the magnitude and also the velocity. And when you reduce the drug and the slope and magnitude start to decrease too. You can see here clearly from the first to the second significantly improved. And when you reduce the drug test they come back again. So, that means there's something changing when they're drug testing in a patient. And we can monitor that by using how red cells respond to PO2 changes.

And the last patient we did is the patient was taking tethered cord surgery, and the green circle was before the surgery, and this green square was the after-surgery a month, and this black line was the healthy controls. You can see after the surgery, the red cell response to PO2 change was improved and this is almost comparable to healthy controls. Meaning the surgery have a really improved effect on red cells response to PO2 changes.

And I'd like to highlight again why this is important because on the red cell's response to oxygen changing, meaning when there's a need of blood flow increase and then these red cells can change their deformability and start to increase the blood flow right away. If that's delayed, then the meaning the blood flow supply will be delayed and that will cause trouble. And for example, in the brain there's no energy reserve.

And the last piece of work we did is this is how we monitor the progress of this patient when they have any surgery or drug treatment. But we're thinking, because the magnesium we find out the deformability change of these red cells, we demonstrated 2019, may be something we can use to improve the red cell deformability. And then that might improve the capillary velocities too at different PO2 levels.

So, we test this salmeterol, this drug. And this drug is known acting on these beta two adrenergic receptors, and red cells have this receptor on the membrane, and they actually can increase the CMP formation and increase deformability. So, we thought that would be the case. We can test it. And we did actually use zero nanometers, one nanometer, and ten nanometers. So, we can see the increase actually is quite dramatic compared to zero cell material. And that's based on nine patient samples we tested.

And we also test this xanomeline, which is acetylcholine receptors. And red cells also have these receptors on membranes too and this can induce the production of GMP and induce deformability and has been reported. And we test that in our system and we can see zero, one micromolar, and 10 micromolar. The increase -- a dose-dependent increase of slope, meaning the red cells become more sensitive to oxygen changes. So, all these samples are -- all these from eight ME/CFS patients. So, we use blood samples from the patient to test it.

So, for conclusion, we demonstrated this PO2-regulated RBC capillary velocity is compromised in ME/CFS, and the accuracy of using this approach for diagnostic can up to 80 percent right now, and we hope we can improve more in the future. And RBC velocity can potentially be used to monitor and assess ME/CFS longitudinally. And also, the two drugs we find out they can improve this RBC capital velocity in ME/CFS. For research priorities, at least for my life. We're trying to develop RBC-based ME/CFS diagnostic devices. So, they're working right now to increase the accuracy. And we have clinical, usable devices in the future, hopefully. So, that's our first priority.

And second is we also investigated the circulating cytokines and capillary velocities because this challenge of ME/CFS was seemingly because of infection. And then there's something in the plasma as previously talked about this micro cloud. So, we are investigating this cytokine red cells behaviors too. And last, we are trying to see if our approach can be used to screen drugs that can improve RBCs' deformability and also sensitivity to PO2 changes. So, we work on these three projects right now.

And this is my group members working on this experiment, and our collaborator Ron Davis, and Mohsen at Stanford and Anna Okumu over there. And Xin Liu was a faculty we collaborated with machine learning approach, Amrit and Mike Gresser. Actually, they proposed salmeterol drug treatment and we thought that's a good idea. We tested it and it works pretty well. And we thank the Foundation for Open Medicine Foundation, NIH, for supporting this research. And thank you.

Vicky Whittemore: Thank you very much for a very interesting presentation. So, in the individual where you saw patients that you tested, that was on just a couple of drugs than on many drugs and then changed the dose, how do you sort out what caused the change? What caused the improvement? And then, you know, they changed the dose on lots of different drugs. So, how do you then sort out what changed the worsening again?

Jiandi Wan: I agree. That question puzzles me a lot too, because I'm a chemical engineer. I'm not a pharmacist or doctor in the medicine area. So, we collaborated with Ron Davis and they provided a sample and I discussed with their team about the patient conditions. We don't know. Actually, the drugs they take are not specifically for ME/CFS either. They are just for that patient. And we found this phenomenon, but we really do not know why that's changing. We're just thinking, you know, something definitely affects this restless behavior. Otherwise, we can see this is dose dependent. Quite interesting how they start to lose the sensitivity to oxygen change, and the other one they start to increase again. But the mechanisms were -- the drugs effect, we don't know.

Vicky Whittemore: Yeah. So, there was a question about -- well, the question -- I'll read the

question then I'll kind of add on my own part to this question. So, do you have any insight into the values in people with hemolytic anemias? We have so many individuals with hemolytic anemia who have ME/CFS and EDS and TCS in the community. So, I guess the overarching question is, how do you again sort out individuals with ME/CFS who may also have comorbid anemia or Ehlers-Danlos syndrome? Is that something that you've looked at in your studies or think will be important?

Jiandi Wan: Yes. So, the thing we find out is quite interesting is we work on sepsis trying to see how the sepsis RBC, ATP release, and oxygen level changes, and so on, so forth. Sepsis red cells from septic patients are completely different with ME/CFS. So, what we saw under the microscope on the results from ME/CFS, they look pretty healthy. You know, the red shape everything is not bad at all, just under the microscope. But the septic patient red cells are spiky, you know, weird shape, all this stuff.

So, I think, you know, normal blood tests for the blood lab testing the RBC mass and volume and, you know, number of the hematocrit over there. But I think our approach is kind of like a functional analysis, how red cells can respond to reduce our PO2 and then start to flow faster in the channel in the capillary. So, I think this has never been done, especially when there's a normal blood cell lab testing cannot differentiate, right? The number is the same maybe the mass is a little bit reduced. But what's a function difference or dysfunction of red cells I think there's no lab testing for doing that yet.

So, we're interested to develop this technology so we can see red cells as a, you know, oxygen carrier that mingle with oxygen and how that change with oxygen in terms of blood flow. So, I thought that's a unique part for this flow testing as a diagnostic device. Yeah. But to answer your question, we didn't, you know, purposely to detect this anemia, you know, the cells number change or hemoglobin change. We didn't do that.

Vicky Whittemore: Okay. And there are a couple of people who have asked when you see an improvement, does that also correlate with an improvement in symptoms? Do people feel better?

Jiandi Wan: Right. So, the second -- the drug testing one, we didn't receive -- the questionnaire in which they have this patient coming out, they didn't see that. But the surgery one was improved, was significantly improved.

Vicky Whittemore: Okay. Do you think ion by IV could improve red blood cell outcomes in your study? I know you're not a clinician. I want to skip that question. So, here's one. Did the increased deformability correlate with improved -- oh, wait, I already asked you that one. Sorry. Hold on. Where did it go? Things jumped around on me here. So, following the law of

continuity or fluid mechanics, how does a red blood cell that fills the capillary accelerate down the tube without creating a vacuum? So, that's an interesting question.

Jiandi Wan: Yeah, that's an interesting question. We actually published a paper in Science Advances in 2019 and just focused on that question, how exactly red cells are going to become more deformable, and how they can flow faster in the capillary. So, you know, for those who are not engineering, I would say imagine you have a small particle go through a narrow channel, but that's a gap between the channel and your particle. So, the gap will become larger and you can flow faster. And there's a lubrication theory in engineering talking about the optimized gap over there. Not too big or too small because there's a flow pressure of theirs too. So, in our calculations, we found out the gap was reduced when the cell became more deformable so they can elongate it in the channel. So, that increase of gap actually helped the cell self-accelerate in the capillary.

Vicky Whittemore: Yeah, interesting. Thank you for that explanation.

Jiandi Wan: Yeah, sure.

Vicky Whittemore: Yeah. All right. Well, thank you very much, Dr. Wan.

Jiandi Wan: All right. Thank you.

Vicky Whittemore: And now for our last speaker, Dr. Systrom, over to you.

David Systrom: Thank you, Vicky. As the title says, I'm at the Brigham & Women's Hospital in Harvard Medical School. I'm an exercise physiologist. And my focus over the last, really, five to six years has been on ME. And more recently, additionally, long COVID, using a specialized test called the invasive cardiopulmonary exercise test, which I will explain to those who haven't been exposed to it yet. All right, so this is a gentleman who agreed to be filmed down in the basement of the Shapiro building at the Brigham & Women's Hospital a little while back, and it shows a few things. I don't know if I can make this go. It's not critical. Oh, there we go, awesome.

So, many in the audience may know that there is a non-invasive cardiopulmonary exercise test, which is quite useful in the diagnosis and follow-up of patients with both ME and long COVID. There's, of course, the two-day study of Betsy Keller from Ithaca, who documents a decrease in VO2 peak on day two that seems to be specific, probably correlated with PEM, post-exertional malaise in ME. What we add to the mix, and the basic part of the test is shown here. There's a mouthpiece in place and a pneumotachograph that measures minute ventilation. There's an expired gas catheter that measures exhaled CO2 and O2. From those things, I'll show you what we calculate. That's the basic part of the test hooked up to a metabolic cart.

The invasive parts are two catheters that are placed around the corner in our cardiac cath lab. One is a pulmonary artery catheter placed in the internal jugular vein, in this case, the right side. And then additionally, a radial artery catheter placed here. And I'll show you what we measure from those things. So, the noninvasive part is shown here. We measure the time-honored VO2 that's the oxygen uptake during exercise. And I should say we do mostly incremental cycling exercises so that the patient can be stable on a bike rather than on a treadmill.

And then as I said earlier, we measure CO2 output and bulk flow of air, which is minute ventilation. From the catheters, though, we take a much deeper dive into the pathophysiology of what ails the patient during exercise. So, there are pressure measurements and they're critical, as you'll see as we go along here. One is the right atrial pressure. And then there's a surrogate for the left atrial pressure, the pulmonary capillary wedge pressure. These are the filling pressures of the heart on both sides. Basically, the heart is a pump. And for it to work properly, one has to prime the pump with adequate volume and therefore pressure.

We measure pulmonary artery pressures and rule out things like pulmonary hypertension. We also measure both pulmonary and systemic gas exchange. So, shown here are a couple of examples. This is the arterial oxygen content. And this is the mixed venous oxygen content which is drawn from the tip of the PA catheter which is sitting in the pulmonary artery. At peak exercise, about 80 percent of the cardiac output is coming back normally, from the exercising legs. And therefore, this becomes a nice measure of the ability of the skeletal muscle under exercise conditions to take up and utilize oxygen that's been delivered to it. So, I'll come back to

all these things as we go along here.

All right. Whoops. Here's sort of a simplified diagnostic algorithm derived from the combination of the non-invasive and the invasive cardiopulmonary exercise test, also known as CPET. So, the VO2 peak can be expressed as a percent predicted. From that, we can determine this non-invasive variable tells us how impaired a patient is. So, the lower the number, the worse it's expressed as a percent predicted. And we can see severe disease. It can be heart, lungs, and other things depressing this down into the 20 percent range. Oftentimes, we're talking about transplanting hearts and lungs in that setting.

I will say that if you do a non-invasive test in ME and you find somebody 80, 90 percent, and even sometimes 100 percent, if you stop with a non-invasive test and conclude there's nothing wrong with them, you may miss a major signal that we actually can determine from the catheters. And I'll get to that. Here's a non-invasive number. This is basically one that is not particularly germane to ME/CFS. It's the bulk flow of air, minute ventilation at peak exercise divided by the maximum voluntary ventilation. That's the ability to move air in the resting state. And that's a high number, it means the lungs are limiting the patient. We generally do not see that in ME/CFS or long COVID, unless the long COVID patient has been on a ventilator and has fibrotic lung disease.

Over here is the rest of the world. And this is the so-called Fick principle. It says that the VO2 at any point in time but will be emphasizing maximum exercise is related to the cardiac output times the difference in arterial and mixed venous oxygen content. Normally, this stays about the same during incremental exercise. This goes up about fivefold, normally from rest to peak exercise. And the difference between these two increases about threefold normally during exercise. And you'll see there are abnormalities both here and here as we go in ME.

The invasive CPET allows us to rule out intrinsic heart disease, both the left heart and the right heart. We can determine whether the heart's limiting by looking at the peak cardiac output and expressing that as a percent predicted, and seeing if it accounts for most of the missing aerobic capacity. And if there is a problem there, we can rule in or out left heart disease by looking at elevations of the left atrial pressure, the surrogate, the pulmonary capillary wedge pressure. And we can do something very similar on the right side. And additionally, we can determine whether there's pulmonary hypertension by pressures by resistance in the lung circulation. I'll say again in ME/CFS, these things, unless there's comorbidity, do not exist.

So, pure ME/CFS without hypertension and coronary disease and without pulmonary hypertension doesn't have these things. But what ME/CFS does have in our world is this and this. And I'm going to spend the rest of the time talking about these two entities. I got into this business several years back when we increasingly recognized using this invasive CPET that was

originally designed to detect early heart and pulmonary vascular disease and differentiate heart from lung disease. That there was a subset of patients being referred to us clinically with exertional intolerance but didn't have anything to do with intrinsic heart or lung disease. So, Will Oldham lead the charge on this particular study. We took a look at our invasive cardiopulmonary exercise test database.

We came up with roughly 600 patients who had undergone an invasive CPET for exertional intolerance. We ruled out heart and lung disease as a cause. And we found a group of impaired patients who were defined by a depressed VO2 peak. And we compared them to a cadre of normal individuals who had come into the lab complaining of exertional complaints, but we couldn't find anything wrong with them. And what differentiated this group of impaired individuals from normal hemodynamically was filling pressures on both sides of the heart. I should emphasize this is in the upright position, and most of the signal is seen at peak exercise, not so much at rest. So, they pedal away, they get to the peak. They're upright. Gravity is the enemy.

You heard a lot about orthostatic intolerance from Dr. Visser and from Dr. van Campen, and this is likely very much related. So, right arteriole pressures are lower. That differentiates these patients and surrogates for left atrial pressures, filling pressures on both sides of the heart. We took a look and attempted to regress some time-honored variables that are thought to reflect overall aerobic capacity. Here is the VO2 peak expressed as a percent predicted. And here's the cardiac output peak at the end of the exercise bout, again expressed as a percent predicted. And there was a relationship. So, the lower the filling pressures on both sides of the heart, right and left, the lower the peak cardiac output, the lower the VO2 peak.

What we learned in retrospect, and this came the hard way, was that many of these patients, if not most of them, met the old IOM criteria for ME/CFS. So, this is how I got into this business. This is where they land on our algorithm. The VO2 peaks were modestly depressed. The cardiac outputs were modestly depressed. They didn't have this, they didn't have this, they didn't have this, but they absolutely had this. Not shown here is that we did an interventional cohort where we actually gave them normal saline boluses, at least a subset of the patients, and asked them to pedal again about 45 minutes later, with the existing lines in place. The right atrial pressures were higher, the VO2 peak was higher, the cardiac output was higher, and they actually felt better despite the fact it was a second bout of exercise.

So, my interim conclusion number one, is that in ME/CFS, there is some evidence of systemic vascular dysregulation during exercise. We call it preload failure. It likely has much overlap. In fact, it does have a huge overlap with POTS and to a degree orthostatic hypotension. And we'll come back to some of that. We took a little deeper dive into all of this, and Dr. Visser kindly showed one of our graphs from this paper. Two summers ago, Philip Joseph led the charge here.

And what we did was two things. We had a larger invasive cardiopulmonary exercise database about three times the original 1500-ish patients. We better defined the existence of ME/CFS by the IOM criteria.

And we also had become increasingly aware that a large number of our patients were coming back with a skin biopsy diagnosis of something called small fiber polyneuropathy or neuropathy. These are skin biopsies, minimally invasive punch biopsies above the lateral malleolus. And they were run over in the Oaklander lab at MGH. So, I will attempt to weave these things together in an effort to convince some of you in the audience that what is at play here, at least in some patients, is neurovascular dysregulation.

This is a slide Dr. Visser showed. We preordained three tertiles of blood flow. That's cardiac output. Remember, this is pulmonary blood flow that we're measuring with the Fick principle, with a mouthpiece in place, on the difference between arterial and venous O2 content. And we regress that during the exercise bouts against increasing metabolism during incremental exercise VO2. It's been known forever, and this is a normal group of about 37 normals, that the normal slope of cardiac output versus VO2 during incremental cycling exercise, about 5 to 6 ml per ml. And so, by definition, there was that middle group.

In the blue here is what we would define as the low flow group. And they are truly the freeloaders. They have low right atrial pressures, and they have a low cardiac output versus VO2. And that is the major reason for their decrement in VO2 max. But what was interesting to us, and this was very analogous to some of the data out of the POTS literature during an upright tilt table test with measurements of blood flow, is that there is additionally a high flow group. Remember, this is pulmonary blood flow. So, high flow, surprisingly high, and associated with that is poor systemic O2 extraction. So, the difference between arterial and venous O2 content is insufficiently low. So, this has all the earmarks in the cardiopulmonary world of left-to-right shunting. We are -- I'll come back to that in just a second.

We had 160 patients who had both the invasive CPET, and I just described, and additionally the skin biopsy for small fiber neuropathy. And when we tally up the definite and the probable small fiber neuropathy cases, there's an intermediate group that's thought to be early and probable. It's about 45 percent of the prevalence of small fiber neuropathy. This is a frighteningly close number of prevalence to that previously published in fibromyalgia and in POTS. So, we think it's very much related. And we ask the question, of course, is this causal? So, small fiber nerves, for those of you who don't know this literature, been known forever to mediate pain. There are no susceptive fibers, but more recently recognized as they play a major autonomic role as well. They regulate blood vessel tone and therefore blood flow, including that during exercise.

So, this is where this additional group ended up here. They all had preload failure, but a subset

of patients that red tertile has impaired oxygen extraction, which has all the hallmarks of left-to-right shunting. And we believe this is peripheral. So, skin, perhaps gut, kidney, organ systems that don't need oxygenated blood and maybe even microcirculatory within the muscle, fast twitch muscle fibers devoid of mitochondria may be perfused preferentially, or at least abnormally, resulting in impaired oxygen uptake. So, on the arterial side, we have a problem of impaired oxygen extraction. I'll say quickly, I can't get to all of this. Also, in the differential of this, as Dr. Visser mentioned, is mitochondrial dysfunction. And I believe the two can coexist.

So, I'm going to make an interim conclusion here that in ME/CFS, we have systemic vascular dysregulation. On the venous side, we have failure to squeeze the big veins and physically push blood back up to the right and therefore the left heart. And additionally, on the arterial side, we have blood going where it shouldn't. And associated with this is a relatively high prevalence of small fiber neuropathy via skin biopsy. So, I think one might begin to make a case that what we've got in ME/CFS during exercise is some element of neurovascular dysregulation.

We took this a little step further by intervening with a drug that is known to mediate nerve traffic, and that's pyridostigmine, a.k.a., Mestinon. It's a myasthenia gravis drug. This is off-label use. And the way we think it works in these disorders, we borrowed this from the POTS literature, is that it enhances the cholinergic step of the adrenergic system. So, that's at the level of the autonomic ganglion. So, we've got this synapse Mestinon retards the breakdown of acetylcholine. And we believe it enhances adrenergic outflow. And in this case, potentially blood vessel tone and therefore blood flow.

So, how do we know that? This was the study. It was prospective, randomized, placebo-controlled approximately 50 patients with well-defined ME/CFS, and they received placebo or a single dose of 60mg of oral pyridostigmine after a clinically indicated invasive cardiopulmonary exercise test identified preload failure. And this is what we found. The effect size is admittedly small, but it was a single dose, and it was 45 minutes later that they were studied after the drug. And we know clinically, most patients with ME who do respond to pyridostigmine do so over weeks to months, not a single dose.

So, here's what happened. If they got drug, their VO2 peak during the second cycling bout went up. Interestingly, if they got placebo, it went down. We wondered if this is the beginning of PEM. It might be. And the reason the peak VO2 went up was cardiac output went up and again down if they got placebo. And we think the reason the cardiac output went up was tightening up of venous constriction, neurovascular function shown here. Up if they got drug, worse if they did not. So, I would make a case that in ME/CFS, there is some evidence that part of the problem, at least during exercise, is neurovascular dysregulation. Because when we give a drug that is known to work neurally, the patients appear to be better.

This is a study that was shown by one of the speakers earlier this morning. We think something very similar is going on in PASC or long COVID. We have preliminary data that suggest exactly the same things going on in long COVID. All right, so that's what I wanted to show you. And I quickly want to address the organizers' questions about what we know. So, I'm going to suggest we do know there is systemic vascular dysregulation during exercise in ME. What we don't know is the subject of today's webinar. And it's all of these things and the relative contributions of all of these things that you've heard about from the previous speakers, all of which deserve prime time, all of which are biologically possible, and all of which may lead to different treatment modalities and the way of precision medicine.

My research priorities, at least vis-a-vis our data, are to determine the root cause of this systemic vascular dysregulation. And very interestingly, I think given the highly touted Amsterdam study on mitochondrial dysfunction in long COVID, we have some similar data in ME that the mitochondrion is abnormal in a subset of patients with that poor oxygen extraction. I believe that the two may coexist, that we may have an O2 supply issue, which is all blood flow related, today's topic. I believe also there is a subset of individuals with ME and long COVID who have intrinsic mitochondrial dysfunction. Currently, the treatments for these two are entirely different. So, deserving of study.

I think you heard in the last talk, and we thank Jiandi for that. I think that his work is a beautiful example of an organic outcome variable that could be used both to diagnose ME. This red cell deformability, and then as a result of treatment to identify who should get what and how much. I think it's a beautiful example of that. So, we need much more of this. And then we need placebo-controlled randomized clinical trials, I believe, because of the dearth of data out there and the number of patients suffering. We ought to be starting with largely repurposed drugs and not waiting eight to 10 years for new drugs. So, I'm going to very quickly show you what we're doing at Brigham with the help of the Open Medicine Foundation.

We're going to be studying and we submitted today the IRB proposal to study Mestinon and pyridostigmine. This is in follow-up of the prospective randomized clinical trial I just showed you versus low dose naltrexone versus the combination. And we'll be doing that over three months. We're going to do 40 patients in each arm. We think it's appropriately powered for some of the outcome variables. Going to be measuring three different questionnaires throughout the three months. We'll be collecting blood for metabolomics, transcriptomics, proteomics, and peripheral blood mononuclear cells.

We will be doing a wearable device that will be on the patient for the entirety of the three months, a Garmin Vivosmart 5, which measures a whole bunch of physiologic variables. So, if somebody gets better, we want to know why, and what's associated with getting better. If somebody crashes during the three months, we want to know what's getting worse.

And then finally, we're going to do what's called a shape metabolic cart test. This is a simple three-minute step test with a metabolic cart shown here. It's self-paced. Patients tolerate it well, it almost never precipitates PEM, and it will give us a variety of physiologic variables. So, that's where we're headed. I'll stop there and see if we have questions.

Vicky Whittemore: Thank you very much, David. So, I'm going to ask you with regard to your first research priority, the cause of the vascular -- neurovascular dysfunction. So, do you think it's an underlying problem that gets exacerbated when someone, for example, has an infection that then -- or underlying genetic predisposition. Sort of why would this happen in someone, for example, who has an illness and doesn't get better compared to somebody who has that same illness and doesn't go on to be diagnosed with ME/CFS?

David Systrom: So, great question, Vicky. I don't think we know the absolute answer. I would say, generally speaking, a large percentage of patients with ME have some predisposing conditions. They are usually not even with whole exome sequencing, a well-defined genetic variant, but EDS is probably the most common one, Ehlers-Danlos syndrome. And the whole spectrum of it not just the specific -- meeting specific criteria, but hypermobile joints in general. So, I think one of the predisposing factors is likely a connective tissue disease variant along the lines of EDS.

And then there's a second hit and that second hit, as we all know, of course, on this call is most often infection, most often virus. But there are other cases at least clinically, where there's no identifiable sentinel infection, but there are other stressors. So, trauma, concussion, etc. So, the concept of a predisposing something and then a second hit is, I think, on pretty solid ground.

Vicky Whittemore: Thanks. So, there's a question. Do you have theories on which vascular bed or beds are the sources of shunting? And do you think it's global, or are there particular beds that are most are more likely than others?

David Systrom: So, great question. And that's actually something we're pursuing. I will tell you, I believe -- this is clinically speaking. There's a subset of patients who absolutely have skin as a major organ system that is preferentially perfused. Many of them have evidence of mast cell activation syndrome. Additionally, another predisposing factor, perhaps. And what I do in the clinic is ask a really cheap and cost-effective question. That is, when you're in the hot shower, do your feet turn purple or red and you get dizzy? And as I think you heard from Dr. Visser and Linda, there's a phenomenal prevalence of answer, yes. So, I think the skin is very capable of accepting huge amounts of blood volume and flow "inappropriately" and depriving the brain and the muscle of needed oxygenated blood. But the same could be true for less easily measurable organ systems such as the gut and the kidney.

Vicky Whittemore: Yeah. Interesting. So, is small fiber neuropathy in ME/CFS potentially reversible?

David Systrom: Another great question. So, we don't know. There is a dearth of data on longitudinal skin using longitudinal skin biopsies to determine what happens to the small fiber density as a function of time or treatment. Gwynn brought up the interesting prospect in some patients of immunotherapy, including IVIg. And I think that, you know, biologically plausible approach where you're interrupting an autoimmune process and perhaps allowing these things to grow back the nerve fibers is laudable. Similar thoughts about the anti-inflammatory effects of low-dose naltrexone and maybe even pyridostigmine which is cholinergic and therefore anti-inflammatory, so needs to be done. Great question.

Vicky Whittemore: So, in your study, how does the group of patients you found to have preload failure overlap with the group of patients who also have POTS?

David Systrom: Unpublished data. We've combined forces with Peter Novak, who's a neurologist at Brigham Faulkner and does extensive autonomic function testing, including the upright tilt table test. In our world, the preload failure patients are more common. We think it captures more of the dysautonomia, but potentially the other vascular abnormalities we've talked about today. And only about a third of the patients with preload failure meet classic POTS criteria.

Vicky Whittemore: All right. Thank you.

Vicky Whittemore: So, I think at this point, I'd like to ask all of the speakers to turn on your cameras and we'll move to a panel discussion. And I'm going to start with this comment and question that came in from Peter, which I think is very good. Let me go up here a bit. So, the question really is, is neurovascular dysregulation sufficient to account for the bulk of ME/CFS symptoms? And what is sort of the chicken and egg? And what is this vicious cycle that patients get into with red blood cell deformability? If I'm saying that right. Cerebral blood flow issues, blood volume issues. So, how can we make sense of all of this that you've all talked about today? Who wants to take a stab at that? Microclots, all of the above.

David Systrom: I can start with just a global statement. I don't think any of these pathways that have been described today are mutually exclusive. I think they may coexist, and I think there may be subsets of patients with predominantly one or the other. With respect to hypovolemia in deference to the Netherlands group and neurovascular dysregulation, I will readily admit I don't know how much of hypovolemia for instance, one might be able to overcome with pyridostigmine. So, you get a neurally active agent that we think helps tighten up veno constriction and improve blood flow. But is it sort of a band-aid on the hypovolemia? It's possible. Or is it treating the intrinsic abnormality? Or do both coexist?

Linda van Campen: Well, to respond to you, David, on that. In line with what we showed with the compression stockings, probably if pyridostigmine improves symptomatology, it probably will improve blood volume and improve cerebral blood flow as well. And we can -- obviously, transcranial doppler can also show that.

Frans Visser: The interesting thing is that we -- well, we tried meshing on in a lot of patients over 300 right now. And the interesting thing is that only two-thirds of the patients do react to Mestinon. And that's almost a golden figure that whatever you do, you have two-thirds of patients who react on something, whether it be pyridostigmine or fludrocortisone or Midodrine or LDN. There's one-third of the patients who do not respond.

And so, I think the heterogeneity of the phenotype is obviously present there. But it's worthwhile pursuing to see what happens with the pyridostigmine and do a sort of fitting test and see before and after what happens. And I think you'll see that it improves not only the cerebral blood flow but also the cardiac hemodynamics.

David Systrom: Absolutely. Well, I think it is quite interesting to think that maybe both can coexist, hypovolemia and neurovascular dysregulation, and maybe to varying degrees in any individual patient, but that in many you may have to treat both.

Frans Visser: Yeah. True. And the interesting thing is, of course, does the small fiber neuropathy improve with the improve with the with the treatment? I think it does. But there was

some beautiful reviews stating that there are approximately 30 syndromes who are related to small fiber neuropathy. So, it's not a unique feature of ME/CFS, but it's a very global abnormality. So, I think that the small fiber neuropathy may be not in a chicken or egg relationship that it's just an egg.

David Systrom: Sort of a final common pathway, I think many believe that both the CNS and the peripheral nervous system are exquisitely sensitive to ongoing inflammation. And that we've heard about from our other speakers and, yeah. And to know what happens longitudinally if we have a drug that works would be very important.

Vicky Whittemore: So, there's a question directed to you, David, but I think to everyone. Do you investigate if autoantibodies are relevant in what's going on with neurovascular dysfunction?

David Systrom: Right. So, we're aware of the work from Germany that has put on the map antibodies against both adrenergic receptors and cholinergic receptors. I will say my read on that and I'm interested in everybody else's thoughts, is that to a degree, it's not uniformly found, but maybe a clue to some individuals. I think it's a little bit of a controversial area right now. But it would be a biologically plausible mechanism by which there could be vascular dysregulation, for instance. I'm interested in others. Thoughts, please.

Linda van Campen: Well, there are studies on microbiome. There are studies on autoimmune things or natural killer cells, and you see all kind of hypes and differences. And the disease presents itself so complexly in different types of patients that I can assume it's not just one type of mechanism entering into this, but in one patient it will be more focused on this. On the second patient will be more focused on that, on the third patient more focused on another thing. And that makes it so complex and so difficult.

Vicky Whittemore: Yeah, absolutely agree with that. Gwynn, do you have a comment or question?

Gwynn Dujardin: Hi. Thank you. I just have a couple of questions that are related to what you've just said from the patient community. First, on the topic of blood clotting. Some patients report having anticlotting diseases like von Willebrand's disease. Can Dr. Pretorius or anyone else comment on clotting in the population and how we guard against that in research?

And next on the topic of blood flow. Can anyone comment on the relevance of menstrual age or menstrual stage on blood flow and blood volume, particularly as ME/CFS is so common in the female population, and many patients I know report fluctuations in symptoms according to the stage of their cycle? Thank you very much, and thanks to everyone who posted comments and questions in the Q&A. I want to let everybody know that these comments and questions are

saved by the NIH. Thank you.

Resia Pretorius: Thank you very much for your question on clotting. I think that is what we are trying to understand with our recent studies in ME/CFS. We have just started looking at the proteomics of the clotting proteins. And hopefully, we will have some information soon on levels of von Willebrand factor and other types of molecules involved in the clotting in ME/CFS. So, we don't have answers yet, but hopefully, we will have that soon. I can just perhaps comment on clotting and estrogen.

And before I give the answer session over to someone else, we do know that estrogen plays an important role in some clotting disorders. We do know that some individuals have got a propensity to clot more when they take the pill or on hormone replacement. So, we are quite interested in the phenomenon of ME/CFS together with such issues, and we are definitely interested to look into that as well. We are just planning such studies as well.

Vicky Whittemore: Have you looked at that in long COVID in --

Resia Pretorius: With estrogen? We have been noting that it seems as if more females are affected, and some of our collaborators have been looking at data and interestingly, a collaborator from the U.K. Sheffield Hallam, we've just had a meeting, a research meeting a few days ago, and she noticed that there was a significant difference between females and male long COVID patients. And it might -- and she thinks it might be related to estrogen. We also know that some long COVID patients have got significant, heavier bleeds during the menstrual cycle. And that is obviously a big problem. And then lots of clots in the menstrual blood. So, it's an interesting phenomenon. I think we need to look into that because it's obviously important for the female patients.

Linda van Campen: So, looking from my training period, from internal medicine, looking at von Willebrand's. I don't think there's a higher incidence of ME/CFS in that group. Maybe it can influence how symptoms present, but I don't think it's a one-on-one. If you have von Willebrand, you are into issue. What about the menstrual and estrogen? We know from clinical patients that premenstrual and menstrual; they have more complaints. How that translates into all the other issues, we simply don't know. At least not from a clinical perspective.

Frans Visser: The only thing we can observe is that patients during the menstrual cycle, in fact during the bleeding period, they have more POTS. So, they have more circulatory abnormalities. But how it translates to microclots or activation of mast cells, we don't know.

Vicky Whittemore: What about the relationship to blood volume and cerebral blood flow?

Linda van Campen: If you kind of state that from what we know, little as we know, we should have known a lot more by now that there is a relation between blood volume and orthostatic intolerance symptoms, cerebral blood flow, and exercise intolerance. The worse the patient is, the worse it gets.

Frans Visser: Yeah. But there's no study about the relation between the menstrual cycle period and volumes and orthostatic intolerance symptoms. That needs to be done. But it's a true phenomenon.

Linda van Campen: If you would fund it, we would love to do it.

Vicky Whittemore: Gwynn, do you still have your hand up, do you have another question or?

Gwynn Dujardin: No.

Vicky Whittemore: Okay. Jiandi, I have a question for you. So, the device written test you're developing I think really nicely shows how you could track change over time. Do you think it would distinguish an individual with ME/CFS from another disease? So, would it be used as an initial diagnostic, or once someone has ME/CFS, used to follow progression of disease or both?

Jiandi Wan: That's a great question. As I mentioned, we are also working on septic patient samples too. What we are trying to do right now is to see if the slope change is significantly different between different diseases. But we didn't do that yet. We only have the control with CFS, has the control with septic patients. And the patient number was not high yet. So, I think in the future that might be the case because based on our machine learning algorithms, the slope is one of the most sensitive parameters to see the difference. So, we're going to test that in the future.

Vicky Whittemore: Thank you. So, we're almost at time, so I'll come back to you, David, for any last words you would like to --

David Systrom: All right. Frans had his hand up.

Vicky Whittemore: Oh, I'm sorry. I'm sorry. I didn't see that.

Frans Visser: I'm sorry. Short question to Jiandi. Did you look at the influence of fish oil on deformability? Because that's a long -- well, 30 years ago, it was studied that the deformability was changed by simply fish oil. And fish oil does not change the symptomatology of ME/CFS patients.

Jiandi Wan: I see. That's good to know. We didn't. You mean, like omega three? That fatty acid? I see. We didn't do that yet. That's good to know.

Vicky Whittemore: Interesting.

Jiandi Wan: Yeah, we can test it.

Linda van Campen: From the diagnostic perspective of ME, don't forget the two-day CPET results from Christopher Snell and our results also that you can have some objective things in the two-day results that can objectively point out that there is CFS/ME.

Vicky Whittemore: Yeah, absolutely. Absolutely.

David Systrom: Yeah. Right. So, Vicky, I guess closing comments would be I really, really, really thank all the speakers and the organizers. You, of course, as well, Vicky, for doing this. This was long overdue. We've had some fabulous schools of thought presented in today's session that I think have the real prospect of leading to treatment, which, of course, is the Holy Grail. And I think we all agree that it's a heterogeneous population. We need better organic diagnostics. Kudos to Jiandi for taking a stab at that and others.

So, we need organic diagnostics that move this whole field out of the realm of, oh, does it really exist? Is it in one's head? Is it simply deconditioning? So, organic biomarkers that we then follow as a function of properly conducted randomized clinical trials, I think is the holy grail here. And everybody on this particular session is contributing to that. So, thank you all.

Vicky Whittemore: Yeah. But that will close the webinar and I'd like to thank everyone. I'm sorry. I thought we all got cut off. I'd like to thank all the speakers especially. And thank you so much those of you from South Africa and Europe and England for staying on with us. I know it's probably late at night for you there now, but we so appreciate you being with us. And thank you to all of -- everyone in the audience who participated. You asked wonderful, excellent questions. And as Gwynn said, we've saved your questions, and we'll be considering these as we move forward. So, thank you again, and have a good rest of your day. Thank you.

David Systrom: Cheers.