Physiology Webinar - December 8, 2013

ME/CFS Research Roadmap Webinar Series – Physiology
Open Session
Friday, December 8, 2023

Vicky Whittemore: Okay. I think we'll get started. Good morning, everyone. It's great to have you here for the sixth in our series of eight webinars, which is part of the ME/CFS Research Roadmap Initiative, to do some strategic planning and identify research priorities for ME/CFS.

I'm Vicky Whittemore. I'm a program director in the National Institute of Neurological Disorders and Stroke at NIH, where I oversee the grant portfolio on ME/CFS, as well as work collaboratively with my colleagues across all of the NIH institutes through the Trans-NIH ME/CFS Working Group.

So, I would first like to acknowledge everyone who's been involved in this process on the NINDS Research Roadmap Working Group of Council and especially the co-chairs, Cindy Bateman and Maureen Hanson, and the chair of this particular webinar, Dr. Craig Heller from Stanford, and also to acknowledge all of the individuals who've been part of this particular physiology webinar planning group. So, thank you all for your efforts to pull together what I think is going to be a fantastic webinar today.

And I would also like to really give a special shout-out and thanks to all of my NIH colleagues who have really worked behind the scenes to help make this successful. And particularly, also call out our colleagues at RLA, the contractor, Holly Riley, Damon Kane, and Martin, our science writer, who really have helped to streamline and make all of these webinars to be really seamless and successful. So, thank you all.

So, we have two webinars left. Coming up on January 5th, we have a webinar on lesser studied pathologies, and on January 11th, on circulation. So, you can go to the Research Roadmap Initiative webpages to both register, see more information, and we have also posted the video recordings and transcripts from past webinars. I believe the one from the most recent one, chronic infections, has not yet been posted because there's a lot of post-editing and cleaning up of the transcript that happens post the webinar. But that one will be posted soon.

So, just some guidance for your participation in this webinar. So, the goals that we set out for each of these webinars, as I said, is to set research priorities for research on ME/CFS. And in particular, on the physiology of ME/CFS is what we're going to be focused on today. So, what do we know? What don't we know? And what do we need to know to accelerate research? And how will a better understanding of the physiology of ME/CFS help us to identify targets for treatments and accelerate the research toward clinical trials and new treatments for ME/CFS?

So, the way the workshop will run -- the webinar will run is that each speaker will give their presentation. And there will be a time after each presentation for a few clarifying questions. And at the very end, we will have a discussion then with all of the presenters. So, we ask that your questions really focus on their scientific presentations, any clarifying questions about the research, and not about your personal health situation. We're not able to answer those kinds of questions on the webinar. So, please keep your comments very focused on the goals of the webinar today.

The -- all of these research priorities from the eight webinars that we will have held by the middle of January will be presented in a report to the NINDS leadership and NINDS Advisory Council at their May 2024 meeting. So, it's really critically important. And the discussions we've had so far in the previous webinars have been really fantastic toward getting us to that goal of narrowing in on the research priorities in this field.

So, for additional feedback, you can send emails to this email address mmecfsresearchroadmap@ninds.nih.gov. The best way to get announcements and updates from NIH is to sign up for the NIH ME/CFS Listserv at this URL, which is just nih.gov/mecfs. And we're a little behind in our putting this out to the public. But we do intend to put out the information to the public, to the community, on a platform called IdeaScale, where we'll get feedback on the research priorities.

So, we're going to, hopefully, launch the first four research priority areas in December for feedback. And again, we'll send information out and updates that that's available and how to access that platform through the Listserv and on the websites as well. So, with that, it's my great pleasure to introduce the chair of the Physiology Webinar Planning Group, Dr. Craig Heller from Stanford. And, Craig, at this point, I'll turn it over to you to introduce the first speaker. So, thank you.

H. Craig Heller: Thank you, Vicky, and thank you to all of the speakers. This is a very exciting webinar that we are about to engage in. ME/CFS, of course, affects the whole body. And physiology is the science of understanding the whole body. So, I expect that we will be hearing many new insights and actionable insights. And without taking more time, I'd like to introduce our first speaker, Michelle James, who's assistant professor in radiology, neurology, and neurological sciences at Stanford. Michelle.

Michelle James: Thank you so much, Craig. And thank you, everyone, for the opportunity to speak on this webinar today. It's nice to be here with you all. I'll be talking about what we know and how we've been using positron emission tomography, or PET, to really shed light on what's happening with the immune response and inflammation in the context of ME/CFS. And it's really an area that is just beginning. So, I'm excited that we're really -- we've got these tools that can begin to really answer these questions so we can learn more about the physiology and, like Vicky said, get closer to identifying great targets that will be helpful for therapy and validating new therapies quickly.

So, my talk part of this webinar is -- the outline here is really aligning with what Vicky mentioned in the beginning. I'll be starting with, what do we know in this area? And then moving into, where are the critical gaps? And then how are we going to fill these gaps in order to really accelerate research and get to a meaningful place with the research priorities as well? So, jumping in, I'll be talking mainly about inflammation and the immune response, as I mentioned.

So, inflammation is really, as you all know, the body's natural defense mechanism to protect and heal, which is a good thing. We need the immune response, it does a lot of wonderful things, and so does inflammation. But if it becomes unresolved and sort of becomes chronic, that's when it can become very harmful. And so, let's just walk through a little schematic here. So, how does inflammation start? There's usually some kind of injury or infection, some kind of trigger. And in the case of ME/CFS, there's a hypothesis that it could be an infection. It could also be something else unknown that, let's say an infection occurs, an immune response is launched. And then certain organs throughout the body, depending on where that infection is, will become inflamed.

And in its transient form, inflammation, as I mentioned, is usually a beneficial thing. It's leading to the mitigation of pathogens, the healing of damaged tissue. These are all really good things. But when it does not resolve, it can become chronic or maladaptive. And that's when we get a lot of cellular dysfunction and cell death eventually, and a lot of these awful symptoms.

In the case of ME/CFS, it's thought that inflammation could really be driving a lot of the symptoms that people are experiencing, unfortunately. Muscle and joint pain being one of those, just the widespread pain throughout the body, memory issues, cognitive decline, brain fog, post-exertional malaise, and a lot of the gastrointestinal issues. And so, there's a lot that we don't understand about inflammation, unfortunately, and in particular neuro-inflammation. Because it's happening in the brain and that's obviously harder to study. You can't easily biopsy the brain.

And so, a lot of folks have been very interested in trying to develop methods so that we can peer

inside the brain and really start to understand what's happening at the molecular level. So, what is neuro-inflammation? Well, it's any inflammation that is occurring in the central nervous system, as the name suggests.

One of the key cell types that's involved is at the microglia, your brain's resident immune cells or resident macrophages in the brain. They're the first cells to jump into action when anything is going awry. And so, certainly, a cell of great interest. They may serve as a sensor for what's going on, just responding very quickly to things. You've also got your astrocytes. You've got peripheral immune cells that actually come in and infiltrate the central nervous system when neuro-inflammation is occurring. You've got a lot of chemokines and cytokines that are being generated and released. It's a very complex process that we still, as I mentioned, don't fully understand, but I think it's critical that we do.

And there are techniques that people have been using for many, many years to try to understand what's happening systemically and in the brain in terms of inflammation. Blood tests have been used and continue to be used to measure markers for inflammation. Cerebral spinal fluid is also another way to assess different markers of inflammation. These are great techniques. The only issue, especially with blood tests, is you don't really get to understand what's happening in the brain itself. And also with CSF, although you're sampling something from the CNS, you're not getting that spatial information about what is happening throughout the whole central nervous system in exact locations, so very helpful but we do need more information.

Imaging which is my area and I think is a fantastic technique. There's a lot of structural imaging out there which has been useful to -- especially to rule things out. So, you may have a CT or an MRI if you come in with symptoms that indicate some kind of neurological disorder, and you'll rule out a brain tumor or a stroke or other things like that. But when it comes to looking at specific immune responses, it's not able to pick up on that if you're just looking at structural information. Structural changes happen far later than molecular changes. And so, there are -- there's a way to look at things very specifically in terms of molecular changes. But post-mortem analysis is obviously far too late.

So, what I think could be very powerful in this field is to develop more and more ways to look at molecular underpinnings of this condition. And that's where molecular imaging techniques come into play. And they really allow us to look in vivo in living systems at cellular and biochemical targets and processes longitudinally. So, that's -- it's very powerful. There are a lot of molecular imaging techniques out there. You may have heard of MRS, magnetic resonance spectroscopy, which is very helpful. It's only able to look in, sort of, very small areas of the brain or throughout the body. So, a little bit restricted, but certainly, very helpful.

I'm going to be speaking about PET or positron emission tomography. Because I think the nice

thing with PET is it's very, very sensitive. So, it gives you that ability to detect nanomolar amounts of different targets or biochemical events happening throughout the whole body and brain. So, it gives you that complete picture. Now, how it works is, we actually put a positron emitter onto a molecule that can track down different immune cells or aspects of disease.

And so, these positron emitters will eject a positron from the nucleus. And that positron actually collides with the nearby electron and produces two gamma rays that are always 180 degrees apart. So, we're able to actually have someone in a scanner and detect -- sort of draw this line of coincidence for where this annihilation event occurred and find out exactly where the PET tracer is binding in the brain or throughout the whole body. So, we can map where these molecular events are occurring. So, very powerful.

There are a lot of different ways of using PET traces to look at inflammation. One of the first and sort of most widely used PET traces in the clinical setting is FDG. It's fluorodeoxyglucose. It's actually a glucose analog, and it helps us to look at metabolism. So, a cell's need for glucose. It's used a lot for cancer detection. So, you know, cancer cells, you need a lot of glucose. But it's also just a general indicator of metabolism.

So, here, I'm showing you a study from 1998. I think it's the only FDG study that I've seen reported for ME/CFS. And it was tagged as preliminary findings. So, I'm wondering if there are more studies like this happening at the moment. But it was interesting to see that this happened a few decades ago now. And there are a number of regions that they showed have hypometabolism, so low brain metabolism. There were, I think, only 16 patients, so a small amount of people in this study, but certainly, some very helpful findings and indications.

There were six healthy controls compared to the patients and also six depressed patients compared to the ME/CFS patients. And the interesting thing was that hypometabolism was found both in the media frontal cortex and in the brainstem. But when you compare to the controls -- but when you compare the ME/CFS patients to the depressed patients -- here we go, 18 patients -- the brainstem was really the only significant area. And interestingly, that also sort of aligned with findings from SPECT studies and its single photon emission computer tomography, showing that there was altered perfusion also in the brainstem. The brainstem seems to keep popping up. So, that's interesting.

And why do I mention FDG in terms of inflammation? Well, it's not specific, obviously, but there is increased metabolism or need for glucose in immune cells. So, people think that potentially, in this case, in ME/CFS or cases where it's not -- we're not looking at cancer -- that increased FDG signal could in fact be immune activity -- increased immune activity. So, that's great. It's an indication that PET could be very powerful for identifying inflammation in ME/CFS. And so, we -- what we'd like to do now that we have that data is to get even more

specific and to identify targets that will tell us specifically about certain immune cells in the brain.

So, here, I'm showing you a slide with other emerging and existing molecular imaging biomarkers of inflammation. So, of course, microglia, I mentioned, is this resident immune effector cell in the brain and the whole CNS. In red, I'm showing you some biomarkers that folk around the world have been exploring as PET targets, so targets to develop PET traces for. I'll talk about TSPO in a minute. But there are a number of targets people are looking at.

Astrocytes, another type of immune cell in the brain, that's driving a lot of these inflammatory responses. There's a really nice new imaging agent that's coming out of the University of Michigan looking at MAO-B activity. So, I think that'll be interesting and could be an area that would be worth looking at for ME/CFS. We've got -- here's the peripheral myeloid cells that I was talking about, so cells that infiltrate the CNS.

A number of markers of monocytes and macrophages, neutrophils, that that could be used and targeted for PET tracers and have been. Then you've got adaptive immune cells, so B cells and T cells. Markers for B cells are shown here. Markers for T cells are shown here. OX40, CD4, and CD8. There're some nice traces that have been translated and are available for clinical use. So, again, lots of great tools out there. People can look at cytokine signaling and also oxidative stress.

There are -- I'm only showing here sort of the main targets. But there are a lot that we could be looking at. And so, this is kind of a summary of some of the promising PET tracers and targets that are being used to detect immune cells. And here, we've got TSPO that's being used in ME/CFS. I'll talk about that in just a minute. You can see the kinds of cells that it's expressed on and some of the example PET tracers that are being used.

There are others as well. It's a long list of TSPO PET tracers. And then you've got other targets here. And what you might notice is TSPO is on a number of cells. And while it has been very helpful, the issue is interpreting that signal. We have to take into account which cells it's expressed on. So, it's a great sort of entry into the world of imaging inflammation. But I think we can get even more specific. And that's what I'm really excited about in this field, is getting very specific with our ability to identify and track certain immune cells and certain -- their sort of activation states, their functional phenotypes in this condition.

So, other targets are mentioned here. And, you know, these are myeloid targets. It's a T-cell target. B-cell targets. So, let's move into the actual data here. So, I'll talk a little bit about the TSPO PET study that's been done in this area. TSPO is a highly hydrophobic protein. It's found on the outer mitochondrial membrane. It's present at very low levels in a healthy CNS, which we

all got excited about and thought "This is good because it's not really there in the healthy situation."

But when there is some kind of inflammatory stimulus or reaction going on, that's when the expression of this particular receptor shoots up. So, we thought it could be a nice marker of glial activation and inflammation. It is also expressed throughout the whole body on different tissues. So, that limits our ability to detect things in a widespread way with greater sensitivity. But it is very helpful, as I mentioned, as our first way of looking at inflammation.

And this was the first study done by Nakatomi back in 2014, and so a while ago now. But people are just starting to try to reproduce this and to look at other PET tracers, which is a really, really promising area. Here, we're showing, in this particular study, which, again, was a small amount of patients, nine ME/CFS and 10 age- and sex-matched healthy controls. They're showing that there's actually a number of brain regions that have elevated uptake.

So, in the amygdala, hippocampus, thalamus, midbrain, pons, cingulate, all about 1.2-fold to two-fold higher uptake. And some of these regions actually correlate really nicely with cognitive impairment scores, depression scores, and pain scores. So, certainly, indicating that this could be a useful method and that inflammation could be playing a key role in driving these symptoms. But we were using a PET tracer back then. That was a first-generation tracer that, you know, isn't really the most ideal for looking at this target. There are more sensitive and specific tracers for this target now.

This is an example of some work that we're doing at Stanford. Others are using TSPO traces, second generation traces. Dr. Jared Younger -- Dr. Vanelzakker at MGH and Dr. Younger at UAB. People are using these traces to now really look at larger numbers of patients. This is an example of using DPA-713 that we're using at Stanford. And we're trying to correlate findings with peripheral fluid-based markers as well and looking at disease severity. So, just started this study.

And I'm just going to show you a little example here. This is the radio tracer we're making of some of the images. Here, we're showing female patients. And you can see that there is, in the ME/CFS patients, more uptake in -- again, in the pons and the part of the brainstem and in the thalamus. So, similar to what was found in some of the other studies compared to the controls. And then if you look at males, the uptake's a little bit different, a lot lower. And we'll see slightly different patterns of uptake but certainly, differences between ME/CFS and controls.

And something that was interesting to me is the parotid uptake. So, in the salivary glands, and there, you know, there are a lot of mast cells that are expressing TSPO. And it made me think, what else is happening in the rest of the body? And so, actually, we just started to image the

whole body and brain with this particular tracer. And this is showing parotid glands in a number of different patients compared to their age- and sex-matched controls.

And then just looking at some of the whole-body data that we've started to acquire, you can see in ME/CFS, there' are actually speckled hotspots throughout all the bone marrow. It's really quite striking in the pelvis and in the vertebra compared to the healthy controls, also in the muscles if you look at the arm muscles, leg muscles throughout the body compared to very quiet uptake in the controls.

So, we've just started this really. And I think that it's opening up a whole area that could be very, very insightful and will help us to learn about the physiology and also like the spatial temporal dynamics of inflammation throughout the course of a patient's disease. It'll also help us to be able to measure someone, sort of, look at their immune response before and after therapies to see if they're responding, so a lot there.

But what we don't know is which imaging biomarker is going to be the best for visualizing the kind of inflammation or other molecular processes that are driving ME/CFS. We also don't know which image approach is going to be the most illuminating for early detection and disease staging and also monitoring therapies. I think PET is going to be very powerful. Can we use it in addition to MRI, with PET MRI scanners and different MRI sequences and types of work that's going on in that area? We don't know that yet.

Are there sex differences in the immune response? Probably. And I think that the data is showing that they very well could be. And so, this is an area of interest that we need to understand. And then how do we translate all of the insights from imaging into new therapies? Like Vicky was mentioning, this is where we need to be going.

So, how do we fill in these critical knowledge gaps? I think we need tissue samples. We need to validate what is -- we're seeing in imaging. We need to create potentially some new animal models. But certainly, looking at spatial transcriptomics to understand what imaging biomarkers could be helpful. And we need streamlined assessment of many PET tracers simultaneously looking at advanced MRI techniques in addition, like I was mentioning.

And then my last slide, these are some of the priority research interest areas I think could be focused on, so biomarker discovery for imaging in particular, biomarker validation in human tissues. So, we need to validate these biomarkers. Developing radio tracers specifically for disease relevant targets for ME/CFS. And then translating these to the clinic, which is a streamlined process for PET. So, that's great.

And then lastly, using machine learning and AI to identify novel markers in our whole-body PET

images. And I know there's a number of groups in Australia working on -- and around the world -- working on machine learning and AI for image analysis. So, lots of exciting areas there. These are my acknowledgements. Thank you again.

Vicky Whittemore: Thank you very much, Michelle. That's really very, very impressive. Very interesting. So, we have a couple of questions that came in. Let me jump over to the questions. So, first of all, Dr. Younger uses a special type of brain imaging that looks at brain inflammation and slightly elevated inner brain temperature. Are you using that type of brain imaging or are -- can you kind of compare-contrast with the type of markers and tracers that you're using?

Michelle James: Yes. So, Dr. Younger is doing excellent work. We are doing some similar things. We have overlap. I'm not measuring the brain temperature per se.

But we are using very, very similar TSPO PET tracers and the nice -- and the same scanner actually, the same PET MRI scanner. And he's looking at moderate -- sort of mild to moderate ME/CFS. I'm looking at moderate to severe. So, we're in discussion often, and we're trying to combine our findings to sort of design the next phase of these studies. But it's really great to be able to have someone like him to talk to and work together to sort of generate data to understand more.

Vicky Whittemore: Terrific. Terrific. So, another question is, what different information would you get from a functional MRI, particularly from the brain? And could it be useful to complement the information you're getting from a PET scan?

Michelle James: Yes. So, the functional MRI is looking at differences in blood flow, blood oxygenation, so very helpful in certain cases, not specific to inflammation per se. It won't tell you about immune cells but certainly will tell you about blood oxygenation. And I think in -- like you were saying, in sort of symphony with this sort of synergizing the two techniques.

And that's why I love PET MRI scanners. Because you can actually collect all of that data at the same time as getting the PET information. So, you're not making someone sit in another scanner, which can be very uncomfortable and time-consuming. We're really sort of very open to talking more with folks about other MRI sequences we could be using to gather information at the same time as the PET. So, that's a great point.

Vicky Whittemore: So, another question, is the bone marrow scanning -- are the bone marrow scanning findings good enough to start preliminary research into what's going on in the bone marrow?

Michelle James: Yes. I think that -- and I -- and I'm not sure there may be other talks today

where people can weigh in on what might be happening in the bone marrow. But I do think that it'd be great to understand that more. And yeah, I personally don't know what it is yet. But I think it's highlighting that there is a big role for cells in the bone marrow that are being activated. And so, yes, I think that would be great.

Vicky Whittemore: Similarly, I think the findings in the salivary glands are also very interesting.

Michelle James: Yes.

Vicky Whittemore: Yeah. Thank you, Michelle. There are some other questions here, but I think we have to move on. Hopefully, we can circle back. So, turn it back over to you, Craig. Thank you.

H. Craig Heller: Thank you. And thank you, Michelle. That was beautiful. Terrific. Our next speaker is Dr. Robert Naviaux from University of California, San Diego. Bob is professor of genetics, biochemical genetics, metabolism, and is associated with quite a few departments, pediatrics, medicine, pathology, and he's co-director of the Mitochondrial Disease Center. Bob.

Robert Naviaux: Thank you very much, Craig. And thank you, Vicky, for the invitation to speak today. I'll be talking to you about our work on ATP signaling, mitochondria, and the cell danger response. And let's see if I could -- oh, yeah. Can I advance? Very good. Okay. These are my disclosures. And one of the ways that we look at the overall physiology of ME/CFS is to try to differentiate the triggers of the acute injury from the difficulty in recovering from those triggers.

And we -- we've all been taught to think about things in a pathophysiologic way. Where we go from a condition of health, you know, to a condition of illness that can be caused by a variety of different things, from genes to infections. And the thing that is important to understand here is that under these conditions, you know, the cells are being acted upon. They're the object of an injury.

But then it turns out that there is a universal path back to health that involves a program that is highly evolved. That involves three separate, you know, basic phases that have a beginning, median, and end. And this is where cells become the subject of the problem. And so, the beginning always starts with inflammation. The middle is the replacement of cells that were lost in the original inflammation or proliferation.

And then finally, differentiation is required. And so, one of the difficulties is that during this time of, you know, of injury, you can be re-injured and exposed to a variety of different environmental compounds. But then I'll go on to show that these stresses will cause the cell to release extracellular ATP. Why that's important is because ATP is the most expensive thing, physiologically speaking, bioenergetically speaking, that a cell makes. And when that's lost from the cell, it signals danger.

So, when this occurs over a long period of time, it leads to incomplete healing and the process of aging. But overall, I wanted to, you know, bring about this dichotomy of diseases of injury versus diseases of recovery. And the disease of recovery are -- involve this process of salugenesis. And we are looking for new salugenesis therapies. But why don't we have any of these? And the answer is because we haven't really known how to look yet.

So, in an effort to compare and contrast pathogenesis and salugenesis, we put together a review that you can look at here. And the big issue is that scientists can't focus on a problem unless they have a name for it. And so, we coined this term salugenesis to contrast it with pathogenesis. So,

to get back to a concrete definition of the cell danger response, the CDR is a multi-system, evolutionary super trait that actually determines organismal fitness and undergoes selection as a unit in the same way that wings and fins do.

It's a universal response to environmental threat. And healing cannot occur without it. And once triggered, the healing cycle cannot be completed until the phases of the CDR are completed and the health cycle is restored. And it begins with mitochondria in the cell and spreads like ripples in a pond, signaling a cascade of information to neighboring cells, brainstem, and vagus nerve and across other -- all other anatomical scales and organ systems.

And then finally, it's -- the important thing to remember is, it's biologically expensive to heal cost energy. It's resource consuming. And until that's resolved, athletes will underperform, and the symptoms of chronic disease and disability will persist. So, just to put a fine point on this, cell danger, in terms of a cell, is the detection of decompartmentalized adenosine triphosphate and extracellular ATP. So, it's a key regulator of the CDR.

This is a cartoon of a cell that illustrates this graded release of ATP through channels in, you know, in the membrane that involve Pannexin and P2X7. But mitochondria is sensing and responding to changes. They're talking to the nucleus. When that intracellular information in terms of metabolites like ATP is released, it can bind in a paracrine way to neighboring cells. It can also bind in an autocrine way to the cells that are releasing it.

When these channels open up under stress, it produces a dissipative loss of ATP that we call purinosis. And when circulating cells like lymphocytes, macrophages, you know, polymorphonuclear cells, move around through the vascular system, if they're releasing ATP, they're signaling to cells that there has been danger detected in the system. And if that occurs, it actively blocks -- I'll go on to show that this has an effect on oxidative phosphorylation, you know, not only as a -- because it causes a chemiosmotic back pressure in mitochondria but also because of the signaling effects.

Okay. Let's see. And so, we went looking, "Okay, are there drugs that might actually help, you know, limit the amount of ATP that's being released from stress cells?" And there are. Suramin is one of them. And, you know, there are other -- or, you know, other groups that are working on, you know, trying to actually find drugs that will also inhibit the directly that -- this block with Pannexin blockers.

Okay. So, the genes needed to regulate healing include a co-evolving group of over a hundred different G-protein coupled receptors that monitor and respond to local and distant danger signals. When we looked at the receptors in our genome that were related to the P2Y1 receptors that bind to ADP and ATP-related nucleotides, we got a long list of things that have a variety of

different natural ligands, you know, including oxysterols, sphingomyelins, lactate, butyrate, viral co-receptors.

That'll be an important one that I'll , I guess I'll finish this slide with, you know, an emphasis on, you know, some of these, you know, neuropeptide hormones that are used as co-receptors. So, the angiotensin II receptor is t a SARS-CoV-2 entry molecule. But it's related to the G-protein coupled receptors that are involved in the overall healing process in response to stress.

Okay. So, we wanted to know what actually happens to, you know, an organism when you expose them to extracellular ATP. So, we did that in a mouse model and looked at the breath of a mouse. We looked at blood over a four-hour period. This is a mouse in a bell jar. We're collecting volatile organic compounds in their breath and analyzing them.

And what we found is there's a dramatic increase in very -- the small molecule carbons from carbon monoxide, methanol, methane, and all the way up to, you know, intermediates of cholesterol synthesis like isoprene, which is a classic organic alkyl compound that is a marker of stress. But when you inject ATP, lots of other things happen too. So, there's -- a CXCL1 chemokine is increased. IL-10, a variety of different molecules are increased, including the stress hormone, like corticosterone, and acutely.

And then when we looked at the whole body -- the whole metabolome, we saw that, you know, over half of the metabolome is changed in the first 30 minutes after ATP injection. They include important molecules like dopamine, inosine, and hypoxanthine, are all increasing dramatically. In fact, just the injection of extracellular ATP produced more changes in the metabolome than any drug we've ever studied.

Okay. And the only other thing that even came close with lipopolysaccharide or endotoxin, which, you know, actually produces many of its effects by triggering cellular release of extracellular ATP. Then we looked at whole body physiology. What are the bioenergetics measured by, you know, oxygen consumption, CO2 production, in mice that were injected with ATP? And we found, dramatically, that mitochondrial oxygen consumption dropped by 74 percent. Okay? So, that's illustrated over -- yeah.

So, the other thing that happens is behaviorally, the animals dramatically decrease their -- you know, their exploratory behavior and become almost instantly, you know, subjectively fatigued . Okay? That'll become important later on. But what we'll emphasize here is that when we measure the amount of oxygen that's consumed, it dramatically decreases in the animals. And the tissues of the animals behave as if they're hypoxic, even though they're not. And the animals are, you know, breathing rapidly. And then there's a recovery that happens as the ATP is metabolized.

When you look at this respiratory exchange ratio of VCO2 over VO2, what you see is it begins to -- you know, begins to drop. And during the active recovery phase in the green oval, which is the period where, over on the left, the oxygen consumption was increasing, there's a very significant rebound activation of mitochondrial fatty acid oxidation. Okay? So, it's critical for active recovery. And that'll be important as we move on.

So, what about other things? So, we had a a mouse model of autism-like behaviors that is produced by a prenatal exposure to a virus-like compound, double strand RNA, the poly(I:C). But when we exposed these eight- or nine-month-old animals to either poly(I:C) or ATP, as adults, what we can do is unmask a difference in the behavior of the animals that had autism-like behaviors or modified behaviors from those that did not.

And, you know, this was associated with an increase in body temperature that lasted for several weeks in mice associated with that postnatal damage associated molecular pattern. And -- you know, and so, one hypothesis that comes from that is that post-exertional malaise or this delayed malaise from stress can be associated with, you know, this final common denominator of stress signaling of ATP.

Well, we didn't want to confine ourselves to mice. So, we went on to look at, you know, a common, you know, immunologic stressor necessary for adaptive immune response. And that's just, you know, the routine immunizations in five-year-olds, or the pre-K. And we looked at the baseline metabolomics before and then two days after. And what interestingly we found is something that's very similar to the mouse situation, where after routine immunization, you know, there's a rise, a fall. And in the blue lines, the -- you know, these typically control -- the children without any other illness return to their baseline within just three to five days.

But the children who had preexisting autism had this kind of memory response, a metabolic memory response or an acquired hypersensitivity that maintained a higher body temperature. It was not febrile, but it was a higher body temperature for a -- nearly a month after the immunization. Okay. So then what metabolites were most changed in that post-immunization period? And it turns out that purines were increased 10 to 800-fold. They were the most changed class of molecules.

But interestingly, the autism ASD and typically developing controls did not have any difference in the level of the purine. So, adenosine, inosine, guanosine, and 7-methylguanosine, they were both the same in ASD and TD. But remember that the physiologic response to, you know, the immunization and the associated purinergic signaling was different despite having the same stimulus in the -- you know, similar to the -- you know, to what we observed in the mice. So, now, we went to exercise physiology and, you know, looked at some papers by groups around

the world. And this is a summary from a paper by De Boer, et al. that looked at how long COVID patients.

So, on the left-hand side, this is a marker. Mitochondrial fatty acid oxidation is measured -- calculated as a function of the increasing amounts of load on a bicycle ergometer. And you can see that the -- on the one hand, the baseline level capacity for mitochondrial fatty acid oxidation was lower. And then with load, it dramatically decreased. In controls, in the black, you can see that the IC50 is about 195. And in the endurance athletes, nearly -- it took nearly 320 watts of sustained power output in order to produce a 50 percent inhibition of mitochondrial fatty acid oxidation.

So, one hypothesis that comes from this is that we know as stress -- cells are stressed, there is a gated and a graded release of extracellular ATP with stress. And that one hypothesis is that the level of performance is proportional to the level of extracellular ATP signaling resistance. And likewise, you can come down on the other side of things and look on the long COVID side. And hypothesize that potentially, that early fatigue is associated with early extracellular ATP signaling sensitivity.

And an obvious question that comes up, well, if it's really pivoting on purinergic signaling, what would antipurinergic drugs do? Drugs that inhibit this acquired hypersensitivity, you know, what would they do? And then, you know, then the follow-up is, could these antipurinergic drugs actually be a concern as performance-enhancing drugs in athletes that are already healthy? So, I'll point out this exertion is really related to fatty acid -- mitochondrial fatty acid oxidation on the acute scale. Okay?

But as soon as the load is released, there's a dramatic increase, a rebound in fatty oxidation that's absolutely required for recovery and adaptation to that stress. All right. So, what about -- let's go back to ME/CFS. In a paper published out of Stanford and Lawson Group in Surgery, actually published that the total ATP production in peripheral blood mononuclear cells in patients with ME/CFS was increased by 2.5 times. Even though the oligomycin sensitive respiration, which is the mitochondrial aerobic ATP synthesis, was the same, okay, the glycolytic capacity of ATP synthesis was increased by about 2.5 percent. Okay.

So, a hypothesis is that potentially -- remember these are circulating cells. And if they're releasing extracellular ATP, they're actually signaling to other bodies throughout the vasculature, you know, and -- that there's danger that's been detected in the system. Okay. And interestingly, there were no actual changes in mitochondrial membrane potential, mitochondrial number, size, shape, et cetera. Many of these other, kind of, key aspects of mitochondrial metabolism were unaltered suggesting that this is a functional change in mitochondrial and cellular geology, I would say, so one conclusion.

The two primary functions of extracellular ATP signaling are, one, to actually cause fatigue and pain. These are used to signal the need for immobility to prevent further injury and basically stop doing what you're doing and to limit transmission of infection by microbial pathogens. And an example from exercise physiology is delayed onset muscle soreness after exercise could be produced by extracellular ATP signaling.

And then the key is to actually start the healing cycle without the -- its initial inflammation. And then the programmatic changes in mitochondrial function, healing cannot occur. So -- and I'll just say that that acquired hypersensitivity is a common denominator in a lot of post-infection fatigue syndromes, not just, you know, in ME/CFS but, you know, also, long-COVID, post-Lyme, post-coccidiomycosis. There's another syndrome, post-chemotherapy fragility also occurs.

So, I won't go into this other than when you're dealing with something that is changing at the bioenergetic level of the cell -- because every cellular function depends on energy -- this relationship of ATP to ADP, AMP adenosine -- which is really essential for sleep, and we'll hear more about that -- changes over a thousand functions that we can measure. Okay? That can all be traced back to the signaling. And I think I'm ending there. And would just recommend that this is actually a testable hypothesis that, you know, we could look at antipurinergic drug therapy in ME/CFS. So, I'll end there. So, thank you.

Vicky Whittemore: Thank you very much, Rob, for the very nice presentation. So, a question I had, I think, that you can help maybe all of us understand. So, glycolytic ATP synthesis, where does that take place, if not in the mitochondria?

Robert Naviaux: Cytoplasm. Yeah. So, the main thing is that you have to always think of the mitochondria and the cell as an endosymbiotic unit. Okay ? They are codependent on one another, and one cannot change without the other changing. And so, one of the primary drivers of glycolytic ATP synthesis is a decrease in mitochondrial ATP synthesis.

So, when one goes down, the other goes up. And, you know, that -- there's a -- you know, an important relationship there. So, I'll leave it at that.

Vicky Whittemore: Great. Thank you. So, there's a question. Is Rob Phair's research of ATP lost during Krebs Cycle unrelated to eATP that you're discussing here?

Robert Naviaux: No. It's related.

Vicky Whittemore: It's related?

Robert Naviaux: It's absolutely -- yeah. So, yeah. Sure.

Vicky Whittemore: Okay.

Robert Naviaux: It's absolutely related.

Vicky Whittemore: All right. Great. Another question. Does mold contribute to hyperpurinergia? Many people with ME/CFS say that mold toxicity caused their condition. Could you speak to that at all?

Robert Naviaux: Yeah. So, it's probably hard to paint all molds with a single brush. There are mycotoxins that have, you know, different functions. But, you know, I will say that microbial infections of many kinds, including those that generate mycotoxins, will have very significant effects on mitochondrial function.

And one of the first and most universal cellular responses to environmental threat of many, many different kinds is to dial back metabolism. Make the cell less dependent on energy resources from around and less dependent on oxygen because those things are unreliable at times of stress. Okay? So, if the cell can maintain survival even at compromised to function in the presence of stress, then it can live to fight another day. Okay?

And so, I know I've sidestepped the overall, you know, mold toxicity question. But I see the -- you know, the meta microbiome in our environment as, you know, part of our ecology. And you -- and, you know, it is one thing that can go wrong. It's not the only thing.

Vicky Whittemore: Right. Right. So, there's a question. Well, first a compliment. Thanks for the very inspiring talk. And the question is, can you summarize how eATP cannot initiate the healing cycle in ME/CFS? What do we understand about that?

Robert Naviaux: Oh, it does. The problem is that there's continued release of extracellular ATP early on. And then chronically, what happens is there's an adaptive hypersensitivity in purinergic signaling. The actual -- if you look at the receptors, you know, that are responding to ATP and ADP and even adenosine signaling, their steady state concentration on the cell membrane is changed.

And then just as a, you know, an example from some of our mouse studies, what we've seen is, surprisingly, P2X7, which is an early marker of inflammation, is actually downregulated, chronically, okay, in the mouse models with autism. But another related ionotropic purinergic receptor, P2X3, is increased. So, yeah. The details matter.

But I guess the important thing for the person asking that question is that continued sensitivity to extracellular ATP signaling makes the individual unable -- it makes -- the mitochondria within cells responding to those -- that signal are unable to progress to the -- you know, through the full -- all the stages of the healing cycle and get back to recovery.

So, it's not, in the chronic end of things, it's not so much the absolute amount of ATP that's released but the -- how the cell has hypersensitized itself, almost like, you know, how a person develops a -- I don't know. They're individuals who have, let's say, sensitivity to monosodium glutamate, you know?

That -- and family members, you know, can go to the same Chinese restaurant, eat the same thing without having any problems. So, that's kind of where we are, is that this -- at the very fundamental cellular level, the differences in response of the cells to that signaling has changed.

Vicky Whittemore: Great. Thank you very much for helping us to understand that and for your excellent talk. So, thank you very much.

Robert Naviaux: Thank you very much, Vicky.

Vicky Whittemore: And back to you, Craig.

H. Craig Heller: Yes. Thank you, Vicky and Bob. That was wonderful. I have a lot of things to follow up with you on . Our next speaker is Robert Phair, who is the genius of Integrative Bioinformatics, a company in Mountain View, California. And his talk is an update on the itaconate shunt and ME/CFS. Rob?

Robert Phair: Hello, everyone. I'm Rob Phair. I've been asked to talk about the itaconate shunt hypothesis in the context of ME/CFS physiology. Integrative Bioinformatics, as Craig says, is a scientific consulting firm. Our full-time job is to help find a cure for ME/CFS. My talk is divided into three quick physiological parts listed here.

Let's start with metabolism as seen through the lens of physiology. The physiologist view of metabolism centers on oxygen consumption. Indeed, ME/CFS research labs have measured oxygen consumption, both in human subjects and in their peripheral blood mononuclear cells, or PBMCs. Here are some published efficiencies of both all humans and PBMCs taken and measured in resting ME/CFS subjects and in controls.

What I mean by efficiency here is the ratio of oxygen consumption or VO2 in ME/CFS to oxygen consumption in a managed healthy control. And these have been reported in the range of 0.9 for the Vermeulen study and 0.8 or so in David Systrom's lab at Harvard. But in cells, the efficiencies are substantially less. And so, you can see right away that not every cell in a patient's body is sick. If they were, the body efficiency would be the same as a cell efficiency.

So, using these reported efficiencies and the equation for a cell-weighted VO2 in a patient, we can calculate that between 9 and 45 percent of a patient's cells are sick, as shown in this little table. So, 9 percent. Depending on the parameters you use from the literature, up to 45 percent of those cells are sick. There's some confirmation of this result that you can find in Andrew Grimson's single-cell RNA-Seq analysis in which he predicts that a fraction of monocytes are diseased. Not all of monocytes, but between 11 and 94 percent of monocytes in his analysis are diseased, with a mean of 66 percent. So, we reached the conclusion that not all ME patient cells are sick. From this, we arrive at a working definition of a sick cell. And we're thinking that that working definition is that a sick cell is one that is making insufficient ATP to do its work.

The whole idea that not all patient cells are sick is shared among several of us working in ME/CFS. Bhupesh Prusty and Bob Naviaux think of ME/CFS as a mosaic dysfunction disease. By which they mean that some cells are sick and some not. Ron Davis describes ME/CFS as a cell autonomous disease, by which he means that some cells are sick and others not. So, in our group, we see that symptoms depend on which cell types are sick and that the severity of the disease depends on the number and criticality of those sick cells. The important point here is that ME/CFS is cell autonomous or a mosaic disease. And if this is true, this has consequences for both data collection and interpretation of experimental data. I think we as a group should discuss

this point.

Now, let's turn to what we know about the itaconate shunts. We can ask, how does the itaconate shunt cause impaired oxygen consumption? The answer is that it does so by creating a short circuit in the TCA cycle. Cis-Aconitate decarboxylase is the key enzyme here. This is not a normal enzyme in the TCA cycle. Under some conditions, which we'll talk about in the next slide, CAD is produced and is imported into the mitochondria. It creates a branch point in the TCA cycle. Instead of allowing cis-Aconitate to proceed normally to isocitrate, it's -- it is instead converted to itaconate. So, this branch point diverts carbon away from the normal Krebs cycle and away from the energy-producing reactions shown in magenta in the normal Krebs cycle loop.

This pathway at the bottom of the slide with the blue molecules is what we call the itaconate shunt. It was a dead end until 2017 when the Mootha Lab at Harvard de-orphaned the enzyme CLYBL and showed that it's capable of converting citramalyl-CoA to pyruvate and acetyl-CoA, thus returning carbon that had entered the Krebs cycle back to the substrates that enter the Krebs cycle. This shunt, as I said earlier, only makes NADH at pyruvate dehydrogenase. And so, it dramatically reduces the efficiency of energy of carbon oxidation because you're not going through all the magenta processes. Of course, some molecules are traveling by the normal pathway. But this reduction in efficiency is produced by using the itaconate shunt. Even worse, the shunts may sequester coenzyme A in itaconyl-CoA and citramalyl-CoA, especially in individuals who have common damaging mutations in CLYBL.

So, you might ask, why would a cell do this? And that question brings us to the control and regulation section of my talk. The itaconate shunt is initiated by the innate immune system. In response to a triggering infection, the pattern recognition receptors shown on the left-side of this slide will initiate two different response pathways. The one indicated by the red arrow is the acute inflammatory pathway and ultimately results in the stimulation of adaptive immunity. The one we're going to follow today is the antiviral state pathway which is induced by the release of type 1 interferons from the infected cell. I'll typically refer to type 1 interferons as interferon alpha in the rest of the talk. That's the first blue box in the right-hand side of the diagram.

I'm just going to talk about the big picture here. This interferon alpha that's released from the infected cell is in effect a message sent to the neighboring cells that is a warning of a nearby infection saying, teleologically, "I want you to reduce energy available -- availability for these potential pathogens." So, the interferon alpha molecule interacts with its receptor, the interferon alpha receptor, and initiates JAK-STAT signaling in the adjacent cells. That signaling mechanism results in the upregulation of perhaps 300 genes. Fortunately, we only need to talk about three of them to understand the itaconate shunt response.

The first one is ACOD1 itself. ACOD1 is the gene that codes versus aconitate decarboxylase, the gene -- the protein -- the enzyme that starts the itaconate shunt. So, that's part of the innate immune response. The second gene we have to talk about is interferon alpha itself, which is also stimulated by JAK-STAT signaling. It is an interferon-stimulated gene. And so, you can see that creates the potential for a positive feedback loop.

You're starting the system with interferon alpha, and you're producing more. This response is supposed to be only temporary. It lasts perhaps hours or days at most. But -- and we're hypothesizing that it fails to turn off because the off switches of this system fail for a reason that we don't yet know. The off switches include SOCS3, indicated here, but also USP18, ATF3, and PIAS. All of these are molecules that evolution has supplied to turn off this positive feedback loop.

So, our hypothesis is that ME/CFS results from a chronic interferon alpha-driven itaconate shunt in effective cells. Naturally, this model predicts a localized chronic interferon alpha accumulation, a prediction that we've tested by measuring interferon alpha in plasma of ME/CFS patients and healthy controls. And what you see in this table is that that's a hard thing to do.

These measurements for interferon alpha and plasma are in femtograms per mil. That's three orders of magnitude below the standard commercial ELISA assays. So, we're using an ultrasensitive Quanterix SIMOA assay for interferon alpha. And the range of interferon alpha we measure in ME/CFS patients is from 1.3 femtograms per mil up to 71 femtograms per mil. The high values correspond to the most severe patients. Nevertheless, any differences between ME/CFS and healthy controls are for now beyond our reach even with the digital ELISA that is based on Poisson statistics.

So, here we encounter one of the vexing problems of a mosaic disease, high, local concentrations of interferon alpha surrounding only, say, 15 percent of the cells may be substantially diluted when measured in blood plasma. An alternative approach to our hypothesis to testing it is based on the observation that many immune cells extravasate from the blood and later return to the blood either directly across annular membranes or via lymphatics, lymph nodes and the thoracic duct.

During their tissue sojourns, it's possible that these immune cells might encounter the high interferon alpha concentration that we hypothesize in disease cells. And so, we've undertaken to measure interferon-stimulated genes in healthy control and ME/CFS PBMCs, peripheral blood mononuclear cells. And you can see that ACOD1 on the right is upregulated twofold in the ME/CFS cells and that the two off switch genes that we're measuring, SOCS3 and USP18, are either unchanged or downregulated. So, this is at least initially consistent with our hypothesis.

We've measured expression of four other genes in the acute inflammatory pathway, TNF, IL-1beta, CCL2, and IL-6. And as predicted, none of these are upregulated in the ME/CFS cells. But so far, as you can see here, we've only analyzed four of the 34 ME/CFS patients from whom we have frozen PBMCs. So, following the Grimson-Hansen single-cell RNA-seq data that are pointing to monocytes as a disease cell type, we've measured ACOD1 expression in CD14 monocytes as well.

And again, you can see that ACOD1 expression in ME/CFS cells on the left bar, is about twice, maybe even a little more than twice, as expressed as it is in healthy-control cells. In fact, in the Grimson Lab's first preprint on their single-cell RNA-Seq data, they say that monocytes are particularly likely to undergo continuous, improper recruitment to one or more tissues. So, it's interesting that in our hands, ACOD1 is induced in ME/CFS monocytes.

Now, I'll finish with two slides on pathophysiology. You can ask "If the hypothesis is correct, what drugs could help ME patients?" Thinking back to the interferon alpha positive feedback loop, we can envision three initial targets. First, we could block the interferon alpha receptor and break the positive feedback loop. This has the downside that the interferon alpha receptor is important for a lot of responses to infection.

Secondly, we could use JAK-STAT inhibitors. This is further downstream of the interferon alpha receptor. And, in fact, some of you will already know that Kenny de Meirleir is using a JAK-STAT inhibitor called filgotinib to treat seriously ill ME/CFS patients. And his last report, the six patients all report improvements. I do not know the details of that study, but it sounds promising.

And finally -- and this is the brainchild of Toto Olivera at the University of Utah. He suggested that we could block CAD itself, cis-Aconitate decarboxylase. If you recall, that's the first enzyme in the itaconate shunt. The idea here would be to restore the carbon flux through the normal TCA cycle and generate or regenerate normal NADH production and therefore, ATP. The first step in this approach was taken by Kelly Hughes at -- also at the Utah. And he expressed human CAD in E. coli, creating a screen for FDA-approved drugs. The idea here is that when human CAD was expressed in E. coli, they would not grow on acetate. And so then by screening FDA-approved drugs and finding that bisphosphonates in particular were able to restore growth in E. coli, he suggested that bisphosphonates could be blockers of human CAD.

Then a medicinal chemist also at Utah, Eric Schmidt, showed that bisphosphonates are in fact authentic inhibitors, competitive inhibitors of CAD. He did this with a classical enzyme assay. Another member of this interdepartmental group at Utah, Ted Liou, has gone so far as to run a retrospective case control study in COVID-19 on one of the bisphosphonates that successfully inhibits CAD. And that's in this slide. And what he's shown is that the probability of long

COVID in patients who have COVID-19 but who are taking zoledronic acid as a therapy for osteoporosis, is almost fourfold less than the probability of getting long COVID with COVID-19 patients who are not taking the drug. And this is true with a p-value of less than 0.05.

So, we're all asked to list research priorities from our own perspective. And I'll do that as my last slide. We're thinking that imaging, sorting, and sensor technologies for the detection and isolation of sick cells are really important. If we are trying to study the mechanism of ME/CFS, we need to be able to study the cells that are sick and preferably without contamination of cells that are healthy. I'm also recommending these ultra-sensitive, single-molecular ELISA assays. The one that we use for interferon alpha is from Quanterix. There are other platforms on the market already.

The reason this is important is that if only a small fraction of patient cells are sick, then biomarkers and mechanistic disease signatures can be all the way down at the attomolar or femtomolar concentrations in blood. These are, of course, not effector concentrations, but they are the mechanistic clues that we may need to identify the underlying mechanism of ME/CFS. These are Poisson statistics-aware technologies because we need to be able to detect rare events.

And finally, I'd like to suggest that we explore the nature of the predisposition to ME/CFS. I think that's a fundamentally underexplored part of this disease. So, I'll thank my colleagues Ann Chasson and Jason Zwolak at IBI, both outstanding software engineers; Chris Armstrong in Melbourne with whom we are doing tracer kinetics; and Ron's group at Stanford of experimentalists who have done the interferon alpha and uh RT-qPCR experiments; the group at Utah that I just mentioned; and finally, the funding from the Amar Foundation. Thank you very much. You're muted, Vicky.

Vicky Whittemore: There. Yeah. Sorry, I thought I unmuted. So, thanks so much for your presentation. So, we have some questions for you here. So, have you had a chance to study the cells of individuals with ME/CFS who've had the disease for years or decades and fluctuate in severity over time? So, you know, have you been able to look sort of longitudinally and study what's happening in individuals with ME/CFS?

Robert Phair: Yeah, that's a really good question. We, at the moment, have only taken one sample from each patient. Many of those patients have been sick for decades, but we do not have the time course yet.

Vicky Whittemore: Okay. And are CADs present in all types of cells or in just particular cells -- specific types of cells?

Robert Phair: The ACOD1 gene is expressed in all cell types, is universally expressed. So, if a

cell type is exposed to interferon alpha and expresses the JAK-STAT signaling pathway, then it will be expressed in all cells. One of the great things about this general hypothesis, from my perspective, is that the interferon alpha receptor is universally expressed. And as a result, that means that every cell on the body is capable of responding. And in some sense, every cell in the body is an immune cell, at least for the innate immune system.

Vicky Whittemore: Thanks. Yeah, thank you. So, what about the molecules that shut off this positive feedback loop? Has anyone tried to measure their concentration and their -- how that's changing in blood and plasma?

Robert Phair: Right. That's exactly what we're about to do with the expression analysis in ME/CFS cells and healthy-control cells.

Vicky Whittemore: Excellent. Okay.

Robert Phair: And we've seen that a little decrease in expression of SOCS3 is apparent in the early samples. But we need to do many more to know for sure.

Vicky Whittemore: So, with one minute left in your Q&A session, there's a request for you to give us the non-technical version. So, can you, , I guess in a couple of sentences, just tell us what the meaning and impact of your research is? The non-technical version.

Robert Phair: I think that ME/CFS is caused by an inability of cells to make ATP, and that inability is the response to this itaconate pathway, which shifts carbon metabolism away from the normal energy-producing pathway to an energy-not-producing pathway. So, the thinking is that if we can block this pathway, we can revert to normal cell function.

Vicky Whittemore: Perfect. Thank you. Yeah. And I think it's incredibly interesting that not all cells are sick. I think, yeah.

Robert Phair: I think that's an important feature.

Vicky Whittemore: It's an important key thing, I think.

Robert Phair: We should all talk about that some more.

Vicky Whittemore: Yeah. Excellent. Thanks so much, Rob. Right back to you, Craig. Thank you.

H. Craig Heller: Yes, thank you. Our next speaker is an individual whom we recruited as a lived-experience contributor. And unlike in our other webinars, the lived-experience contributors have ME/CFS, our speaker, Dominic Stanculescu from Uppsala University in Sweden, working with Jonas Bergquist, is the spouse of an individual with severe ME/CFS and has devoted his time to searching for common denominators in other diseases. So, Dominic, I'll turn it over to you.

Dominic Stanculescu: Thank you very much, Craig. Just a small correction. I'm not at Uppsala, I'm an independent researcher.

H. Craig Heller: Oh, okay. Sorry.

Dominic Stanculescu: No problem. Thank you. So, I'll present on the overlapping mechanisms in intensive care unit, ICU, and ME/CFS patients. But I am presenting on behalf of Jonas Bergquist and myself. Next slide, please. Firstly, a quick overview of the presentation. I'll start with an introduction to chronic critical illness, also called chronic ICU in short. Then I'll describe our initial findings regarding overlapping mechanisms in chronic critical illness and ME/CFS patients. And finally, I'll conclude with some of the lessons learned from critical illness research for ME/CFS. Next slide, please.

The critical illness is the physiological response to virtually any severe injury or infection such as head injury, burns, cardiac surgery, severe viral infection, and heat stroke. There is the acute phase in the first hours or days following the severe trauma or infection. It's characterized by mechanisms that shift energy and resources to essential organs and repair. Then patients that survive this acute phase will either start recovering or enter into what is called a prolonged or chronic phase.

For unknown reasons, they do not start recovering and continue to require intensive care. And with the one researcher, they're neither dying nor recovering. And here the symptoms of chronic critical illness include profound muscular weakness, cognitive impairment, pain, vulnerability to infection, et cetera.

The treatment of chronic illness remains an active area of research. Important here is that with the physiological processes during the acute phase are considered adaptive, there's generally consensus that the mechanisms during the chronic phase are maladaptive. They hinder the recovery. Moreover, also relevant is that of the patients that eventually recover from critical illness, their cognitive and/or physical disability can last for months or even years for unknown reasons. And the name for this is post-intensive care syndrome. Next slide, please.

The mechanisms which are considered central to chronic critical illness relate to the vascular, intestinal, and endocrine systems. They are hypoperfusions and endotheliopathy, intestinal injury, suppression of the pulsatile pituitary function, and also low thyroid hormone function, meaning activity at the tissue level of the thyroid hormone. And critical illness researchers often focus on one of these mechanisms and its relationship to inflammation. I'll present each of these mechanisms in the next slides. Next slide, please.

First, the hypoperfusion and endotheliopathy. So, during the acute phase of critical illness, researchers observe a redistribution of led away from the splanchnic area to the critical physios. It was considered adaptive in response to the stress of the severe injury or infection. But during the chronic phase, researchers observed inadequate oxygen circulation, hypoxia, endothelial dysfunction, even coagulation issues. And these are considered maladaptive. The mechanisms behind these apparent dysfunctions are not fully understood.

Researchers described ischemia/reperfusion contributing to tissue injury. This is in turn believed to contribute to organ dysfunction. They also described an initial cytokine surge leading to disproportions of endothelial structure and function. Some implications might be or that have been found are altered cerebral blood flow, increased blood-brain barrier permeability, and even cerebral hypertension. Next slide, please.

A further mechanism essential to chronic critical illness is intestinal injury. So, again, we have the acute phase. That's the stage for the chronic phase. And as I already mentioned, during the acute phase, researchers observed a cytokine surge and the -- and splanchnic hyperfused -- hypoperfusions. And then during the chronic phase, researchers described a vicious inflammatory cycle centered around intestinal injury. Here too, the mechanisms are not fully understood. Again, local ischemia and reperfusions. And redox imbalance leads to intestinal injuries.

And researchers have found a shift in the composition of intestinal microbes contributing to erosion of the mucus barriers. And some of the implications are the leaky guts, the travel of toxins from bacteria into the bloodstream, inflammation in the gut, leading often to decreased secretion of gastrointestinal hormones. And many researchers really consider the gut the motor of critical illness. Next slide, please.

A third mechanism central to chronic critical illness is a suppression of pulsatile -- of the pulse of the pituitary secretions of the endocrine gland at the central level. And essentially, there's an initial increase in the availability of hormones such as cortisol, which is considered adaptive. And then through negative feedback loops, this leads to a suppression of the pituitary secretions. Whereas, in critical ill patients that begin to recover, the hormone ACTH essentially normalize within 28 days of illness. And chronic critical illness, these pulsatile pituitary secretions of

ACTH growth hormone and TFH remained suppressed.

That adhere to the mechanisms and maintains the suppression are not fully understood. They're complex, but in -- essentially, they are inflammatory pathways maintaining the suppression. And the suppression is irrespective of the nature of the original inflectional trauma. And it is increasingly also considered maladaptive and requiring treatment irrespective of the initial infection trauma. The fact that the pulsatile secretion of the pituitary is suppressing the chronic phase was only discovered in the early 1990s with measurements of the frequency and amplitude of secretion via measurements of circulating hormones every 10 minutes over 24 hours in patients.

The suppression is not readily observable with single or average measurements of circulating hormones because the average or the circulating hormones are a function of both hormone release and hormone elimination from the bloodstream. Some implications, so, loss of ACTH versatility, compromised patient's ability to cope with external stressors, excessive inflammatory responses, loss of growth hormones, versatility results in muscle weakness, and also, interestingly, changes the activity of enzymes that determine thyroid hormone function. Next slide, please.

Right. The fourth mechanism essential to chronic critical illness is low thyroid hormone function, is one of the most studied mechanisms in critical illness essentially during the acute and early stages of critical illness -- excuse me -- yeah, the quick depression of thyroid hormone activity. They're responsible of peripheral mechanisms like changes in the half-life, the transport, the conversion, and cellular uptake of thyroid hormones. And this depression thyroid hormone function is considered protective downregulation to help conserve energy resources during the acute phase.

However, then these peripheral mechanisms plus central mechanisms alter the function of the HPT axis during the chronic phase, which are considered maladaptive and hamper the recovery of the patients. The term here used is non-thyroidal illness syndrome, NTIS, also euthyroid sick syndrome. Also important point here is that that the peripheral mechanisms, which I described earlier, can lead to important depressions in thyroid hormone activity at the tissue level is -- even without a responding minor change in blood concentrations of thyroid hormones. Researchers say that changes of thyroid hormone and thyroid hormone in circulations may be just the tip of the iceberg of depressed thyroid hormone function in target tissues. Next slide, please.

On this slide, you again see the mechanisms that are central to critical illness that I just described and some of the implications as well as errors to indicate interlinkages between them as they are described in the literature. The mechanisms have largely been studied in isolation, yet in the aggregate, that probably -- the answer to why illness perpetuates in the chronic phase. Next

slide, please.

A quick summary from the literature on chronic critical illness. So, in response to the stress of severe infection injury, the vascular system, intestines, endocrine axes, and thyroid hormone function experience profound alterations. Researchers believe that self-reinforcing interlinkages between these mechanisms, as well as visual cycles involving cytokines and inflammation, may perpetuate illness irrespective of an initial severe infection or injury. Next slide, please. But in the second part of the presentation should provide the overlap of mechanisms in chronic ICU and ME/CFS patients. Essentially, we were asking the questions, do mechanisms that prevent recovering chronic ICU patients also underlie ME/CFS? Next slide, please.

First, what do we know? And in our papers, we summarized the initial evidence of similar pathological mechanisms in ME/CFS. In interest of time, I won't go into the details. Next slide, please. I just want to mention that there is evidence from studies of the each HPA -- of the adrenal axis going back to the 1980s, showing that the pituitary ME/CFS patients does not respond as expected to stimulation test. The pituitary has a blunted response. Also, the magnitude of the HPA axis dysfunction becomes more pronounced with illness duration and associated with symptom severity.

Regarding low thyroid function -- hormone function, a paper by Ruiz-Núñez in 2018 found that on lower T3-to-rT3 ratios, two different types of thyroid hormones in the active and non-active form in ME/CFS patients. This -- as is found in critic -- chronic critical illness. And she writes that this is probably just the tip of the iceberg of genuine T3 deficit in target tissues in ME/CFS. The takeaway here is from this initial analysis, and we hope it gets expanded by others, if the elements that perpetuate hypermetabolic and inflammatory state and chronic critical illness also found in at least a subset of ME/CFS patients. Next slide, please.

The second. What don't we know in terms of the overlaps between ME/CFS and chronic critical illness? I think foremost, we do not know if there are alterations in pituitary pulsatile secretions in ME/CFS and which was critical to understanding the failure of patients to recover in ICU. And we don't know the implication for metabolic and immune functions, more for the severity of ME/CFS patients. And we also do not know much about the inter -- existence of interlinkages between these mechanisms in ME/CFS and the implications for the perpetuation of illness. Next slide, please.

Third, what are research priorities? What do we need to know? In our paper, we highlight five research priorities. Firstly, a comprehensive study of the pituitary pulsatile secretions from -- in ME/CFS patients. The study could really determine if there's a relationship between pituitary pulsatile secretions and the severity of the illness. We also highlight the need for a comprehensive study of the HPT axis or the thyroid hormone axis in ME/CFS, both the central

and peripheral functions. The study should determine if there's a relationship between thyroid hormone function and the severity of illness in ME/CFS. Next slide, please.

We also need to better understand the interlinkage between mechanism and the reciprocal relationship with inflammation, which chronic illness researchers have described, essentially, the linkages between intestinal injury and pituitary suppression and the role of the ghrelin hormone in influencing gross hormone secretions, linkages between pituitary suppression and low thyroid hormone function, linkages between low thyroid hormone function and endothelial function, and linkages between all the mechanisms and mitochondrial function. Next slide, please.

We also need study of the relevance of chronic illness treatments trials for ME/CFS. In one of our papers, we described some of the treatment trials and also the overlaps of treatment trials, which in ME/CFS and their potential as therapeutic avenues for ME/CFS. Finally, we also need a study of the commonalities in illnesses induced by physical infectious and/or emotional stressors including ME/CFS, chronic critical illness, which I described today, post-intensive care syndrome, cancer-related fatigue, post-viral fatigue, heat stroke, fibromyalgia, et cetera. Next slide, please.

To finish, I want to highlight some of the main takeaways from our papers, some lessons from critical illness for ME/CFS. An initial adaptive response to severe injury and infection can set the stage for maladaptive mechanisms and chronic illness. The same maladaptive mechanism can occur and persists regardless of the initial injury and infection. The interlinkages between mechanisms and reciprocal relationships with inflammation may explain the persistence of illness in the case of chronic critical illness, that a single or average measurements of circulating tropic and non-tropic hormone concentrations can fail to discern the dysfunctions of the endocrine axes, lessons from the critical illness research.

And there's initial evidence suggesting the existence of similar mechanisms in ME/CFS and chronic critical illness. And finally, we say that the collaboration between critical illness and ME/CFS research that would surely lead to improved understanding of those conditions. Next slide, please. This presentation is based on a series of papers that Jonas, others, and I collaborated on in 2021 and 2022. Next slide, please. Finally, we want to thank the support of the Open Medicine Foundation. And thank you for your attention.

Vicky Whittemore: So, thank you so much, Dominic. That was really excellent. So, some questions that have come up, I think, helping us to understand all this. Could you summarize the consequences of TSH reduction on the immune system? So, what is that interplay and what types of cells are involved in that?

Dominic Stanculescu: Yeah. So, the thyroid hormone function -- in this case I talk about

thyroid hormone functional, and the activity of thyroid hormone does impact the immune system. There's clear evidence of that also in the critical research field, but also in other fields including the natural killer cells where it was also a focus --

of much of ME/CFS research in the past. So, there is clear effect of, sorry, depressed thyroid hormone dysfunction on the immune system. But it also goes the other way around. So, there's a reciprocal relationship between, yeah, immune system and thyroid hormone function where the -- in that case actually the HPT, active in there, overall.

Vicky Whittemore: Thanks. Very interesting. Could you please describe any mechanisms regarding cortisol in the framework that you're outlining the interactions?

Dominic Stanculescu: So, in chronic critical illness, cortisol, the availability of product cortisol initially, why it was, one, considered -- believed that that was because more cortisol was produced, but it's not actually the fact. That it's the binding -- it's less bound in the -- in circulation and also less eliminated during the acute phase. And this -- the increase in cortisol then through negative feedback loops, has an impact on the entire endocrine axis. It would suppress it, yeah. At the later stages, it is believed that this suppression was maintained due to changes of cortisol receptors at also as a central level -- new cortisol receptors at the central level. Yeah, I hope that answers the question there.

Vicky Whittemore: Yeah. Thank you. So, I think a high-level question here is what are the treatment trials you think are most promising? And that, you know, if I were to give you money to say, "What trial would you do first? What -- or what studies do we need before we get to trials? What would that be?"

Dominic Stanculescu: So, regarding the trial, I think there's a researcher actually in Belgium at a university here who -- from -- chronic critical illness researcher who has, since the 90s, in an experimental way, on chronic critical illness patients, has worked to what they call reactivation of the central endocrine glands, so the pituitary. And she does that by -- her name is Van den Berghe -- she does that by supplying the pituitary with the hormones, which the pituitary would normally receive from the hypothalamus.

And they did it by supplying only the hormones for one axis, like HPA axis, then HTS axis, or the growth hormone axis or the HPT axis, which involves the thyroid one at a time. But then found that by doing a combination, they get the best results. But I think this -- but it was only then done on critical illness patients. It was done for five days to see what sort of effect it would have. Yeah, I think that's one area that -- that's very interesting to explore. So, the reactivation of the pituitary, they call it.

And another interesting area is the -- some researchers really describe this vicious cycle between inflammation, oxygen stress, and low thyroid hormone function. And so, to break that cycle and to take out oxidative stress or supply thyroid hormones . So, looking at those interlinkages between these different elements and trying to break that cycle is also another area where chronic illness researchers have looked into, which would be interesting.

Vicky Whittemore: Yeah, it's incredibly complex. Yeah.

Dominic Stanculescu: Yeah. But -- certainly complex -- but I think, , maybe one takeaway as like the less technical message as was asked for the previously, is yeah, chronic critical illness, there are similarities perhaps.

And they do involve several systems. And we can look at the research that has been performed in that field for the last decade, to see how can that inform ME/CFS research.

Vicky Whittemore: Yeah, absolutely. So, thanks so much, Dominic. That was really, really outstanding. I'm going to follow up with you about the research in Belgium that you just mentioned. So, that's very interesting.

Dominic Stanculescu: I'd be happy to.

Vicky Whittemore: Yeah. Thanks so much.

Dominic Stanculescu: Thank you very much, Vicky. Bye.

Vicky Whittemore: Bye. Sorry, Rob. We didn't get to your question. I just saw your hand. We have to move on, but we'll come back to some discussion later. Back to you, Craig.

H. Craig Heller: Thank you. And thank you Dominic. Very interesting. One of the characteristics of ME/CFS is called non-refreshing sleep. And it's a little bit difficult to analyze what non-refreshing sleep is because we don't know the functions of sleep. We know many possible functions. So, we thought that it might be interesting to first try to define what refreshing sleep is or how it's defined. So, we've asked Rebecca Robbins who is assistant professor at Harvard and a member of the Sleep and Circadian Division there. And we've asked -- she recently led a consensus report on what is refreshing sleep. So, we asked Rebecca to join us to give us a summary of her study.

Rebecca Robbins: Thank you so much for that warm introduction, Dr. Heller. It's great to see you and be with you all. I have to apologize. I have a very hard stop at 1:00 p.m. So, I'm going to be speaking a little bit quickly but hopefully hitting the major points. Next slide, please. Running through broadly as others have, what we know, what we don't know, what we need to know, and some priorities for future research. Next slide.

So, what do we know? The gold standard measures of sleep include objective assessments, quantitative, that we collect in laboratory settings, very controlled environments. And those can measure quantitative parameters, brainwaves. This person you see is undergoing a polysomnography overnight sleep recording. We're detecting brainwaves, respiratory rate, and a number of other quantitative parameters. In addition to measuring in the laboratory and controlled settings, there's a really burgeoning interest in sleep tracking. Some data published in 2018, although this number likely has gone up from them -- from that point, 25 percent of U.S. adults report interest in tracking and current behaviors and practices related to tracking their sleep with some sort of metric, some sort of device, a smartphone, a wearable device, et cetera. Next slide.

Now -- oh, thank you. Next slide. Put in some builds. Now, this is a growing term in the field, and that is sleep health. It's trying to shine a light on the fact that the quantitative assessment that we've spent so long talking about and measuring in the field of sleep duration and sleep staging. There are other dimensions that are really important to capture when it comes to sleep and, as Dr. Heller mentioned, shed light on its function and its implications for our daytime success. And so, this more holistic measure captures the idea of satisfaction with your sleep, how happy you were, and how alert you are as a consequence of your sleep. Next slide, please.

And one example in another disorder where insomnia patients will, on objective recordings, very atypically report any differences from an -- a patient without insomnia. But where there are striking differences is in these self-reports. So, someone who's suffering from insomnia will likely report their sleep to be very poor quality and the refreshment after waking very low. Whereas someone that doesn't have this condition will not report those things. But in many cases, the objective assessments of their sleep actually look really, really similar. So, just again,

important to shine a light on the qualitative and the restorative self-reports upon waking. Next slide.

What do we need to know? Next slide, please. Is capturing some of these qualitative assessments is a really important piece that gives us a much more broader understanding of sleep and potentially its implications. Now, with a medical librarian, we went to the literature. We looked at the articles on PubMed that mentioned the term restorative sleep. And 366 articles returned -- were returned to that search. But only 10 actually measured this notion of restoration or feelings of refreshment. There's a really rich literature on the other hand of the qualitative assessment of sleep quality. But those are often studies that are done with maybe a single item measure asking people to report their -- the quality of their sleep on a 1-to-5 Likert scale. But we really believe that there's more richness that needs to be captured. Next slide, please.

So, two key questions that kind of remained were, what is refreshing sleep? Clearly a lot of scholars -- and I think colloquially we use these terms a lot, you know, "Oh, I slept -- my slept -- sleep was so refreshing." Or "I feel so restored after my sleep." But we really don't know what that is. So, seeking a definition and then measurement for this construct. Next slide.

We embarked upon this in a paper. We conducted what's called a Delphi procedure to obtain a consensus from seven expert sleep scientists and clinicians. And through a series of iterative stages according to the Delphi procedure, we reached the following definition. And that is that restorative sleep is the aspect of sleep that's associated with improved subjective alertness, cognitive function, mood, energy, and/or wellbeing relative to the immediate pre-sleep period. The degree to which these aspects of daytime function show more or less improvement depends on the population being studied. So, as you'll see, a lot of our future research really calls for looking within populations that might be burdened by certain conditions or disorders. Next slide.

With our expert panel, we conducted a thorough review of the literature and the available qualitative assessments of sleep quality or feelings of refreshment. And we developed with our expert panel this nine-item questionnaire. It's the REST-Q questionnaire. You have that available in the link below. It's in the supplement section of the paper that we published in this consensus report. Nine items assessing different domains of restoration, a refreshment from sleep, feelings of tiredness, feelings of sleepiness, whether you're in a good mood, you're groggy, you're energetic, alert. And then we have some guidance on how to measure that construct. Next slide.

The next step in this project -- in this single paper, we have all these -- all this information and more. We looked -- we delivered the REST-Q to a large, nationally representative sample of adults. Now, these were largely healthy individuals without chronic conditions. But in the sample of adults, we found that reports of restorative sleep are very low. Reports of low or

somewhat were much more common, the reports of high feelings of restoration upon waking from sleep. So, it really shines a light on this potentially being a broader phenomenon but -- across the population, but also relevant to subpopulations such as those that we're discussing today. Next slide, please.

This is a correlation. This is available in the paper, but we looked at the relationships between the REST-Q and other single measures of sleep quality. So, that was the five items or the single-item question with the five-step Likert scale. How of good quality was your sleep? Very poor to excellent. Those with insomnia, sleep difficulties, either difficulty falling asleep, that's onset difficulties or maintenance difficulties have to do with waking and struggling to fall back asleep. And one of the kinds of main takeaways here is that many of these items are correlated and in the expected direction. So, that gave us confidence in our scale.

But the message here is that there's really still a lot of variances that's not explained by sleep duration alone. Only 30 percent correlation to REST-Q or 60 percent with those single-item measures of sleep quality but still suggesting -- and the sleep difficulties, you know, 40.4, correlation 0.41, really suggesting that the qualitative component, how restored, how refreshed a patient feels, is a really important aspect of sleep health. Next slide.

Just a couple of other analyses here we looked within a couple of these domains. And then categorizing the REST-Q scores, that was the score on your restorative sleep questionnaire, either low, somewhat, or high. Again, behaving in ways that we would expect, giving us further confidence about this measure but again, really not kind of a perfect relationship between high sleep quality and high restorative sleep. Responses here you see in panel A really suggesting that this is a nuanced construct that does deserve attention. Next slide.

So, what are some of the priorities for ME/CFS research? The first, I think, it would be interesting. Although I'm not an expert in this field, I just refer to you all. But the power of the REST-Q scale. So, it does have a couple subcomponents, the reports of grogginess, alertness, productivity. So, it might be interesting to explore the prevalence or reports of some of those subcomponents in this patient population. Number two, exploring the prevalence of restorative sleep using the REST-Q overall. So, looking at the prevalence of low, somewhat, or high scores in this subpopulation of patients. And third, looking at the relationship between REST-Q scores and then other metrics that might be important in this population, quality of life, treatment, et cetera. So, those are a couple ideas.

We'd love to open it up to a quick, few moments of discussion. And I'm so sorry to be a little bit pressed for time. Next slide I think just has my email address. Yes, if you have any follow-up questions, happy to take one or two questions now.

Vicky Whittemore: Thank you so much Rebecca. And I apologize that we're running behind.

Rebecca Robbins: No problem.

Vicky Whittemore: Certainly, appreciate you being here.

Rebecca Robbins: I just have a deadline. Thank you for having me.

Vicky Whittemore: Yeah. So, I don't see any questions. Are there any questions from the panelists? I have a question if no one else does. So, I often hear, talking to individuals with ME/CFS, that they're tired but wired. And so, they'll be exhausted, have trouble going to -- feel exhausted and have trouble going to sleep. And then wake up and not be able to go back to sleep.

Vicky Whittemore: So, I think, are those kinds of measures the kinds of things that REST-Q would be able to pick up or I guess measure how restorative the sleep was?

Rebecca Robbins: Really good question and sounds very similar to reports that we hear from patients who are suffering from insomnia, a very wired brain despite feeling physically exhausted and really struggling to power down. The REST-Q is really capturing the daytime consequences of those nighttime difficulties.

Vicky Whittemore: Okay.

Rebecca Robbins: So, in those cases, that sounds a little bit symptomatic. And Dr. Heller, please jump in. I'd be curious to your thoughts on this. But if that's a chronic issue, if it's happening most nights of the week, then it might be something to consider speaking to a healthcare provider about and/or maybe considering CBTI, the gold standard for insomnia treatment. And then we might see a boost in the REST-Q scores once those issues are addressed.

Vicky Whittemore: Yeah. Excellent. Thank you. Dr. Heller, any comments or questions from anyone else?

H. Craig Heller: No. I think we'd love to follow up with Rebecca after our next talk. So, maybe at our general discussion. So, I'm just wondering if you think that applying REST-Q to diagnostics of ME/CFS, would that have any value?

Rebecca Robbins: I think very much to understand the lived experience and its interrelationships to so many aspects of quality of life, treatment adherence, progression, et cetera. I think that would be a really interesting construct to capture. I would love to speak

further about that.

H. Craig Heller: Thank you.

Vicky Whittemore: Excellent. Thank you so much, Rebecca.

Rebecca Robbins: Thank you very much for having me.

H. Craig Heller: Okay. Moving on to our next talk. So, if we can understand the difference between refreshing and non-refreshing sleep, we might then wonder what mechanisms might be involved. And our next speaker, Dr. Maiken Nedergaard, who's professor at University of Copenhagen and also professor at University of Rochester, has been the leader in discovering and explaining, investigating what is proposed as one of the most recent functions of sleep.

I think we all recognize that sleep may have many functions, but some may be more important than others, and especially in the context of particular diseases. So, Dr. Nedergaard is the leader in the field of the glymphatic hypothesis of sleep function. So, Maiken, I'll turn it over to you.

Maiken Nedergaard: Thank you so much. And I will go straight. I have a lot of data to share. And I'm actually really excited about being here because this is what we are working at very actively. Let me just go down here. Let me see whether I can share it probably. Let's see here. I just need to get access to -- I don't think that Craig is aware that we are actually working very actively in this field and going beyond the glymphatic system but really try to understand the microarchitecture of restorative sleep.

So, if you look at the very basic question I would try to address is why is restorative so important for how we function? And our very basic answer is it's because it fulfills a function we just cannot do when we're awake. And in that scenario, I would say that the glymphatic cleaning system is only active during sleep.

So, what is the glymphatic system? It's very simple. It's basically like a dishwasher that turns on when we fall asleep. And the brain is organized such that the arteries are surrounded by perivascular spaces, and they link actually to subarachnoid space where we have CSF. And the brain has been so clever that it's actually using arterial pulsatility to pump fluid in -- along the perivascular spaces. And that would enter the brain parenchyma supported by the water channel aquaporin-4 that's expressed as astrocyte endfeet. Once the CSF enters the brain parenchyma, it would drag any solutes or any metabolic waste -- that can be lactate, it can be amyloid, it can be tau -- along with it. And it would accumulate primarily along the perivascular spaces. But also, it flows along the cranial and spinal nerve.

Just see here. I just want to show how it actually looks like because it's fairly dramatic. Here, we are looking at a mouse, and we want to look at glymphatic function. So, we're injecting into the subarachnoid space a green tracer when the mouse is awake and a blue tracer -- a red tracer when it's asleep. We have here labeled the vascular compartment with a blue indicator. And what you would see is, here, if you look at the sleep state, you will see that the red tracer enters the field and dip down into the penetrating periarterial spaces here. In the awake state, almost nothing happens.

If you're not convinced by that, I also want to show you that we have moved to microscopic imaging. And the beauty of microscopic imaging is we can look at the whole surface of the brain in either sleep or anesthesia or awake. And if we look at the sleeping mouse here, first, you will see that the tracer enters along the middle -- this is anterior -- the middle and the posterior cerebral artery. And it will fill the entire surface of the brain within just 30 minutes. If you, on the other hand, look at an awake mouse, you'll see that the awake brain is almost entirely capable of suppressing in-flow of the tracer that is injected into CSF.

I think this is remarkable because it's basically stating that neural activity is in control of the brain plumbing system. Just see here. What drives it? And that's, of course, the key because I think this is a key for restorative sleep. Here, Laura Lewis' group -- she's now at MIT -- had actually defined a driver that can only occur during sleep. And what she defined is that she looked at the very slow wave activity in patient using BOLD imaging.

So, every time you see this right here lightning up, that's because you have a burst of electrical energy because all -- basically, all the cells in cortex are firing at the same time in synchrony. And what she observed was, if you looked at fluid flow in the fourth ventricle, she consistently, after that burst of firing, saw that CSF moves in the fourth ventricle. This is the intercorrelation where she's linking the BOLD. So, this is an increase in neural activity. She's showing it's completely intercorrelated with CSF flow in the fourth ventricle.

What is the BOLD signal? The BOLD signal is basically increase in blood volume and blood oxygenation. So, we use that as a surrogate of neural activity. And based on this analysis, Laura defined a very beautiful connection and that is that slow wave EEG activity drive a blood volume increase that, in turn, drives the CSF flow. And why is that so? That we are actually going back to the very old principle that state that we have three compartments within the skull. We have the brain, we have blood, and we have CSF. We cannot compress brain. So, if blood volume increases here, the only thing that can be moved is CSF. So, they are exchanging each other. So, basically the brain has as a clever biological engineering defined that it used blood volume changes to drive the CSF flow.

What -- why are we interested in it? We are interested in it because we know that norepinephrine is a very important vasoconstrictor. So, we start to ask "Are these incredible waves that Laura Lewis sees, are they actually driven by changes in local norepinephrine in the brain?" To do that, we are using mice where we can more easily detect various physiological variables. So, we are using a norepinephrine snapper to detect norepinephrine in here, in prefrontal cortex, along with detecting the activity in calcium in locus coeruleus. That's the main producer of norepinephrine.

And if you look at this very simple trace, you can see here we have spiking activity detected as

calcium in locus coeruleus neuron. And you can see it's really correlated very tightly with norepinephrine release in the prefrontal cortex. And we can subdivide this into different brain states by just measuring EET and EMG. And what we noted right away was that we see during non-REM sleep here in light blue, we see this oscillation that we do not expect because everybody expects that norepinephrine is slow. So, if you enlarge that, you will see that we find that locus coeruleus firing. This correlative will increase this norepinephrine during non-REM sleep. And we can subdivide based on the EEG measurement, this norepinephrine oscillation into oscillation that does not cause EEG changes, those who cause EEG changes, and those that actually wake the mice up.

So, is this important? Could it be explanation on restorative sleep? Because norepinephrine is a vasoconstrictor, and it might drive glymphatic flow. First of all, we want to see, what can we learn from the literature? And if you first look at -- this is basically the norepinephrine oscillation broken down into the different components. And if you look here in close up, we can see that the sigma power is completely inversely correlated to the LC activation and the norepinephrine release.

So, why do I show that? I showed that because the EEG sigma power has multiple papers been shown to be linked to increase memory performance after sleep. It's basically a sign of healthy sleep. So, we thought we could use this as a first proxy. So, this is just additional correlation that shows how closely correlated norepinephrine and sigma power are. So, they're inversely correlated.

So, in order to define whether the sigma power -- well, it is implicated in the restorative action of sleep. We wanted to manipulate norepinephrine release and look at the sigma power and also memory performance. So, what we did was to use a genetic manipulation of locus coeruleus -- heavy-inhibited locus coeruleus. I don't have time to explain. It has been published, and we mentioned norepinephrine and sigma power.

And what you see is when you turn the light on and we're inhibiting the locus coeruleus activity, we can very quickly prolong this norepinephrine oscillation. And that actually triggers an increase in sigma power. This is plotted here. This is an increase in sigma power when we inhibit locus coeruleus. And we have -- if we use a novel object recognition test, we have a significant increase in memory performance.

Opposite, we can inhibit the norepinephrine oscillation by channelrhodopsin. So, we are here inhibiting that the locus coeruleus fire by, basically, having a positive feedback loop that detect norepinephrine and signal back and inhibit -- activate locus coeruleus when norepinephrine is at a certain level. And what we see here is that we can very significantly decrease sigma power, and we can also significantly decrease memory performance in these mice.

So, I think we can conclude that the norepinephrine oscillation involved in both in sigma power and in memory performance. But what happens if we do more extreme suppression of the norepinephrine oscillations? And here, we used an old work. It's desipramine that has been used as an antidepressant agent. And if you gave desipramine, you would see an increase in norepinephrine. And that increase in norepinephrine, basically because it's not taking up, but that would inhibit the oscillations.

So, if we look at the norepinephrine oscillation amplitude, it's decreased. And you can also look here that sigma power is very significantly reduced after we give this norepinephrine reuptake inhibitor. Most importantly, we can inhibit memory performance in the novel uptake recognition test. So, this is basically shaping the picture. Or at least, I want to try to convince you that norepinephrine oscillation during non-REM sleep appears to be important for memory information.

But what about the glymphatic system? And here, again, I want to go back to that norepinephrine is an important vasoconstrictor. So, how can we then look at these norepinephrine oscillations and the glymphatic system? First of all, we want to use the -- a concept Laura Lewis proposed. And that is that vascular volume changes drive the glymphatic system.

So, what did we do? We, again, went back to fiber photometry here, and we measured norepinephrine. And you see the beautiful oscillation during non-REM sleep here. Then we, instead of measuring another neurological marker, we are actually measuring blood volume by checking albumin. So, albumin is very much present in blood.

And if you check that by fluorescence style, you can measure blood volume very accurately. And what is clear here, even low magnification, is that, yes, norepinephrine is a very important constrictor because every time norepinephrine goes up, we have a constriction of the vascular volume. Opposite, when low -- when norepinephrine folds here during REM sleep, we have a huge decrease in vascular volume. And this is shown in a little bit higher magnification here to convince you that they're inversely correlated. So, it looks like norepinephrine is doing what we expected it to do. It changes vascular volume repeatedly during non-REM sleep.

What about the CSF flow because that's what we are interested in? The CSF flow surrounding the blood vessels that constrict and dilate is what -- is actually driving glymphatic clearance. So, how do we study blood volume norepinephrine and CFS volume? We can do that if we inject a cerebral spinal fluid tracer, as I showed you before, into CSF. So, here we have the CSF tracer. This is a very long recording, over, I think, eight hours. And you could see it slowly enter the tissue. We're here looking at ventral cortex, and it slowly disappeared.

We can measure the vascular volume. And we can basically subdivide the timing into different brain state by measuring EEG and EMG. And what do we find here? We find, as we expected and as predicted by Laura Lewis, said CSF and blood are inversely correlated. Every time blood volume decreases, we have an increase in what's called CSF volume. We know that that happens because of norepinephrine oscillation. This is just to show how it looks like. And we can actually -- if you take many, many animals correlate, we can make this correlation during non-REM sleep that norepinephrine -- and here, we are plotting the decrease here.

So, the norepinephrine oscillation that keeps oscillating, and the timing is about 20 to 50 seconds for each of these very slow wave activities. We get an increase in blood volume and a decrease in CSF. But note that the CFS signal increased. And this over many animals. I think about 22 animals, it increased. So, these changes in volume of the blood are actually driving CSF flow. And the same time, we can correlate it's the sigma power. We know it's a very good measure of memory performance after sleep.

So, the next question is -- this is, of course, looking at the pineal. We are using fiber photometry, looking at the little, tiny area of cortex. Can we look at global brain clearance and try to correlate it to this? Yes, we can do that. And here, we have used a new technique we have developed fairly recently. So, we used SPECT scanning. And how does it work? They basically just inject a very small tracer in the brain. And then we return the mice to the cage. They have both EEG and EMG electrodes, so we can measure the -- what you call the microdialysis that reflects the norepinephrine release. And then when we scan them five hours later, we can see that this little tracer injected into the brain is not only a spike in brain, but it's also spike to the kidney. And actually, the mice are peeing it out. So, this is a pee pad.

And if you measure the total radioactivity, we can very accurately calculate how much of the -- this little SPECT tracer has left the brain. And if you do that, I think it's pretty exciting. So, what we find is that if we look at a tracer in the brain here, it has nothing to do with how long the mouse actually slept in non-REM sleep. And this is all validated by EEG electrode. It has nothing to do with delta power. We expected that, but it did not. Here, we are looking at natural sleep. I would just want to emphasize that it's nothing to do with how often the mouse actually wakes up during the period. But it's correlated inversely to the number of micro-arousal sleep that is the same as the norepinephrine oscillation.

So, this is basically data in very strong support of Laura Lewis' concept she proposed. So, the frequency of micro-arousal sleep correlate with glymphatic clearance. So, we have -- for -- I actually made this very simple little model trying to explain what we believe happens. So, we believe that norepinephrine similar to -- in the macroscopic stages where too low norepinephrine is harmful for focus on retention, and too much also is harmful.

We actually believe that we have an inverted U-shaped curve that were -- we have an optimal number of norepinephrine oscillations here that perfectly well drive glymphatic flow and sigma power. But then if you gave too low amplitude of the norepinephrine oscillation, sleep is not restorative. If you, on the other hand, get a very high level of -- high number of this oscillation, it's not a restorative sleep because it does not drive glymphatic fluid well.

You can ask, because I've listed a whole bunch of things here. Much of it is work in progress. But we actually do have data suggesting -- and this is published from Penn Sleep Clinic -- that acute stress is greatly enhancing the frequency and shortening the duration of the norepinephrine oscillation. And we do know that sleep aid or, as I showed to you, norepinephrine reuptake inhibitor basically eliminates or almost eliminates the norepinephrine oscillations.

So, what are the priorities in my mind? The priority is really to take information like this and try to use biomarkers of restorative sleep. And that could be -- of course, this would not only drive glymphatic flow, at least no norepinephrine oscillation. But there are also changes to metabolic pattern and the electrophysiology of the brain. There's probably more to add to it.

So, this is really -- taking this new knowledge and understanding is the duration where these patients are sleeping, but sleep is not restorative because we are not having sigma power to enhance memory performance. And we are not having glymphatic flow to clear out beta amyloid that is clearly toxic to the brain. So, this is all I have to say. And I hope I didn't go over time, but this was -- so, thank you so much.

Vicky Whittemore: Thank you so much, Maiken. So, have there been studies of glymphatic flow in individuals with heavy CFS or the norepinephrine oscillations? Do we know anything about that?

Maiken Nedergaard: Yeah. In patients, there have been probably 500 papers now on glymphatic flow. And basically, any neurological disease you can list has decreased glymphatic flow very clearly. Glymphatic flow is basically -- require a very healthy brain. And any event that happen in a human life, that could be aging, it could be traumatic brain injury, it could be inflammation, would decrease glymphatic flow and also, protein accumulation, of course.

Vicky Whittemore: So, there's a question, could clinical cerebral and somatic oximetry -- sorry -- oximetry monitoring has any utility in determining non-restorative sleep or other sleep disorders caused by impairing systems?

Maiken Nedergaard: Yeah. That's a great question. And that's very feasible of it -- probably a very good approach to non-invasively detected. So, the idea is that we believe that it's

norepinephrine oscillation that drives the restorative sleep. Norepinephrine would be released not only in brain, but also systemic. And thereby, constrict vessels in the finger and cause a decrease in oxygen tension. So, Jeff Duyn at NIH had actually used that in his study showing the same type of oscillation as we are showing here.

Vicky Whittemore: Yeah. Very interesting. Thank you. Bob Naviaux, do you have a question?

Robert Naviaux: Yeah. I was wondering if you can detect changes in cellular volume that might be happening because mitochondria changed their volume within cells in -- you know, in accordance with what's available as substrate.

Maiken Nedergaard: Yeah. So, norepinephrine is very important volume control. Like, it's controlling especially as to show the volume. That's just the paper published on it. We have data we have not published. But norepinephrine is -- I have not looked -- nobody has looked at mitochondria, to my knowledge. But norepinephrine would block the sodium-potassium ATPase. So, every time you have norepinephrine release, astrocyte would expand. And that's actually the reason that glymphatic flow is slower in wakefulness or interval state. Because if you have expansion of cellular volume, you get a decrease in extracellular volume.

Robert Naviaux: Okay.

Maiken Nedergaard: And thereby, the resistance to fluid flow goes up.

Robert Naviaux: Right.

Maiken Nedergaard: So, norepinephrine is a key regulator of glymphatic flow, and it is by volume changes.

Robert Naviaux: Yeah. So, I mean, one of the things that we see in looking at, you know, MEG images, you know, MEG analysis of patients with traumatic brain injury and then also in children with mitochondrial diseases, that they can have wakeful delta wave sleep. So, in other words, we begun to think of, you know, non-REM, you know, delta wave sleep as the interval of which the most brain healing is occurring. And if it can't be done during sleep in some people with brain injury [laughs], then it's extended into wakefulness and, you know, in order to presumably achieve the same physiologic aims.

Robert Naviaux: Okay.

H. Craig Heller: I have a question of the relationship between your two talks. Maiken, you

have shown that the glymphatic clearance includes substances such as lactate. I'm wondering if there have been any measurements of ATP. Certainly, we know in the brain, ATP is a major factor in controlling sleep through hyperpolarization of saliva cortical neurons. So, you could imagine that brain fog could be -- and non-restorative sleep could partially be the effect of not adequate clearance of the extracellular ATP.

Maiken Nedergaard: Yeah. So, ATP is clearly higher in non-REM sleep than it is in wakefulness. I think it's as much as 1 million mol. And astrocytes are primarily regulated by purinergic signaling. This is what I used to study [laughs]. So, I was very happy to hear all this talk about ATP. So, purinergic signaling is a very ancient signaling. And astrocyte is, basically, simple epithelial-like cell. So, clearly, all of this is related. But there hasn't been -- it hasn't been explored to a great extent, mostly because it's hard to manipulate ATP. All the pharmacology is very dirty.

Robert Naviaux: Yeah.

Maiken Nedergaard: Any ATP receptor antagonist or agonist would also affect the degradation of ATP. So, you never know what you're actually doing.

Robert Naviaux: Right.

H. Craig Heller: Of course, you know --

Robert Naviaux: Well, you know a little bit what you're doing but --

Maiken Nedergaard: Yeah. I would doubt that. I doubt that.

Maiken Nedergaard: You need to go in and measure ATP, that biosensors to measure ATPs released now from [unintelligible]. So, ATP releases can be measured very accurately.

Robert Naviaux: That's also the kinetics of its metabolism, too, you know, and under these different diseases, you know, like, ATP, to ADP, to AMP, to adenosine. And there are some actual CNS-permeant, blood-brain permeant antagonists now, the purinergic receptor antagonists that might be tried to see their effect on glymphatic flow, cycling, and norepinephrine, you know, micro-arousals.

Maiken Nedergaard: Yeah. But I would definitely mention ATP before I trust the data. I've worked on purinergic signaling for a long time, and it's a very messy field. Because we have ATP, ADP agonist, AMP does little. And adenosine is a very important inhibitor of basically everything. So, it's really dirty.

H. Craig Heller: Anything would've been the --

Maiken Nedergaard: But what I do remember was there was actually -- 10 years ago or so, those whole field that injected ATP into blood in a fatigued patient, and they all felt better for a while. I don't know whether you -- I can find that literature if you don't know it well but --

Robert Naviaux: Yeah. So, Craig -- well, a lot of this actually goes back to, you know, people have tried ATP infusions in cancer patients.

Robert Naviaux: Kind of thinking, you know, low energy, maybe if I give them energy, you know, with ATP, that the cancer patients would get better. And those -- you know, it would really never -- it never showed any benefit. And honestly, I think, you know, they may have induced the -- a counterregulatory phenomenon that, you know, might not have been, you know, desirable. But -- yeah. I'd like to see the effects on sleep. I'd like to see anything you've got.

Robert Naviaux: Okay. Thank you.

Vicky Whittemore: Great. Thank you very much.

H. Craig Heller: Thank you.

Vicky Whittemore: Yeah. Thank you very much, Maiken, for an excellent presentation. Thank you for joining us. So, we're due for a break now. We're running about 15 minutes behind. So, Craig, a 10-minute break?

H. Craig Heller: I think that sounds good.

Vicky Whittemore: Okay. So, it's 1:00. We'll come back at 1:40. So, thank you, everyone.

H. Craig Heller: Okay. Well, thank you, everyone. Welcome back. I hope that was a 10-minute refreshing period. Our next speaker is Karl Tronstad from University of Bergen in Norway. We've been hearing a lot about metabolism and energy. And Karl is going to speak to us on metabolism and ME/CFS. Karl?

Karl Tronstad: Thank you, Craig, for the kind introduction. And it has been really interesting to listen to the other presentations that has been held today. And I think there are quite a lot of common elements and overlapping elements and -- with different perspectives. So, I will come with some of the focus that we are working on and -- yeah. We will see about the discussion afterwards.

So, I'm in a research team here in Bergen. I'm in the Department of Biomedicine at University of Bergen. And I collaborate closely with clinical partners at the Haukeland University Hospital. So, they are doing clinical investigations on ME. And we are working laboratory studies and we're collaborating on this translational path.

So, we are trying to find mechanisms and treatments and biomarkers for ME. And our underlying working hypothesis is that ME is due to an acquired error in the immune system and possibly similar to autoimmunity and that this creates some downstream effects. And at the clinic, they are also investigating strategy for a therapy to reboot parts of the immune system. And all this was started by Olav Mella and Øystein Fluge at the hospital. They work at the cancer ward. And they got some patients that had ME for a long time and then blood cancer. And they got better from the ME as well when they got the cancer treatment.

So, based on these observations, they'd made interest, and they made this hypothesis that there can be some immune-linked mechanism behind it. And they've done several clinical intervention trials, and they have established the biobank and also done observation of studies in patients. And in -- around 2015, my group started to collaborate with them. And we have been involved in the mechanistic investigations and also the comprehensive analysis of blood samples in association with the clinical trials they have been doing.

And then -- and now, I'm going to jump into what -- some of the things we are building on and some of the things we have found out in the cohorts that we have been investigating relating to metabolism. And then I will touch both on things we do know and don't know during this. So, basically, we have four elements that I will address during the talk here. We had done multiple studies in the field that have found changes in blood biochemistry in ME patients.

Now, we still haven't got that common metabolic phenotype for ME. But there are many indications that there is an underlying element of energy stress. And ATP, we have heard quite a lot about today. And also, there is quite significant evidence that tissue hypoxia is relevant and

involved, and that also we have heard quite a lot about today. And we have also, in our study, seen that there are metabolic subgroups that may be of clinical interest.

Now -- and there have been found changed levels of metabolites in blood and different mechanisms have been circulating. And these are not mutually exclusive. They are overlapping partly, and they may even be different parts of a -- the big picture, different pieces. But still, it is a problem that we don't have a kind of a common phenotype to relate to. And I think this is often due to the limitation we have in the field with underfunding and then only being able to do small-sized studies. Because we see the heterogeneity in patient groups, but we are not able to really follow up because we don't have enough patients and samples to do that.

But still, we have been focusing on this main axis of energy metabolism. And now, we are talking inside the cell and the intracellular ATP. And there are several studies. Here are some of them that have found changes related to this energy, main energy pathway, both related to lipid and amino acid metabolism and TCA metabolites and also redox status, and different other aspects as well. And some of these also linked -- have link to the immune system.

And the other sets of data have shown that there is a sign -- and there are signs of impaired blood supply to tissues and hypoxia effect, that this may contribute to the phenotype as seen in the patient, the phenotype of fatigue and physical impairment. And I think -- here are some examples of the -- maybe the strongest evidence we have now that this is a real thing in the patients that there are reduced oxygen delivery to issues, there are lactate overproduction. And this may -- these are linked to physical impairment.

And these data also show that this physical impairment is not only explained by deconditioning alone, being an effect of a long-term disease. It is something extra, and it is something that is maybe difficult to detect because this overlaps also with -- in part with symptoms of deconditioning. But this is something stronger. And that will take you into what hypoxia will cause inside tissues themselves.

Now, amplified hypoxia effects, they cause a metabolic shift towards glycolysis and reduced oxidative phosphorylation inside the cell. And this is due to the lack of oxygen. Now -- and the consequences will, of course, be metabolic compromises, more lactates. There will be a struggle for ATP. And this will be amplified during activity or during an energy-requiring state.

Now, the possible causes may be reduced oxygen supply to the cells due to vascular effects, for instance. But somewhat the same phenotypes are expressed in conditions of mitochondrial defects or diseases. And there are also some acquired diseases -- metabolic diseases that touch upon this kind of effect.

Now, the negative physiological impact will be lowered anaerobic threshold. That means that patients cannot do the same amount of work as they're healthy because they reached the anaerobic threshold and lactate production before. And then it can also lead to exercise intolerance, that actually there is such limitation that they cannot perform, and also possibly post-exertional malaise effects. This can be some kind -- different types of effects.

But the body has different ways to try to protect against damaging effects of this harmful condition and caused by the energy stress that it causes. These are regulatory programs, adaptations that are there to try to secure and maintain energy supply. And these are inherent programs activated, for instance, during starvation and exercise when there is an energy challenge in the body. And this is more of a shifting of fuels or energy fuels.

And it also have -- has a systemic impact. Different organ systems are contributing to this. And this is a rescue for -- to counteract the effects of hypoxia or energy stress. And then -- and they kind of activate alternative pathways to try to fuel this main access of energy production. The positive -- this has positive physiological impact. It tried to kind of protect. And therefore, it may have mitigating influence and symptoms in ME. But it's also influenced by multiple factors that are individual and context dependent. So, it is quite complex. It is kind of a counterbalance to the energy stress that has been created.

So, we can look more into the main inherent program activated when you have a hypoxia effect. First, the lack of oxygen will darkly inhibit oxidative phosphorylation because oxidative phosphorylation requires oxygen. But it will also activate the hypoxia-inducible factor 1-alpha, which is a transcription factor that is stabilized and activated when oxygen is lacking. And this will, for instance, induce angiogenesis to try to repair the lack of blood flow or oxygen supply to the tissue.

In addition to angiogenesis, the HIF-1-alpha will also mediate different effects at different levels on the main energy pathway. It will promote increased glycolysis. It will also promote inhibition of the pyruvate dehydrogenase enzyme. And this is because the pyruvate needs to be converted to lactate and be secreted because of the high flux of the glycolysis, and since the oxidative phosphorylation is inhibited under these conditions.

And the PDH kinase 1, we have found, is upregulated in ME patients. We found that in a study in 2016. And this is a kinase that has an inhibitory effect on the PDH enzyme. So, this is a direct sign that this axis or this hypoxic response is activated in the patients. Here are just -- here is the paper. We saw that. We mentioned amino acids in the blood of patients. We saw that particularly amino acid that fuels into the TCA cycle and the acetyl coenzyme A, they were reduced.

I mean, we made the hypothesis that pyruvate dehydrogenase may not be working as good as unhealthy. And we found that several inhibitors, as you can see on the right-hand side here, were upregulated. And these are inhibitory signals to the PDH enzyme. So, this is one indication that you have a kind of an anaerobic type of metabolism.

But earlier this year, there came another interesting paper from Wang and their co-workers. They found that a protein called WASF3 was overexpressed in ME patients. And they investigated the mechanism for how it was upregulated or could be upregulated. And they did this -- did it through cell and animal experiments. They induced endoplasmic reticulum stress and found that this increased the WASF3 expression and that this WASF3 overexpression led to mitochondrial inhibition and that this could explain exercise intolerance and fatigue.

So, this was a very interesting finding and mechanism. But there is also another mechanism whereby WASF3 is activated. Because in the gene of WASF3, there is a hypoxia response element which is targeted by HIF-1-alpha. So, this is actually an overexpression of WASF3. It's a part of the inherent hypoxia program. And it makes sense that upregulated WASF3 will contribute to inhibit oxidative phosphorylation under these conditions.

So, it would be interesting to do more to try to follow that path and see if there might be some more into the mechanism. I think findings like these are important because it gives the researcher some clues to make good in vitro models that they can use to really dig into the mechanisms. And for the patients, I also think that such findings are very important. Because it supports the notion that they are in a continuous exercise mode and also that they need to avoid reaching their anaerobic threshold to be able to control their symptoms.

Now, the -- in addition to -- when you have the hypoxia response activated and if this takes some time, there will be adaptive responses in the body that have to protect. And they will typically counterbalance the effects of the energy stress that is related. For instance, they will try to decrease glycolysis because glucose is a limited source. And they do this by inhibition of the PDH enzyme.

So, actually, both hypoxia and energy stress contribute to inhibit the PDH enzyme. But this time, it does it for another reason. It does it because it will also induce alternative fueling of energy substrates that go into the acetyl-CoA pool and then can fuel the TCA cycle. And this, of course, requires oxygen that there is oxygen available and oxidative phosphorylation can contribute. If there are limitations here, you will have an accumulation of acetyl coenzyme A. And then this will promote ketone body production. And actually, we have seen in a subgroup of ME patients that there are increased ketone bodies.

Here are some of the molecular factors that contribute to these kinds of adaptive effects. Some

of them have been investigated in relation to ME. But we know that cell types are an issue or that we know -- we need to know more about which cell types are relevant to study these kinds of mechanisms. But of course, the muscles are -- could be targets and also other organs. Liver, for instance, which is very important in these kinds of adaptations.

We have seen that there is an increased expression of PDK4 in the patients also. So, this will contribute also to the PDH inhibition in the patients. Another thing is that deregulated metabolism may occur. And especially in the lack of physical activity, this can be an amplified problem. So, it is important also to be aware of this that the dysregulated metabolism may actually add burden to the symptoms.

And we have done a metabolomic study based on the samples from the clinical studies. And we measured in 83 patients, and 35 had the controls, and we found quite many significantly changed metabolites. Many of these were amino acids and lipids. But we also saw this data heterogeneity. And we used this in order to cluster three different subsets of the ME patients that we saw three, and we call them metabotypes, metabolic phenotypes.

We can see it here in the PCA plot that there are three different subgroups, and we looked in detail what happened in these. And then we'll take the short version here. We did see common features of energy strain which were quite homogenous in the entire patient group. But then we saw these three subgroups that expressed different metabolic phenotypes. One paired similar to fasting in metabolism. It would increase the non-esterified fatty acids and ketone bodies and low triglycerides.

Another one and two, we called it -- it had a similarity to dyslipidemia or some mild insulin resistance. But it is important to notice that there are no diagnosed metabolic disease or diabetes or anything like this in -- among the patients. But they had something similar or low grade of this. They had high triglycerides and low free fatty acids.

And the third metabotype was -- it had some overlapping elements with the other three -- other two. But it was also more similar to the control. And we put it in a map like this where we think there is an underlying energy strain that may be caused by tissue hypoxia. And it can also be stress molecules involved here to amplify this and then -- that it is extortion sensitive.

And then overtime, you have these adaptions and compensations, which can be individual and influenced by many factors and have -- yeah, have a longer term impact. They can contribute -- both the common effects and the contextual effects can contribute to symptoms and severity, and maybe also the variable effects during -- overtime in the patients.

So, what do we need to know and what should be the research priorities? I will show you some

thoughts about how I think about this. I think we have quite some knowledge gaps to fill. Although we have quite many indications that something is wrong with blood supply, it can be hypoxia. There are -- energy stress. There are adoptions. There are different things. But we are still lacking a complete view of the molecular architecture of these things. And we need to find good ways to study those. And I think it's partly due to the small-sized studies we have been able to do because we have not been able to really see if there are multiple subtypes in any context and what this means.

So, we need to see how it is connected and really see if, in fact, oxygen delivery and uptake can be an issue and how this can be exacerbated by exertion. And very few studies have looked into post-exertional malaise and the molecular architecture of that. And also, the link is, of course, to the immune system dysregulation and how it is related.

I also think one thing, one aspect that has been very interesting is that several groups have reported that there is or are some active components in the blood that seems to be ME-specific and that can mediate effects on cells cultured in the laboratory. So, we did a study where we cultured healthy muscle cells and exposed them to ME serum. And we did see that there were signs of energy stress when they were cultured in this serum for some days. And other groups have found other things that indicate that there are some serum factors.

We don't know if it is a specific factor or several specific factors or if it is a combination of the cocktail effect of all the changes. But I think this is something we need to look into. We do not have good model systems for ME/CFS. But exposing cultured cells for MEC serum is maybe the most relevant model we have today.

Also, I think with a map like this, there are different ways we could think that supplements that medication could support parts of this. A systems group, they called it treatable neurovascular dysregulation, and they are doing clinical trials to address this. I mean, it's very interesting. And this will target the hypoxia-related problem and will remove then some other problem. It is also possible that we can target factors on this adaptive side in trying to mitigate or reduce the severity of symptoms. Because I think there are many aspects there that can, in some way, help the patients.

And these molecular regulators are very relevant in other diseases as well. And many of them have been relevant and targeted in cancer treatments. So, there may be from mycological ways you could -- we could use to try to do that. But it will probably not remove the disease, but it can help probably to -- again, some of the symptoms and maybe milden them.

And we think, you know, to be able to remove the disease, we have to go one step upstream and target the immune system, at least in a subgroup of patients. But still, there are problems there as

well because there are no objective biomarkers or markers down or objective outcome measures, which we really need. And also, there are -- we need more data and solid data, robust science on several parts, on the missing links in a working model like this.

So, in the big picture, I think the challenges are -- I guess most will agree to this. We have been discussing in different settings before. But the diagnostic uncertainties to and the lack of therapeutic approaches to insufficient evidence-based -- so, there are many things, which may rely more on the clinical aspects but -- which also, of course, affects how we can model the disease and how we can address the different questions in the laboratory.

And I think we need larger COA controlled studies and then harmonization of protocols in the cohorts in order to be able to get more comprehensive and solid data and validate findings, and really find out what are the mechanistic aspects of it. So, this was a little -- we're taking it really to the big picture. And finally, I would like to thank my collaborators, and thank you.

Vicky Whittemore: Yeah. Thank you very much, Karl. That was very comprehensive and really pointed out a lot of clearly important avenues of research we need to take. We're overtime. So, we're going to move on.

Karl Tronstad: Sorry.

Vicky Whittemore: Thank you, though.

Karl Tronstad: Yeah.

Vicky Whittemore: No. That was really very, very interesting. Over to you, Craig, for the next speaker.

H. Craig Heller: Sure. Our next speaker is someone who has been involved in ME/CFS research for a very long time, my colleague and friend, Ron Davis, at Stanford. And he's going to tell us about a new idea that he has, the relationship between BH4 and NO in ME/CFS. Ron.

Ronald W. Davis: It sounds like I can unmute. Make sure I'm unmuted.

H. Craig Heller: Yeah, you're unmuted. Now, you're muted. You were unmuted. Now, you're muted.

Ronald W. Davis: Okay. I think I'm unmuted now.

H. Craig Heller: Yeah, there you go. You're on.

Ronald W. Davis: Okay. Thank you. So, I want to talk about some of the new experiments that I've been doing. And I -- and this is going back to an old experiment, but I guess it's some insight. And that was the -- working with Hector Bonilla at Stanford and the clinic, and Laurel Crosby did all the analysis to find that Abilify was a very effective treatment.

And the evidence is that there's a large response in this study. And it's not a double-blind study, but it's about 75 percent of the people responded. My son is very severe, and we gave him Abilify. There are no side effects at the low dose of the 2 milligrams that was used. So, we kept giving it to him even though he didn't respond. And it took about three months, two to three months before he got any response.

That's a bit of a warning for a lot of the things that people try in treating these patients. If you give them a drug and there's no response and then -- in a few days, it doesn't mean it's not working. And I don't understand what's going on, but something had to be modified that was very slow to be modified. Abilify is thought to increase dopamine. So, that prompted us to look at how dopamine is made. Next slide.

So, these are the reactions that make L-DOPA which then gets converted to dopamine. And it's first made from phenylalanine. And phenylalanine gets converted to tyrosine and then tyrosine is converted to DOPA. These are all oxidation reactions. And they require BH4 to carry out that reaction. And of course, another one is the precursor to serotonin which is the same kind of oxidation reaction from tryptophan. So, these are possibly very important in the phenotypes of those diseases because of the -- of these hormones have big effects.

In the case of my son, we wanted to test this. Because if the BH4 is low that we will get a slow conversion from phenylalanine to tyrosine, and then loss of tyrosine, which goes to DOPA. And so, we gave him a bolus of phenylalanine, about the amount you're getting one day of food. And

then we followed that -- we're taking blood samples every hour and looked at the results from analysis of that blood. And his rate of phenylalanine to tyrosine was very slow. And tyrosine loss was slow. That actually qualified him to get KUVAN, which is a synthetic BH4. And that is in PKU. In other words, if BH4 is low, you can also get an accumulation of phenylalanine.

Interestingly, a lot of patients are fairly high in phenylalanine. Some people, earlier on, thought it was a basal-like biomarker, but I don't really think so. It's not necessarily consistent with everybody. Next slide. So, phenylalanine to DOPA pathway is very important. And BH4 levels are very important, I think, in this disease. BH4 is a little difficult to work with because it's so easily oxidized to BH3 or BH2. And if you take a blood sample, you have to be very careful to stabilize it immediately. The loss of BH4 after a blood draw is you lose about 1 percent of it every minute. So, a lot of times, people simply don't do the experiments correctly, I think. Next slide.

So, another reaction of -- involving BH4 and, again, an oxidation reaction very similar to the ones above is from arginine. And arginine ultimately gets converted to citrulline and nitric oxide. And this, the nitric oxide I've circled that, I think, may be very important. And its nitric oxide actually might be what's really going on in the patients as a result of the low levels of BH4.

So, we have to consider both of those. If you look at patients and look at the arginine to citrulline ratio, it's often high in patients indicating that this reaction is not happening very effectively. Next slide. So, BH4 deficiency is actually an old idea. There's no hypothesis about it, but it's never really been followed up on. And I think it's largely because it's kind of hard to detect. BH4 is usually detected by mass spectrometry. A lot of labs don't have adequate facilities.

We have set up to measure the quantitation of BH2, BH3, and BH4 by HPLC because there's a new high sensitivity detector that will allow us to detect the presence of BH4. We have detected it. We've looked at it in one patient only at the present time, and it is quite low. It's about 30 percent of what a healthy person has. That could have significant consequences. And we are planning to set up a major study after IRB is all approved to measure BH4 in lots of different -- and lots of ME/CFS patients and controls.

One of the interesting things that you find in the community, we often go to the community to get ideas. The Abilify came from a patient and that turned out to be quite valuable. But there is a bacteria that supposedly makes BH4 based on patients making yogurt from this bacteria and saying that they get great effects from it, and they argue that it must make BH4. That strain is available in Amazon, so it's easy to work with. We've analyzed the genome sequence of this organism, and it does not have the enzymes to make BH4.

So, we don't know what it's making and what effect it has on the patients, and that is something that we're trying to understand. It's possible that it makes some intermediate that can feed into the BH4 pathway once it gets into human. We'll have to look at that. But it would be very useful if it is, because it makes it easy for patients to get access to it. The Kuvan is actually quite expensive, and it -- you have to show that you need it in order to get insurance coverage. Next slide.

Well, the other thing is that -- that we've noticed is that patients are very low in iron, manganese and copper in the hair. And a lot of people don't like this because they say, oh, hair can't get contaminated, but in this case, we're looking at not high levels. And that's where this idea that hair is not a good thing because it can get contaminated with heavy metals and so forth. We're looking at low levels, and I don't think hair is easily decontaminated. And it's very common and it almost is a diagnostic marker for seeing these three metals being very low in the hair.

Well, how these metals get into cells is through a metal transporter. But that metal transporter has to be nitrosylated, and that requires BH4. So, it's possible that this low level is caused by the failure to have enough nitric oxide available to nitrosylate the transporter and get these into the cells. If you look at the blood when the hair is low, the blood seems to have adequate amounts, but that's not an indicator of what's in the cell.

We've been trying to develop an assay for looking at the cellular level of these metals, but it's complicated by the fact that it's hard to wash away anything that's surface bound. But this also could be a very important part of this because, for example, these models are extremely important in all sorts of reactions in a cell and the mitochondria, namely, manganese is responsible for eliminating reactive oxygen species. Next slide.

Well, one of the questions we've had is why does this disease last so long? And so, I think one of the issues is that we think this is a really a cellular autonomous disease. And that actually makes it difficult to study, because when you take out a population of cells, you may have a mixture. And Rob Phair has already mentioned this, a mixture of basically disease cells versus healthy cells. And so, the quantitation has become complicated and complicated to sort out. One possibility is to try to find some technology where we can separate out sick cells from healthy cells. And there's a number of ideas that we have for maybe doing that.

The other thing is that crashes are probably very important in this disease. And it's possible that energy depletion from exhaustion causes the crashes. And these crashes could maybe continue to reactivate the disease. I mentioned that because this is an n of 1, but I've talked to a lot of patients that have spontaneously cured. And one of them really believed the crashes were causing her to stay in the disease. And she went for a whole year without crashing, which is extremely difficult to do. But she is now completely over the disease. And that would be a great

treatment, but it's really hard for patients to do. Next slide.

Now, I want to also mention antibodies because that's another complication of studying this disease. We've seen a lot of patients that have come to us and saying that they were originally diagnosed with MS. But then later said they didn't have MS, they had ME/CFS instead. But there are some similarities. But one of the drugs for MS is Copaxone. And patients have often reported to us that Copaxone makes them feel better. And why would that be if they don't have MS?

So, we decided to explore this by looking to see if antibodies from patients can actually digest the myelin basic protein. And the answer is yes, it can, and it's very specific. Health controls don't do this. The concern, of course, is some other random protease that is present in the patients and it's causing the digestion. But you can look at this in terms of specificity, does it degrade other proteins, and mainly can it be inhibited by Copaxone? And the answer is yes. So, a small study of our -- we find that about half the patients have this degradation of myelin basic protein degradation, and they possibly then have MS.

And what we think is that patients can have both. And I think that's actually pretty relevant in terms of a lot of the physicians I've talked to say, I know you can't have two diseases by the same reaction and same exposure, but I think that's incorrect. And so, that's going to complicate things. But I don't think the autoantibodies that are present in these patients are always the same. And so, it's going to create some great complications because there may be -- because a lot of the heterogeneity we see in a lot of the studies. Because the MS is causing effects, and the autoantibodies are causing effects and they're not always the same. Next slide.

So, these are just our documentation that this cleavage is very specific. And over on the far right you can see this is the Copaxone addition and we get inhibition of the cleavage. Next slide. Here's another thing that I think could confuse things a little bit. And that is the activation of EBV. So, these are patients from Jonas Bergquist. And what we looked at here is the presence of EBV or herpes virus. We actually screened for 20 different herpes viruses. EBV was the most common. Next common was HHV6. But about 80 percent of the COVID patients have active DNA replication of EBV, and that's the complete genome. We assay multiple regions of the genome in this assay. That could be a complication in terms of Long COVID.

So, we know that EBV can cause ME/CFS. Is the reactivation of EBV the cause of Long COVID? That has to be figured out. Next slide. Well, we see the same kind of phenomenon in trauma patients. These are old samples from trauma. And we see that trauma patients can activate EBV as well, but not all trauma patients, and we're going to continue to study this. It may be very important to treating trauma patients. Next slide.

This is the group that we work with. I mentioned Robert Phair, who's talked. We really worked with him extensively. And I also want to thank all the donors. The donors are mostly patients. Vinod Khosla has been a major donor for us, and the Open Medicine Foundation has. Thank you.

Vicky Whittemore: Thank you, Ron. So, I encourage people to put questions in the Q&A or for any of the panelists to raise their hands. There's a question here. I'm not sure if you want to take this one or not, Ron. This is really to everyone. The question is why do ME/CFS symptoms so often get better during pregnancy? I've heard that they can also get worse, but I don't know if you want to answer that or anyone else on the panel.

Ronald W. Davis: Pregnancy has a big impact on antibody production in general. I'm not an expert in it, but I would be guessing that it -- you don't want it to have antibodies against the fetus and reject the fetus. And so, there may be mechanisms of suppression of autoantibodies. That may not have any direct effect on ME/CFS, but it may have effects on all the autoantibodies that are present. And it depends on what they are, how that will affect the patients. But I would suspect something like that. I've also heard that getting pregnant can actually cure ME/CFS, and that's even more interesting. But if that's true, that ought to be also looked at. Thank you.

Vicky Whittemore: So, I think your findings in ME/CFS are very interesting. Have individuals with MS been -- let me rephrase my question. Have they identified the same sort of MRI changes in the nervous system in individuals with ME/CFS that you see in MS?

Ronald W. Davis: There's some old stuff. I don't know of any more recent things. And it ought to be done again when we should do it with patients that we find these autoantibodies versus ones that don't have and look at the imaging. And Michelle James would be a great collaborator for doing that if she has time with all the other things that she's doing. But there are some old studies which spots that, similar to what you see in MS. And people were saying that's caused by ME/CFS and that's what I wanted to mention those things. Because you've got to be really careful now with this kind of thing that everything that you see of ME/CFS patient may not be directly from ME/CFS, but maybe from the secondary effects of the autoantibodies.

Also, interesting to me is that when you have a trauma event, you see a lot of autoantibodies. And so, that may be a very natural reaction of activation of innate immunity trying to protect the body. And autoantibodies can maybe protect if you have an infection that is reactive to it. But then as you heal, they get shut off. And in the case of ME/CFS, they're not getting shut off. The trauma also could be of useful modeling to look at how to maybe shut them off.

Vicky Whittemore: Right. Thank you very much, Ron. We'll move on to our next speaker. Over to you, Craig.

H. Craig Heller: Sure. Thanks, Ron. That was very interesting, lots of new insights. Our last speaker is Dr. Ludovic Giloteaux. I hope I got that right, who works with Maureen Hanson at Cornell. And he's going to tell us about some very recent and new data on extracellular vesicles. So, Ludovic, all yours.

Ludovic Giloteaux: Thank you. Can you see my slide?

H. Craig Heller: Yes.

Ludovic Giloteaux: Yeah. All right. Well, thank you for the introduction, and thank you for the opportunity to present this about extracellular vesicles. And I'm going to try to convince you that those little entities are really, really great. So, extracellular vesicles that I'm going to refer to EVs during this talk have been a topic of interest for the past 20 years now. And those entities represent a new dimension in cell-to-cell communication.

So, this quote from Clotilde Thery, which is a pioneer in the field of research since 1998, tells you pretty much everything about EVs. They are a small membrane molecule that carries signals to distant parts of the body and where they can impact multiple dimensions of cellular life. Sorry.

So, let's dig into it. What are EVs? As indicated by their name, they are extracellular structures with a lipid bilayer membrane that are usually spherical in shape, and they can be found in all body fluids, tears, lymph, blood, breast milk, urine, sweat, et cetera. They are usually differentiated based upon their biogenesis, either by exocytosis, and we refer to them as exosomes, or by budding of the outer membrane for micro vesicles or blebbing during apoptosis, forming apoptotic bodies.

So, on the right panel, you can see the size of these EVs compared to the size of a cell. But they also carry different functional proteins, either on the surface or as a cargo, for example, cytokines, heat shock proteins, and they also carry nucleotides such as RNAs, mainly being microRNAs and transfer RNAs.

So, why study them? Well, mainly because of all their functions, whether being physiological functions where they can use coagulation, regulate immune responses, or involve in communication between cells. But also, for their functions in pathological conditions. So, EVs from tumor cells can suppress immune cells or they can facilitate angiogenesis. But also because of their diagnostic and therapeutic applications, they can be drug nanocarriers that can stimulate the immune system in patients, and they can be used as biomarkers. It's been shown that EVs secreted by these cells have different RNA and protein content.

So, why study them in ME/CFS? Well, with the hypothesis in mind that they are abnormal contents, signals in EVs of ME/CFS cases, and that EVs are involved in cell to cell signaling. They can have profound effects on the functions of the cells resulting in abnormalities in ME/CFS patients' immune function and metabolism.

So, what do we know about EVs and ME/CFS? Well, there are less than ten articles that have been published on the subject since 2018. Three of these papers highlighted in red are coming from our lab at Cornell, and with the most recent edition that has been just accepted a week ago from today in the Journal of Extracellular Vesicles and coming from our lab. And I'm going to talk about it a little bit later.

So, here I summarized in the table those papers, all this publication. So, in half of this publication, cohorts from the study were females only, as you can see here. But in the other studies that included males, males were underrepresented compared to the females, ranging from one male for every three to six females. The subjects were recruited in Spain, U.K., Japan, but mainly in the United States. And EVs were isolated from serum in two of the studies, or from plasma in all the other ones.

So, ultracentrifugation has been the gold standard for EV isolation since the beginning of the research. But other methods were used in these studies, such as precipitation, or size exclusion chromatography, or affinity-based column methodologies. So, our group did precipitation in the past. But we switched to size exclusion chromatography to get a pure quality of EV preps with less protein contaminants for subsequent mass spectrometry analysis.

So, when you're studying EVs, the International Society of Extracellular Vesicles, ISEV, requires a full characterization of the isolated particles, including sizing, imaging, analysis of specific surface markers. So, sizing and quantification can be done with nanoparticle tracking analysis, flow cytometry, Zetasizer. Imaging is being checked by transmission electron microscopy or cryomicroscopy to look at their morphology. And the non-EV surface markers are verified by immunoblots, the cargo being -- can be analyzed using multiplex or mass spectrometry.

So, in this last column, it shows the analysis that we had done in each of these papers, but I'm going to go more into detail now. So, what do we know about their number? So, in all the papers, but one that I mentioned previously, it was found that the concentration of EVs is consistently higher in ME/CFS patients as compared to controls as you can see in all these box plots that have been taken from all these publications mentioned. And this result is consistent with other studies in other diseases such as Alzheimer's, Parkinson's, and HIV.

So, Castro-Marrero in 2018 were the very first to demonstrate that the concentration of

circulatory EVs were elevated in ME/CFS patients as compared to controls. They also find the EVs to be smaller in ME/CFS. Two years later, the group of Dr. Elisa Oltra in Spain were the first to analyze microRNA profiles from 15 ME/CFS patients and 15 controls, and they looked at 800 microRNA using the NanoString platform. They found nine microRNA in EVs that were significantly different in ME/CFS. When they looked at possible targets of those microRNA, they found neuronal and endocrine system pathways to be involved.

In 2020, Eguchi and colleagues were the very first to do proteomics analysis in EVs using mass spectrometry. And they only analyze three ME/CFS patients and three controls. And the protein identified and upregulated in ME/CFS patients were related to focal adhesion and actin skeletal regulation. Our group in 2020 looked at the cytokine profiling of plasma-derived EVs by measuring 45 cytokines in EVs from patients and controls. So, we found that the cytokine profiling could not distinguish patients and controls.

But in this study, we were also interested in how the cytokines played together or didn't play together, as I may say, by looking at cytokine-cytokine associations. So, on this complex graph, colored dots are the cytokines in both groups, pink for the controls, ME/CFS in blue. The gray lines are the positive associations in between the cytokines and the black lines are negative association or correlation in between the cytokines.

So, this analysis reveals very -- and you can see that there is a very dense network in the control group as compared to the ME/CFS group. So, this analysis revealed very different cytokine correlation patterns in ME/CFS as compared to the controls. More recently, Bonilla and colleagues did not isolate EVs, but stained them directly in plasma with surface markers that identify the EV cell type of origin. And they found higher levels of B cell and platelet originating EVs in ME/CFS.

Last year, Tsilioni and colleagues in 2022 were the first to examine the effect of exercise on the cargo of EVs in ME/CFS and controls. They showed that mitochondrial DNA associated with serum EVs was increased in ME/CFS following exercise, after exercise here. They also showed that EVs isolated from patients after exercise stimulated a significant release of the pro-inflammatory cytokine IL-1β from cultured microglial cells. Very recently, in collaboration with the Lipkin group at Columbia University, here at Cornell, we analyzed a larger cohort of subjects consisting of 49 patients and 49 controls.

So, samples were already analyzed for plasma cytokines and plasma proteomics at Columbia, and our lab isolated EVs from the same samples. Again, we found higher levels of EVs in patients, but we found also that IL-2 was significantly higher in EVs from ME/CFS subjects. We also find higher CSF2 and TNF-α correlated with greater physical and fatigue symptoms. And by measuring 15 plasma proteins and five EV proteins, we are able to discriminate with 86

percent accuracy ME/CFS cases and controls.

So, in summary, what do we know about their cargo? Well, that EV cargo is different between cases and controls, with one study showing EV microarray profiles to be different in ME/CFS. Our group showing cytokine networks to be dysregulated. That focal adhesion and actin related pathways are highly expressed in EVs from ME/CFS patients. And that mitochondrial DNA associated with serum EVs is increased in ME/CFS following exercise, which brings me to my next topic about what do we know about EVs, ME/CFS, and post-exertional malaise?

It's been well established in studies conducted mainly in healthy males, that EVs are rapidly released as soon as exercise begins. And that all EV classes have a higher concentration post-exercise, and that specific proteins refer to myokines or exerkines were found in EVs following exercise and altering different pathways. So, based on that, we ask ourselves, is the proteome of EVs different in ME/CFS, and does it change after an exercise challenge? With the hypothesis in mind that altered EV signaling in ME/CFS patients after an exercise challenge may contribute to the physiological, immunological, and metabolic alterations that occur during post-exertional malaise.

So, it is possible that patients EVs are carrying aberrant signaling molecules, that signals that should be transmitted in EVs post-exercise are missing in patients, and or that the EV response to exercise is temporarily dysregulated. So, therefore, our objective and the originality of this paper was to study to characterize the EV proteome before and after exercise in female ME/CFS patients compared to healthy sedentary controls. And again -- so this paper was just accepted for publication last week. And if you're interested, I really recommend you to go check it out.

So, for this study, we have included 18 female ME/CFS patients and 17 age- and BMI-matched healthy sedentary controls. All subjects provided blood samples, prior cardiopulmonary exercise test, 15 minutes post-exercise, and 24 hours later. When the healthy control subjects have recovered from exercise but most of the CFS patients are experiencing PEM. All blood samples were collected prior to March 2020. EVs were isolated by size exclusion chromatography and characterized using nanoparticle tracking analysis. Transmission electron microscopy and mass spectrometry, and targeted proteomics was used to determine the protein content of EVs.

We were able to detect 862 proteins, but we analyzed 301 after filtering out proteins with more than a third of missing values. Random forest was used to impute remaining missing values. So, as I mentioned before, we quantified the EVs with nanoparticle tracking analysis. And our results of significantly higher EV concentration at baseline are again consistent with the other ME/CFS publication that I previously mentioned. Fifteen minutes post-exercise, they were still significantly higher levels of EVs in ME/CFS as compared to the controls, but not 24 hours post-exercise.

Also, we observed that the total concentration of EVs increased significantly post-exercise in the controls from baseline 15 minutes, 24 hours later. And this post-exercise increase in EVs is not -- so, we didn't see it in ME/CFS patients. The disposed exercise increasing -- this is consistent with other studies that were done in healthy subjects -- with healthy subjects. We then looked at differences in the EV proteome in ME/CFS patients versus controls at all the three different time points baseline, 15 minutes, and 24 hours. We used a bootstrapping approach to statistically compare the two groups at each time point.

So, the plot shows the median log2 fold change of ME/CFS versus control. Sorry. The fold changes ME/CFS versus controls. The red dots show proteins that are significantly different after FDR adjustment of the 95 percent confidence interval. We found the most differences at 15 minutes post exercise. And looking at an enrichment analysis for those most definitely abandoned 15 minutes post-exercise, we found that the significantly enriched pathways primarily included platelet degradation, activation and aggregation, or other platelet functions such as hemostasis, clotting cascade. The proteins are also involved in several immune-related pathways and muscle contraction.

We also looked at changes in protein abundance in EVs over time within the ME/CFS group and the control groups. So, the controls shown on the right in red had 197 differentially abundant proteins in EVs at 15 minutes post exercise, whereas we only found 63 in the ME/CFS group, as shown by the blue dots in this volcano plot here on the left. Forty-five of the proteins were significantly altered in both groups, as you can see in the Venn diagram. Changes in the EV protein abundance post-exercise has also been previously demonstrated in healthy and athletic males but had not been previously studied in females.

We also look at correlation with the change in symptoms after exercise. So, the change in symptom score -- let's hear delta myalgia, for example, is calculated by the score for the symptom at 24 hours minus the score at baseline. So, zero hours. Thus, a negative delta score indicates an improvement in that symptom following exercise, while a positive score indicates that the symptom got worse 24 hours post exercise. So, we found seven proteins in EVs in which the change in protein levels correlated with the change in myalgia or muscle tenderness and pain, such that an increase in the protein level, for example, this one, corresponding to an increase in the symptom post-exercise.

So, to comment on some of those, we found tropomodulin TMOD3 and tropomyosin TPM4 that are involved in muscle contraction, and thrombospondin, which was shown to be increasing ME/CFS in plasma in ME/CFS by the Doctor Alan Moore's group. We also found a very strong, significant correlation between post-exertional malaise, 24 hours post exercise, fatigue, and EV protein levels.

So, in conclusion to this study, healthy sedentary controls in the proteome changed drastically 15 minutes post exercise. EV signaling post-exercise is highly dysregulated in females ME/CFS patients, and there are many strong and significant correlations between changes in EV protein levels post exercise and ME/CFS patients' symptoms, including myalgia. And again, I invite you to check out this new paper. I'd like to acknowledge for this study our study participants, our collaborators, and the funding agencies.

And to end my talk, what don't we know and what we need to know on extracellular vesicles and ME/CFS? Here are the lists of the research priorities. So, where do they come from and where do they go? What about other biofluids and other tissues? I mentioned the paper published so far looked at serum and plasma. We should look at CSF urine tissues such as muscles. Correlation between plasma proteomics with EV proteomics should be done. And we have shown that the EV signaling can change very rapidly upon provocation. So, more longitudinal studies are needed with different types of provocation such as cognitive testing, for example. We need more sex stratified analysis in both males and females with -- and larger cohorts. And also, look at the different cargo analysis, such as the lipids or metabolites. And with that, I'd like to thank you.

Vicky Whittemore: Thank you very much, Ludovic. That was really very excellent.

Vicky Whittemore: So, I'll start with the first question. And if I miss this, I'm sorry. So, at baseline, are EVs the same in ME/CFS versus controls prior to provocation?

Ludovic Giloteaux: In our study--

Vicky Whittemore: Yeah, in your study.

Ludovic Giloteaux: In our study, they are, like, the same.

Vicky Whittemore: Before the provocation, but then they rapidly increase.

Ludovic Giloteaux: The main differences we are seeing are like, after post-exercise, a different post-exercise.

Vicky Whittemore: Right. So, yeah, I think it will be really interesting to really understand where these EVs are coming from, because my understanding is that the technology, I'm not sure it's all the way there, but is improving such that you can identify sort of essentially what tissue type or where those EVs have derived from. Is that correct?

Ludovic Giloteaux: Yeah. So, what we did in this paper -- I couldn't show everything because we have a lot of figures. We did a lot of analysis. For the sake of time, I couldn't present everything but what we did, we compared those differentially abundant proteins, post-exercise to reference databases. So, this is not telling the source of the EVs in the plasma, but it links the protein cargo, actually, of the EVs to the tissue and the cell types that they may have originated. And we found very, very interesting differences actually.

For example, in the -- unique to the controls, we found that smooth muscles and myoblasts were involved, which were not seen in the ME/CFS group. And for the ME/CFS group, we saw that many, many immune cell types were enriched, such as CD4, T cell memories and non-classical monocytes that were not seen in the control group. And again, there are -- so, I've been lucky enough to go to several of these international conferences on EVs. And there is always a topic where they are trying to find markers by using like immunoaffinity to capture them and mainly from brain origin. So, there are several potential markers right now to be used in future experiments. Ideally, it would be to have a marker for like the muscles one and analyze them, but not that I'm aware of here.

Vicky Whittemore: Okay. Right. Thank you. Rob, do you have a question?

Robert Phair: I do. I'm wondering whether the micro vesicles have selective incorporation of cytosolic samples for selective incorporation of membrane proteins at the time of formation, or

are they random samples of the cytosol or the membrane of the cell of origin?

Ludovic Giloteaux: Yeah, that's the question I wondered too if it feels selective or not. What if -- you know, the site of inflammation -- if there is a site of inflammation, requires a very specific site of -- very specific proteins, other cells are programmed to load in those vesicles so they can go travel to the site of inflammation. I don't know exactly if there is a program for that.

Robert Phair: Have people looked at micro vesicles formed in cell culture when you have only a single cell type as the source?

Ludovic Giloteaux: Oh, yeah. There have been experiments on that. Yes.

Robert Phair: And are those micro vesicles that are formed a very different composition or are they all the same?

Ludovic Giloteaux: They were. So, they were pretty much the same, I think.

Robert Phair: Thank you.

H. Craig Heller: Are there any experiments which show a direct relationship between cell damage and EV production? I would imagine that would be an organ culture system or an animal study.

Ludovic Giloteaux: I don't --

Robert Phair: I could answer a little bit about that, Ludovic. There are extensive publications from the cancer literature on EVs and cancer cell cultures and non-neoplastic precursors of certain, you know, cell types. And, you know, it's very true that basically, you choose any kind of stress and whether it's exercise or, you know, ionizing radiation or hypoxia, EVs go up. So, EVs are the little thought bubbles the cell is sending out saying, "I'm in trouble," you know. And, you know, whether part of that signal is to help, you know, or to just pass on that signal is, you know, kind of probably cell dependent.

Ludovic Giloteaux: Yeah, as soon as the needle goes inside the arm to draw blood, actually, EVs are released right away. You just look at it and exercise. Excuse me?

H. Craig Heller: How can you distinguish between cell damage and signaling? In other words, is getting rid of damaged proteins, damaged membrane, and so forth, is that the cause of EVs, or are they as -- is just signaling?

Ludovic Giloteaux: I mean, they could act as trash, yes, to get rid of like, the very bad proteins, or they could act as, like the signaling proteins. Oh, let's bring beneficial proteins to help coagulation processes, for example. But that I don't know when it happens, when is it a trash or when is it not.

Robert Phair: And you know, there's cancer cell experiments where inhibition of thymidine kinase or, you know, active radiation is used to stimulate the EVs. And then you purify the EVs and then put them onto untreated cells and they can start to die. So, there's an innocent bystander effect that can be documented, you know, early in the cancer, you know, side of things.

Vicky Whittemore: Thank you, Ludovic. We have just about five minutes left in the webinar. So, I'll ask all of the panelists to turn on their cameras and see if you have any questions for one another or any additional conversation you would like to have.

Robert Phair: I'm kind of curious, Ludovic and Maureen about, you know, how far you've gone down the lipidome end of things. Because, you know, there's strange things with surface volume ratios that happen when you get down to nano scale, you know, vesicles. And we used to purify these a lot from human urine. And what we found is if you calculate the surface area that is excreted in extracellular vesicles and the urine, it's like a third of our whole -- it's like a half a meter, you know, squared of surface area membranes that are excreted in the urine. But the total included volume was five microliters.

So, you know, they're -- so, lipids are potentially a big part of EV signals. And we know that sphingolipids and you know, sphingomyelins and ceramides are enriched in EVs at least in the exercise and I'm assuming in other areas too.

Ludovic Giloteaux: Yeah. We didn't do anything yet on the lipids analysis on our EVs so far.

Robert Phair: Is there evidence that EVs are specifically targeted to target receptors or targeted cell types?

Ludovic Giloteaux: Well, they do have some receptors very specific on their surface membrane, yes. So, some of them will be targeted to very specific cells. And it's when all the uptake is going to happen when one EV is going to go attach to this very specific cell and deliver his cargo.

Maureen Hanson: So, I have a question for Rob, actually. With regard to your talk about the sick cells, these cells are sick. They have higher interferon alpha. I'm wondering if it's possible that they're not sick, but they're doing what they're supposed to do, that they are fighting against a chronic infection. Do you have any evidence? I mean, do you have any comments about that?

Isn't that a possibility?

Robert Phair: I think it's a possibility. There's a question here about whether it's interferon alfa that's signaling an innate immune response or whether the cell is secreting interferon alfa to pass information to other cells. But the notion of a chronic infection is certainly one of the mechanisms that could initiate the pattern recognition receptors and keep the innate immune response going.

Robert Naviaux: Yeah. There's an interesting connection with the ecology of planktonic cells in rivers that under stress, you know, the eukaryotic algae or even, you know, you know, certain bacteria will adopt smaller forms that are dramatically decreased in their metabolic rate. How much, you know, oxygen they consume? You know, how many other, you know, chemicals from the environment they consume? So, what's interesting is that the conversion from healthy growing cells to survival cells is a deterministic -- like, it's a very reproducible thing. But as they flow down the river, it takes -- they keep assuming it's dangerous. Okay. The cells basically, they have this evolutionarily -- you know, one would assume, you know, advantageous it's to be, you know, program to be skeptical.

And because anybody that opens up and becomes a rapidly growing cell in a polluted area of the river dies. So, it's actually healthy to even if there are no infections going on, to, you know, continuously -- it just, you know, continuously wait until you receive either more safety signals or signals that, you know, the nutrient environment is sufficient in the -- and toxins are absent. But that's a stochastic event. So, deterministic and stochastic out is, you know, how a lot of those cells do it. And I wouldn't be surprised if that's connected, you know, if our cells are doing that in ME/CFS.

Vicky Whittemore: Well, at this point we're at the top of the hour, so we need to sign off. I just want to thank everyone for participating in this webinar, especially our speakers and in particular also Dr. Craig Heller. So, thank you very much, Craig and the whole webinar planning group who organized this webinar. So, we look forward to seeing you at the next webinar on January 5th. Thanks, everyone.

H. Craig Heller: Thanks, everyone.