

ME/CFS Research Roadmap Webinar Series – Chronic Infections
Open Session
Thursday, November 30, 2023

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Vicky Whittemore: Okay. I think we'll get started. Welcome, everyone. So, I'm Vicky Whittemore, a program director at the National Institute of Neurological Disorders and Stroke, where I oversee grants on ME/CFS, and work together with my colleague Joe Breen and Dr. Koroshetz to coordinate the Trans-NIH Working Group. So, it's our pleasure to present today the fifth webinar in the series.

We've been working with a really excellent group of investigators, clinicians, and individuals with lived experience, as well as members and leaders of patient advocacy groups as part of the NINDS Advisory Council ME/CFS Research Roadmap Working Group. So, you can see the list of all the individuals here who are on the working group. The co-chairs are Lucinda Bateman from Bateman Horne Center, and the other co-chair is Maureen Hanson from Cornell University.

So, I'd like to thank and acknowledge the working group that planned this particular webinar. The chair is Maureen Hanson. And these individuals have all served to work with Maureen to organize this webinar. I'd also like to thank my team at NINDS, who have been really fantastic in helping to really coordinate things behind-the-scenes. And also acknowledge our partners at RLA for all of their logistical and -- help and coordination for all of the webinars as part of this webinar series.

So, as I said, this is the fifth in a series of eight. So, after this webinar today, we have three more, one December 8th on physiology, January 5th on lesser study pathologies, and one January 11th on circulation. For more information about any of these research roadmap initiatives, you can go to the website. If you go to NINDS website and just type in ME/CFS research roadmap, you'll get -- that will take you to information about this working group and all of this -- the webinars. Video recordings and transcripts from past webinars are posted on the Research Roadmap website, which is there. You can get to it through this website or also, you can get to it again through the NINDS website.

So, just some guidance for participation in the webinar today. The goal of the webinar is really to identify research priorities for research on the role of chronic infection in ME/CFS. What do we know? What don't we know? And what do we need to know to accelerate research? And how will this information help us to inform and identify new targets for treatment of ME/CFS?

So, questions for each speaker will be addressed immediately after their presentation. So, put your questions in the Q&A. We are unable to answer questions that pertain directly to your individual health situation. So, please refrain from putting those types of questions in the chat or

in the Q&A. We're really looking here to talk about research priorities moving forward. At the end of this whole process, after all eight of the webinars, we'll be putting together a report that will go to the NINDS advisory council and NINDS leadership and will be presented at their May 2024 meeting.

For additional feedback, you can send an email to this email address, mecfsresearchroadmap@ninds.nih.gov. And the best way to stay informed about things from the activities that are happening at NIH is to sign up for the Listserv at this URL (www.nih.gov/mecfs). And we will be soliciting information and feedback from the community on the research priorities using a tool called IdeaScale. And we'll be sending out information. We're in the process of getting that set up for the first four webinars. So, as soon as that's ready to go, we'll be sending information out about how to access that platform and how to provide feedback on the research priorities.

So, with that, I would like to introduce Maureen Hanson, one of the co-chairs of the whole ME/CFS Research Roadmap initiative, as well as the chair of this planning group. I'll turn it over to her to introduce the first speaker.

Maureen Hanson: Okay. Thank you. So, I'd like to introduce David Holcomb, who is going to give our lived experience session. So, David is a caregiver to his wife and two sons that have severe ME/CFS. He recently retired as a software architect to be able to devote more time to researching potential treatments for his family, while also raising awareness of the disease through ME/CFS San Diego, the 501c3 organization that he co-founded with his wife, Debbie. So, David, go ahead and tell us your experience.

David Holcomb: Thank you, Maureen. So, I'm going to start out with a short history of the family's health history and then go on to discuss some of the challenges and learnings that we've had along the way. So, my wife's childhood history, she had some recurring infections as a child. But by the time she was in high school and college, she was a relatively healthy individual until 1984, when she got a bad case of mono and ended up having to take a semester off of college.

After she returned, in her senior year, she was experiencing symptoms from type one diabetes that wasn't detected or diagnosed until she failed her first drug screen for her first job out of college. Then about three years later, she ended up getting endometriosis. And I'm a little curious if either the type one diabetes or the endometriosis or both were triggered by a virus, perhaps the Epstein-Barr virus that caused the mono.

But then later, she was healthy until 1992, when she got a bad flu-like illness during her second year of going back to graduate school at UCSD. And she never recovered from that. It turned out to be disabling ME/CFS. During the illness, the only thing that showed up on tests were high liver enzyme levels. And I'm wondering if that was perhaps reactivation of the Epstein-Barr virus causing that. But we then went to specialists. She did get detected that she had Hashimoto's thyroiditis, but her thyroid levels were normal. She was diagnosed with irritable bowel syndrome. She had all the rest of the standard chronic fatigue, you know, symptoms.

And I came across a book from Katrina Burns called "Running on Empty." And that was, you know, I was like, "Hey, dear, this sounds exactly like what you're going through." Because, you know, we were -- actually, when she got ill, two months later, we were getting married. And she was an avid hiker prior to this, and we were going to be doing some hiking in the Black Hills. And she just couldn't go. And that was the first real indicator that for me, that, wow, something's wrong here.

So, we found a specialist in Southern California, Dr. Jay Goldstein, and went to him, and he was able to confirm the diagnosis of fibromyalgia and ME/CFS. So, we searched for treatments and cures for three years without any success, and we realized that we had planned on having a family and that time was ticking. So, we talked to a number of specialists, and they assured us that it shouldn't be genetic nature to this. So, we went ahead and started having kids. We had our first son in '96, our second in '98. A couple of miscarriages. That turned out it was probably likely due to low progesterone because when that was supplemented, we had a successful pregnancy in 2005 with our third son.

Actually, with the birth of our second son, she had, with the epidural, a CSF leak back in '98. And interestingly enough, she had a spontaneous CSF leak in 2009. It took a number of months to realize that it was orthostatic in nature, and then finally was able to tell the doctors, "Hey, I

think I have a leak ." So, she self-diagnosed that.

Then our kids were healthy until around 2013 when something went through the house. We don't know what it was. We didn't notice it at the time. But our oldest two started having -- missing days of school, having stomach problems or head problems, that sort of thing. But we didn't -- it wasn't -- it was mild enough that we didn't -- you know, even the fact that we knew about chronic fatigue syndrome, we didn't necessarily, you know, identify it at that time. Another thing that happened shortly after was that my youngest was tested for type one diabetes and had two autoimmune markers. And so, a little bit parallel, perhaps, with what my wife went through.

Then my kids, you know, continued with full-time schooling but had to back off some of their extracurriculars until 2016 when, as a fifth grader for my youngest, senior for my middle child, and in college for my oldest, an illness went through the whole family. And it was a bad head cold that moved into the chest, and it triggered a full-fledged ME/CFS in all three of my kids and a decline in my wife, Debbie. And so, that was hard because I did not -- I was definitely in denial. I didn't want to accept that this was going on.

But I was the coach of my youngest son, both basketball and soccer. And he was the Energizer Bunny midfielder. And he kept -- he started asking me, "Hey, can I play goalie? Can I play goalie?" Because he was just getting so tired. And that was my first -- I recognized at that point that something was going on. So, they got diagnosed. And in 2018, my wife started having significant orthostatic issues. And that was finally tested to be -- determined to be low blood -- brain blood flow on tilting. My middle child also got diagnosed with that later.

In 2019, however, my oldest son recovered and was able to transfer to UCSD and complete his degree there. So, there's some good news. And then of course, coming along with that, an MCAS diagnosis for all of the three kids. It seems like we collect comorbidities, you know, kind of like merit badges. Some of the challenges we've had over the time, doctors. I have to go with my wife to every doctor's visit. We call them dates. And that's just in order to have doctors take her seriously. You know, when we went to the early doctors at UCSD, they showed us a differential that said, "Hey, if they're suffering these symptoms, send them to a psychiatrist."

When my kids were sick, we had Kaiser as our insurance, and Kaiser refused to diagnose them with ME/CFS. We had to go outside and go to specialists to get that diagnosis. School accommodations were always a challenge as their illness progressed. You know, initially they were going part-time, or we had to switch them to part-time. And we had to get that done through many meetings where Social Services would be involved. And they would be, of course, threatening us with truancy. And, you know, there was always that concern that, you know, we'd heard the bad stories about your kids getting taken away from you.

And I'm curious if ME/CFS caused by COVID is perhaps one of the causes of the increases in school avoidance that we've heard about recently. Social Security disability, of course, is a challenge, getting through that. They still use old policies from prior to the IOM report, where a psychologist is supposed to be used to evaluate the severity of the disease.

For all those things that I just mentioned above, a biomarker or test, ideally one that indicates severity, would be really helpful. Whole genome testing had that done when the price dropped below \$1,000. Ironically, that was filled with its own challenge because the Veritas, the company we used, had a problem with their mitochondrial DNA pipeline where they compared it to the wrong reference. And that popped up a, you know, a normal haplotype as a pathogenic variant. So, we had fun tracking down that wild goose chase. All of our genomes have been supplied to Personal Genome Project. So, they're available.

Moving on. Some of the learnings. Getting sick, avoid it. It can make your current ME/CFS symptoms worse. And overall, this disease is strange, inconsistent, and hard for people who don't have it to understand. Different parts of the body and brain are impacted at different times. So, sometimes my wife and kids can do one thing, and other times they can't. Sometimes they can do activity A, but they can't do it -- to me seems very similar activity B. Sleep, of course, is a challenge. Sleep initiation, particularly for my kids, they're tired but not sleepy. And so, their sleep cycle shifts, and they end up having to go around the clock probably once every three months.

The energy envelope, it's not marked, knowing, you know, pacing is easy to say, but you're always challenged with where that limit is. And then sometimes in life, there are opportunities to make memories to have still a little bit of a life even with this disease, and you know you're going to pay a short-term cost. But the question is, is there going to be a long-term cost?

And finally, identifying those causal relationships is very difficult, especially for my family. My wife disconnects from her body to get through the day-to-day pain. And so, when I'm trying to get feedback from her on whether a treatment is working, it can be difficult. And my kids, of course, are kids. They're inconsistent with their medications and feedback.

Concluding thoughts. I'm preaching to the choir here, but ME/CFS needs a lot more research funding and a lot more researchers just based on the disease burden and economic impact. We need to actively work to reverse the incorrect messaging in the past decades, and we need to make ME/CFS and other infection-associated chronic conditions a subject in the medical education and on health care licensing tests so that physicians are made aware of this disease.

And finally, we need to produce a detailed research roadmap to encourage, guide, and coordinate

new and existing researchers in the field. And that is hopefully what we will be working on more today. So, thank you for your time.

Maureen Hanson: Thanks very much for your perspective. Your experience is sadly similar to a lot of other patients and parents of patients. I guess we will move on to the next speaker. So, the next speaker is Michael Peluso. Michael is an infectious disease physician scientist at the medical school at the University of California, San Francisco, where he is assistant professor of medicine. He has research funding to study the HIV reservoir, but recently has done some important work concerning the persistence of SARS-CoV-2 that we'll be hearing about. Michael, go ahead.

Michael Peluso: Hey, everybody. Thanks so much for the invitation. I'm really happy to be here with you. I just want to say, before I start, that I really agree with a lot of David's points, especially the importance of including these conditions in the sort of medical education training that we go through. It's just -- it's like very important. And there's not enough of that that we learn about in medical school and residency.

So, yeah, as was mentioned, I'm an infectious disease physician and clinical researcher here at UCSF. And at the beginning of the pandemic, sort of became interested in what might have turned out to be the long-term impact of COVID and have been studying that for the last three years.

So, I'm going to talk today about some work in viral persistence but just to say that most of my experience in long COVID comes from our research program in San Francisco, which is called LIINC, which stands for Long-Term Impact of Infection with Novel Coronavirus. And LIINC launched really in the earliest days of the pandemic, to understand what happened to people after they had COVID, based on the assumption that it would probably be more complicated than it had been billed at the time as a two-week illness.

So, we opened our program in April of 2020, and within two weeks of opening, the program began to encounter participants with what would now be called long COVID, although we didn't have a name for it at that time. We worked to sort of systematically record and bank specimens on people that we were seeing, and since that time have enrolled -- actually, at this point, nearly a thousand participants, conducted thousands of visits, done really detailed longitudinal phenotyping, banked tens of thousands of specimens and supported dozens of collaborations.

And since early 2022, we've also been fortunate to be a part of the NIH's program, the national program called RECOVER, which is basically really, really synergistic and asking complementary questions to what we've been doing in LIINC but just on a much greater scale, which I think is really important. And so, we're part of both programs.

So, I'm going to begin with what we know. And I'll touch briefly on ME in all of these sections but talk mostly about long COVID. So, we know that infection-associated chronic conditions are common. I probably don't need to explain that to this audience. There's a really nice review paper from a couple of years ago, looking at different conditions associated with various infections. ME is probably the prototypic and likely, you know, perhaps until long COVID, the most common.

And I'll touch a little bit on ME-long COVID overlap in a few slides, but lots of other -- there are lots of other pathogens that cause infection-associated conditions that we really don't understand all that well, including gastrointestinal pathogens like Giardia, which is a, you know, a protozoal

pathogen, other viruses like Ebola which got a lot of attention about ten years ago in the West African outbreaks, and we're still learning a lot from. And to me, the fact that all of these infection-associated conditions have overlap in their presentations really provides a lot of external validation that there must be some overlapping underlying biological mechanisms that we should be able to figure out.

Lots of people are affected already. This is a figure from a paper that Lisa McCorkell from the Patient-Led Research Collaborative and I published in Nature last month. Currently, there are estimated to be over 65 million people with long COVID right now. The CDC estimates that there are 24 million people with pre-COVID ME. That's probably a low estimate. And that number is honestly, probably closer to the 65 million with post-COVID long COVID. And, you know, if 5 percent of COVID cases over the next ten years get long COVID, that's on par with current estimates of the burden of things like heart disease. And these numbers are already on the same level as other high priority medical conditions like cancer and stroke and Alzheimer's and HIV. So, I don't think that people should really make the argument anymore that not enough people are affected for this to be important. That's clearly not the case.

We know that ME often has an infectious trigger, and the subsequent talks today will get into more detail on this. But just to say there are multiple, you know, series of ME outbreaks after non-specific viral illnesses. There's some controversy regarding whether multiple different pathogens can cause ME, or there's a single or a set of pathogens that can cause ME, which is nicely outlined in Dr. Hanson's recent paper here. It's likely that enteroviruses, which again, we'll hear more about, are probably the biggest culprits. And there is evidence for enteroviral persistence, which I'll touch on. But reactivation of DNA viruses like herpesviruses may also play an important role.

So, in my opinion, some, but not all long COVID is similar to ME. There's a lot of symptomatic overlap, but there are also cases that appear to me to be quite distinct. There are various estimates that you could find in the published literature about long COVID-ME overlap. I think they're all quite flawed because there are very few true epidemiologic population-based studies. They're usually just cohort studies. We tried to sort of jerry rig a ME case definition in LIINC, and our upper estimate for the participants in LIINC who have ME overlap is about 18 percent among people who got COVID prior to ever being vaccinated. And there are other cohorts that have tried to do this which have found a lower incidence, probably around 2 to 5 percent.

But actually, you know, this is a question that I think RECOVER is going to do a really nice job of trying to delve into because the cohort is so large. And they followed many people from shortly after the time of infection. So, it removes a lot of the sort of biases that might exist in cohorts like LIINC. And so, I'm really hopeful that we'll learn more about this from RECOVER. And my personal take on this is that there is post-COVID ME. But really at this point, we need

to consider this to be COVID-related ME, because the attribution to SARS-CoV-2 may turn out to be very important because we actually know the infectious trigger, for sure, in these cases. So, I know this is a somewhat controversial area, but that's just my opinion on it for the time being.

So, we also know that what happens during acute infections is really important in long COVID. And so, these are some data from a CDC-sponsored study called Find COVID, which has been working closely with us in LIINC for the last three years. And what they did in this analysis was they looked at viral shedding during the first three weeks after SARS-CoV-2 infection and tested people basically every day. And what you can see here is that those who are destined to develop long COVID or PASC, at four months have a longer duration, higher peak, and longer duration of both positivity and actual infectious virus on cytopathic effect assays than people who are destined to fully recover.

So, this suggests that what goes on, even in the first couple of weeks, can really affect people's long-term trajectory. And I think that this provides some mechanistic understanding, this other important clinical observation, which is that, at least in some populations, early antiviral treatment really makes a difference in long-term outcomes. So, these are data from the VA cohort that showed that antivirals for people who qualify in the first, you know, five days of symptom onset changes, both long COVID outcomes and other outcomes like heart attacks and diabetes and strokes and blood clots out to six months. So, I think that those two studies together really suggest that stuff that goes on early matters in the long-term, but that's not really all that matters.

So, we know that there is precedent for persistence of RNA viruses. This is a nice paper from Diane Griffin from Hopkins from last year that basically explains why this might happen. And so, we typically think of these viruses as coming and going. But there's clear evidence that at least some of these viruses can persist beyond the acute phase and can be recovered from people with infection-associated chronic conditions.

So, people used to ask me as an HIV doctor at the beginning of the pandemic, what's the difference between HIV and SARS-CoV-2? And I would say HIV is a virus that persists. It integrates, it persists forever, and SARS-CoV-2 is transient. It should come and go. And I think that that framework has now sort of been challenged and is falling out of favor, at least in some cases.

This is a really nice review paper from Amy Proal who's a microbiologist at the PolyBio Research Foundation, where she cataloged all of the studies over the last few years that have examined and found evidence for persistence of different components of SARS-CoV-2. And I'll touch on some of these in the talk but encourage you to check out this paper. I'm just waiting for

it to advance.

So, these stories actually began with a focus on populations that were immunocompromised. We knew from early on in the pandemic that some people shed virus for a long time, and that particularly, immunocompromised patients could shed virus even from their nose, for a very long time. This was a really nice study that was done in Europe, looking at people with inflammatory bowel disease, doing gut biopsies six months after they had COVID, and they were able to identify SARS-CoV-2 RNA in a large proportion of people at six months.

Interestingly, they couldn't culture the virus, and they couldn't recover it from stool. And it was not related to the severity of the initial illness, but it seemed to be related to long COVID in the sense that a large proportion of people who had evidence of viral persistence reported post-acute symptoms, including GI symptoms, whereas those who did not have evidence of this generally did not have symptoms. So, this was an early look that I think was really provocative that there could be persistence, particularly in the GI tract.

And then studies started to emerge looking at this in immunocompetent populations. So, this was a paper from Michel Nussenzweig's group at the Rockefeller that came out early in the pandemic, where they were looking at the evolution of B cell responses to COVID, and the responses continued to evolve. So, they were looking for why this might be. And they found on staining again of gut biopsies that they thought that they were identifying components of SARS-CoV-2 RNA in the GI tract of people who presumably felt fine. There's no clinical data about these individuals, but a really provocative finding.

And then many of you might be aware of this really nice paper that came out from Dan Chertow's group at the NIH in Nature earlier this year, where they performed autopsies on people who died after COVID, not necessarily related to COVID. And what you could see here on the right side of this figure is that they were able to identify SARS-CoV-2 RNA in a variety of tissues, including neurologic tissues for up to six months post-COVID from people who died from other causes. So, again, not a study of long COVID, but suggesting that this is a true phenomenon.

So, measuring tissues is really hard, and there have been efforts to look for antigen or virus in more easily accessible spaces, particularly the blood. David Walt's group at Harvard has done a lot of important work in this area, and their first look at this was published in CID last year. David developed the single molecule array assay and then further developed it to look at SARS-CoV-2 antigens. And they studied a pretty small cohort of about three dozen people with long COVID and found that a really high proportion had intermittent detection of full-length spike in plasma for up to a year.

It's hard to draw a lot of conclusions from this paper because the sample size is very small. It was mostly people with long COVID. They didn't have good comparisons to people who recovered or to true negative samples. And the clinical data were pretty limited, including a lot of people were tested after they had subsequently been vaccinated. So, hard to know whether this was really attributable to the infection. But after we saw this paper, we decided to develop a collaboration with David's lab, which has been really wonderful, and so, have been working to apply this assay to samples from LIINC.

So, what we did was we sent 250 pre-pandemic control samples. It is impossible for these people to have had COVID because the blood was collected in like 2015 to 2018, and the blood was collected and processed in the same way by the same labs as 172 people post-COVID across 600 timepoints, between 30- and 450-days post-infection. And really importantly, the vast majority of these samples, the blood came out of a person's arm before a vaccine ever went into them. And we did the same exact assay in David's lab. And what we found is quite striking.

The false positive prevalence in the pre-pandemic samples was 2 percent. So, it was very low. And we clearly found increased prevalence of detectable antigen in plasma during all of the post-acute time periods up to as far as we looked, which was at 14 months. Even more compelling to me was that there was a relationship with the severity of the initial illness. So, people who had been hospitalized for COVID were twice as likely to have this than people who had not been hospitalized. And even among those who were not hospitalized, those who self-reported that they were very, very sick during the acute phase of COVID were four times as likely to have it as people who self-reported mild infections. So, that is plausible to me. And so, I really believe that this is happening in a subset, not everybody, of people.

So, the summary of this section, what we know. We know that infection-associated chronic conditions are common, that both ME and long COVID are likely to have infectious triggers. What happens during the acute phase of those infectious triggers probably matters, but that components of these pathogens probably persist in some people beyond the acute phase. So, what we don't know and what we need to know, most importantly, is antigen persistence responsible for these conditions, or is it just something that is happening?

So, the subsequent speakers will talk more about antigen persistence in ME. But just to say there's similar work from 10 to 15 years ago, looking at gut biopsies and identifying what is thought to be persistent enterovirus in the GI tract of a subset of people with ME. And we're very interested in this in long COVID. So, I mentioned this initial paper from David Walt's group last year. They really only evaluated the most kind of severe cases of long COVID in that people were presenting for clinical care, and they didn't really study people who had fully recovered from COVID. So, really hard to draw conclusions.

There are a lot of other small studies that have tried to draw conclusions. So, this is some work from Navneet Dhillon's group in Kansas, which did ddPCR and spike ELISAs in blood of people with and without long COVID. You can see here that the groups are quite small, but it seems that there is some signal where this is more likely to be detected in people with long COVID, although very difficult to draw firm conclusions given the small sample.

And then on the right is a study, another study from Europe, looking at circulating S1 in people prior to vaccines, where they found that the proportion of people with ongoing long COVID where this was detected was about 60 percent, and that was about twice as high than in people who had fully recovered from COVID. But again, the sample was so small that they couldn't really draw firm conclusions from this. But that's, to me, quite a provocative observation.

So, as you can imagine, we are now, you know, interested in looking at this in LIINC. We did an early study about a year and a half ago in collaboration with Ed Goetzl at UCSF, where we looked at participants in LIINC with and without neuropsychiatric Long COVID, so who had post-COVID anxiety, depression, and other neuro and neurocognitive symptoms. We looked three months post-COVID at proteins found in exosomes, which are little sort of packaged vesicles that are derived from different cell types in the body. So, we looked at exosomes that are thought to be derived from neurons and astrocytes in this study. And what we found, to me, again, was really striking.

So, there were some backgrounds false positive rate in pre-COVID controls. Those who had COVID but fully recovered had more detection of spike in these exosomes. Those who had long COVID, but no neurologic symptoms had a higher level. And on the highest level was found in those with long COVID with neurologic symptoms. So, again, this was a small study, about 40 people, but a really provocative observation.

But not all of the studies are positive. So, this is a recent study from a really well-regarded group in Sweden, where they looked at people with neurocognitive long COVID and looked in both plasma and spinal fluid, and basically found no evidence of antigen persistence in this cohort. Now, that being said, if you look at the sample size, there were 25 people with long COVID and six without long COVID. And if you apply the prevalence proportions that I talked about at the beginning that we're seeing in LIINC, you would actually only expect one or two people in this study to have this. So, I think it's a negative study but doesn't necessarily prove the negative.

So, as I mentioned in this collaboration with David Walt, we're now looking at this in LIINC. And so, in that same cohort that I showed at the beginning, we also have detailed long COVID symptom data. And in this cohort, there's -- about half of the cohort has sort of moderate, at least moderate long COVID and about a third of the cohort had COVID but fully recovered. And so, we're applying those same assays to see if we can see a signal. And we're starting to see

something, I think.

So, we see that antigen persistence in the post-acute phase is most common in the most symptomatic long COVID cases. So, about 40 percent of people with a lot of symptoms over time, more than nine symptoms, over time, seem to have this. Not 100 percent, but 40 percent is a fairly sizable proportion. But we're also seeing it in some people who say that they are totally fine. And so, it's, you know, again, about twice as common in people who are really severely affected, but it's not unique to that group. And so, my take on this is that it may be more common in people who are more symptomatic, but it can be detected even in people who have fully recovered.

This is a sample size of about, again 170. And so, we really need to scale it up. And I think the next slide will illustrate why, which is that we're actually able in LIINC to look at the relationship between antigen persistence and specific long COVID symptoms. And the takeaway here is that all of the point estimates are on the right side of the line, which is that basically, all of these symptoms are more common in people with evidence of antigen persistence, but all of them currently cross the line. So, none of these are statistically significant observations yet. There are some that appear to be approaching significance already. And so, we're building out this analysis now to try to see whether we can confirm that there's an association between antigen persistence and any specific long COVID symptoms.

Based on this work, you know, we don't think that the answer to everything is going to be in the blood. And so, we launched a gut biopsy program about a year and a half ago, where we're performing flexible sigmoidoscopy in people with long COVID. And what we actually do is we take pre-pandemic control tissue samples and mount them on the same slide as the post-COVID patient sample. And then we do RNAscope and then various immunohistochemical stainings, and mounting everything on the same slide eliminates the potential for batch effects. So, I'll show you some of our preliminary data from this project.

So, what you're seeing -- so, SARS-CoV-2 RNA would be in green on this slide. So, what you're seeing in the two control participants at the top and the bottom is no green. So, there's no background staining in these participants. But then on the adjacent tissue section from people with Long COVID, there's a lot of green. So, the top participant is a person with long COVID who's six months post-COVID. The bottom is a person with long COVID who's two years post-COVID. In both of these cases, there is no known re-exposure or reinfection.

The signal is all located in the lamina propria, which is sort of the underlying connective tissue layer, rather than the epithelium. And what's really interesting is that it appears to be concentrated in these areas that are dense with CD68 staining, which is a myeloid lineage marker. So, it suggests that there's monocyte or macrophage activation more so than lymphocyte

activation in these cases.

So, on the initial five biopsies we did, we had similar findings from this in four of the five. We did 18 more biopsies over the last four months as part of this initial project. And that's all going through the analytic pipeline right now. We should have results next year. And we're now studying people who have fully recovered to see whether this is specific to long COVID or not.

So, there's a whole other talk that can be done on how antigen persistence causes long COVID, but there are a variety of other mechanisms that have been invoked in long COVID, things like inflammation, immune dysregulation, EBV reactivation, microbial translocation, dysbiosis, clotting, autoimmunity. The reason that viral persistence is so attractive to me is that it could exist upstream of all of these things and be sort of the most proximal driver of everything that subsequently happens.

And so, I'll just touch on a couple of points that I think are really important in terms of how this could actually cause disease. So, the first is really, really interesting work that's come out of the Gladstone Institute here in San Francisco from Katerina Akassoglou's group. She's a neuroscientist looking at the molecular interaction between spike protein and fibrinogen, where the spike protein actually conformationally changes the fibrinogen, makes it more likely to polymerize as fibrin. And that's a mechanistic explanation for why people might have these micro clots that have been found by Roger Pretorius's group in South Africa. So, this spike fibrin interaction and then the relationship between fibrin and microglial activation is very, very interesting to me and I think is going to turn out to be important.

And then many of you will be aware of this really nice paper from Maayan Levy's group at Penn that came out in Cell last month. We contributed LIINC samples to this analysis, and they basically invoked how viral persistence in the gut, for example, could interfere with tryptophan absorption and serotonin metabolism, resulting in changes in vagus nerve stimulation and potentially causing some neurocognitive symptoms of long COVID. So, viral persistence, to me, is upstream of a lot of these mechanisms.

So, in summary of this section, we know that antigen persistence -- we need to know if antigen persistence can cause long COVID in at least some people, and we need to know why this could occur. So, why does antigen persist? Is it because of immune escape? Is it because of a local microenvironment that prevents immune surveillance? And then how does viral persistence cause downstream effects? And then ultimately, what we need to know is does treating persistent SARS-CoV-2 infection result in improvement? And that's a really, really important question.

So, there are three -- to me, there are really three or four research priorities. The first is better

biomarkers, as was mentioned by David at the beginning. So, the current biomarkers are great, but there are a lot of limitations. It's unclear what level of sensitivity is needed. It's unclear how specific these measurements are. Many of the positive values are just above the assay limit. So, there's concern that we need more sensitive assays. It also might be that the timing of the assay matters because we don't see this consistently in the same people over time, which suggests that it may be related to sporadic release related to like a meal or stress or a hormone cycle or something.

And then we need better tissue-based markers as well, but these are really, you know, sort of limited in availability and accessibility. This is so important because going back -- you know, putting back on my HIV hat, before plasma HIV RNA existed, which is the viral load that we measure in blood, which we totally take for granted now, the primary endpoints of clinical trials for HIV treatment trials really relied on observing a lot of people for a long amount of time to see any benefit.

So, these studies took a really long time to get answers and were really, really labor-intensive. And it prevented a lot of early investment in HIV research. But once this was validated as a biomarker, all of these endpoints vanished. And there was just this explosion of investment and development of therapeutics for HIV. And now we have dozens of HIV drugs that we can offer our patients. And that's really because of the impact that good biomarkers had. And so, we need better biomarkers for long COVID.

Tissue studies are also going to be really important. That includes looking at areas that are fairly easily accessible in living people. And so, we are launching a large tissue program here at UCSF that's supported by the PolyBio Foundation and is going to be looking at gut, lymph node, CSF, and bone marrow tissue. And that'll be starting next year. But not all tissue is accessible in, you know, in living people. And it's going to be really important to have good autopsy studies. You know, as you may know, RECOVER is running an autopsy cohort that I think is going to be really, really important in figuring this out, and I don't think that the -- you can't really overemphasize the importance of tissue-based studies, because I think a lot of us really think that this is going to turn out to be a tissue-based phenomenon.

And then the last thing that I really believe we need is more experimental medicine. I'm not talking huge randomized controlled trials. I'm talking of foundational proof of concept work. And so, when I talk about experimental medicine, what I mean is that you identify a pathway, viral persistence that you think is causing downstream effects in people with the condition. And then in a controlled manner -- the controlled aspect of this is very important -- you try to disrupt that pathway using drugs that are easily accessible. And you study people really, really intensively.

And the purpose of this is to probe whether manipulating that pathway actually has a downstream effect. It'd be great if it was a slam dunk, and it had a downstream effect on symptoms. But even if you can change the levels of different biological markers, that could be really, really important to learn. And so, there are some experimental medicine studies going on now, but not nearly enough. And we've identified a variety of different pathways. And so, I really think we need more investment in these sort of phase two type clinical trials, which can be launched and implemented relatively quickly to probe these pathways.

We launched at UCSF and LIINC, an experimental medicine program this summer. The first trial that we were doing is of a SARS-CoV-2 monoclonal developed by a biotech company called Aerium Therapeutics, which is based in Boston. This is funded by the Patient-Led Research Collaborative with additional support from PolyBio. And what we're doing in this study is we're taking 30 people with long COVID attributed to a variant that the monoclonal would be thought to work against, so before September of last year, and doing a blinded study, two to one randomized, where people get a single infusion of this monoclonal and then be followed really, really closely over time with lots of different measurements.

This is a phase two study. So, the primary outcome is safety. Monoclonals have never been in a randomized trial given to people with long COVID, but we're measuring a lot of patients reported outcomes as well as objective outcomes like neurocognitive testing, six-minute walk tests. And then layered on top of this is additional studies of viral persistence with things like gut biopsy, lumbar punctures, imaging studies. So, we've nearly completed recruitment for this study, and we're expecting that it'll read out at the end of next year.

There are other really important studies evaluating virus persistence. There are three studies in the U.S. of Paxlovid that I'm aware of, smaller studies at Stanford and Yale, and then a large study called VITAL, which is going to be taking place within the RECOVER Initiative. And I think these are going to be really important. I'm particularly excited about the one that's going to be happening via RECOVER because it is large enough that it will be able to study different phenotypes of long COVID. And I think that that's going to be really important because it may be that virus persistence only drives certain subtypes of long COVID.

And then we at UCSF, our second experimental medicine study will be an antiviral study of the Shionogi antiviral called Ensitrelvir, and that'll start next year. So, summary of this section. How are we going to find this out? We need better biomarkers. We need more funding for large, really carefully designed studies of viral persistence, particularly studies that can leverage specimens that were banked before things got really complicated with vaccines and reinfections and lack of testing and all these other circulating infections going around. We need more tissue studies and autopsy studies, and we need more investment in experimental medicine.

And then just to say, Lisa McCorkell and I were given the opportunity to comment on this in Nature last month, and I encourage you to check out this commentary which is arguing this case, basically more investment in both clinical and research infrastructure, continued coordination of the research agenda, and more investment in clinical trials. And so, you know, I think that there's too much negativity around what has been accomplished in terms of understanding long COVID over the last three years. And I think people really need to understand that we've actually generated a substantial amount of momentum that needs to be sustained.

So, in conclusion, I believe that long COVID is really our best chance to figure out an infection-associated chronic condition. I hope that there will never be another opportunity in our lifetime to do this. I hope that there will never be another pandemic of this scale. I think a really clear research agenda has emerged around the concept of viral persistence as an upstream cause of all of the other pathways that have been invoked in long COVID, and we really need more investments specifically targeting this pathway. And I think that the answers that we find in long COVID are really important. Because the hope is that these will then be translatable to sort of, you know, further jumpstarting research in ME to better understand the drivers of non-COVID ME.

So, I'm going to end there with some acknowledgments. I want to acknowledge the other PIs of both our local program and our RECOVER program, foundation funding from PolyBio and Patient-Led Research Collaborative, the NIH, including RECOVER, and of course, our volunteers and our Community Advisory Board. Thanks for the opportunity to speak with you today.

Vicky Whittemore: Thank you so much, Michael, for really excellent presentation. So, there's several questions in the Q&A that I will read out. I would also encourage any of the panelists to raise their hands, and I'll call on you. But let's start with some of the questions in the Q&A. So, I think you did cover this, whether your research includes people post-vaccine. I thought that the significantly large decrease in the number of people with long COVID was pretty striking. Do you want to comment more on that, Dr. Peluso?

Michael Peluso: Yeah. So, you know, the majority of our cohort was enrolled pre-vaccine. And so, I do think that we saw a lot of -- a lot more long COVID in that era. But, you know, we have gone through this exercise where sort of each breakthrough in COVID, when vaccines were developed, when Omicron happened and may have been milder, when antivirals came out. At each stage, people have sort of declared that this is going to be the solution to long COVID, you know, including post-COVID ME. And I know plenty of people with -- who got vaccinated and got COVID and subsequently got long COVID.

I know plenty of people who had Omicron and got Long COVID. I know plenty of people who

got treated with antivirals and got long COVID. So, even though it might be less common, it has not gone away. We do not have a specific -- I wasn't sure if you were asking about sort of vaccine injury. We don't have a specific program around that, but there are groups that are investigating that, and I think it's an important and relevant question.

Vicky Whittemore: All right. Thanks. So, the next question is something that we talk about a lot. So, and being part of the discussions and RECOVER on the NIH side, we've discussed this a lot. How to compare individuals who've had COVID and long COVID with individuals who've had ME/CFS for decades. I mean, it's really a challenge to think about, are we comparing apples and oranges when we do the -- would think about doing those kinds of studies?

Would you comment on that, Michael?

Michael Peluso: That is a really important point. And the fact that we're at least acknowledging the point up front, I think is also important. That's sort of part of the reason why I think it's -- you know, there are sort of lumpers and splitters in all areas of medicine, and I think that the field of understanding for long COVID is still at the point where we really need to be splitters and sort of think about groups differently.

I, you know, I think, there's also -- you know, there's a subset of people who had ongoing ME or had ME and got better and then subsequently got COVID and relapsed. And I think that that's also another really important thing to study because there might be clues to both conditions in studying such individuals. I think the power of cohorts that are as large as RECOVER, you know, 15,000 people, in contrast to smaller cohorts like our local cohort, is that there are -- you need to build up substantial numbers of people of those types of phenotypes. And, yeah, I think looking at all of those groups is going to be really important, but I don't have a good solution to the problem that you mentioned.

Vicky Whittemore: Thanks. So, Nancy, I see your hands raised. Would you like to unmute and ask a question?

Nancy Klimas: Michael, that was really a splendid talk. Thank you very much. I was wondering, as an HIV guy who is doing long COVID, could you talk about the role of co-infection and what do you think the data is with this EBV during primary or --

reactivation of EBV might have to do with reactivation of COVID?

Michael Peluso: Well, yeah. So, yeah totally. So, you know, again, going back to David's comments at the beginning, you know, I did medical training in the U.S., and we were really conditioned to be quite dismissive of herpesvirus activations outside of specific, extremely

immunocompromised contexts. And this has really made me revisit that framework. There are now three or four studies that have looked at this from different angles, initially from Jason Goldman and Jim Heath in Washington, study from us in San Francisco, a study from Sinai and Yale, Akiko Iwasaki's group that all showed this basically similar findings, which is that either direct or indirect evidence of EBV reactivation, likely during the acute phase of COVID, seems to be more common among a subset of people with long COVID.

In our study, we see strong relationships between early antigen IgG, so suggestive of marker of reactivation and specifically fatigue, not other symptoms, specifically fatigue. And we see a relationship between very high level EBV responses overall and neurocognitive symptoms. And I think that this is an incredibly important thing to delve into because there's been so much progress made in understanding the relationship between EBV and other conditions like MS over the last five years. I think that there is something here to be found.

Now, that does not mean that I think that the current treatments for EBV would necessarily work, and I don't typically prescribe EBV treatment to my patients. And I've heard that for a subset of people, it does work. And there's some nice work in ME that was done maybe 10 years ago suggesting that there might be some signal there. But I think that looking at herpesvirus reactivations in long COVID is going to be really, really important.

Nancy Klimas: Thank you. Thank you very much.

Vicky Whittemore: We have one minute left. So, I'll ask a quick question. Are you doing genetic analysis of the individuals in LIINC?

Michael Peluso: Yeah. So, we are not geneticists, but we have contributed to several genetics analyses, including whole genome sequencing analyses. People have to opt into that. So, not everybody wants to do it. And then just to say, you know, that's -- enrolling people in RECOVER, that's also an option that people can opt into as part of RECOVER. People, I don't think, get their individual data back, but I think these genetic analyses are going to be really important. And there's some good work already looking at asymptomatic COVID and genetic markers. And there's a preprint looking at long COVID and genetic markers. So, I think that we will learn from that, but can't change genetics.

Vicky Whittemore: Tell us -- give us information about who has genetic susceptibility.

Michael Peluso: For sure. Yes.

Vicky Whittemore: Great. Thanks again so much, Michael. That was really good.

Michael Peluso: Yeah. Thanks for letting me be a part of this.

Vicky Whittemore: Thank you. Right. I'll turn it back to Maureen, for your presentation.

Maureen Hanson: Okay, thanks. I have to share my screen. Okay. Can you see my title slide?

Vicky Whittemore: Yes, we can.

Maureen Hanson: Good. Okay. So, I'm talking about chronic infection in ME/CFS, avoiding the herpesviruses, which are going to be the subject of the next talk. I'd like to say that I had not seen Michael Peluso's presentation before, but it's very interesting how it resonates with what I'm going to talk about. One thing that I could tell him is that we did publish a paper a few years ago, Arnaud Germain and myself, with an estimate that the worldwide prevalence of ME at that time was 65 million, which is very close to what we're hearing is the prevalence of long COVID.

The other thing that I would like to say about his talk resonating with mine is that he mentioned that he thinks we should be referring to COVID-related ME, and not just conflate the two. And I agree with that, and I will mention more about that in my talk. So, these are what we were asked to do. We were asked to talk about what we know and what we don't know. What I'm going to actually say is that I think some of the things we think we know we might not actually know and that we need to learn whether what we think is correct or not.

So, I'm going to start by saying which pathogens have been associated with onset of what I'm going to call pre-2020 ME/CFS? This list of pathogens shows ones that have been published in various papers or -- describing, you know, that they were related to the onset of pre-2020 ME/CFS. There are some additional pathogens that I feel there's inadequate information, or there's disagreement in the literature, including mold, Borna disease virus, and Borrelia species. But all these species on the left, they've been associated with onset of pre-2020 ME/CFS. And which of these pathogens associated with pre-2020 ME/CFS can cause long-term infection? Well, in fact, it's the same list. All of these pathogens are known to be able to cause long-term infection.

But if you ask which pathogens associated with pre-2020 ME/CFS have not definitively been shown to cause a syndrome that includes post-exertional malaise, we have a list of most of those pathogens. So, studies on these pathogens as causal for chronic fatigue syndrome have actually used rather weak diagnostic criteria. And so, whether you think these pathogens are causal of chronic fatigue syndrome depends on your definition of chronic fatigue syndrome. So, I really feel that loose chronic fatigue syndrome diagnostic criteria result in conflating multiple types of chronic infections or other illnesses. One of the more notorious criteria is the Oxford criteria for chronic fatigue syndrome, in which fatigue is the principal symptom. It has to be disabling. It has to be present for six months. But other symptoms don't have to be present, and particularly post-exertional malaise doesn't have to be present, but not even myalgia or sleep disturbances. So, this is a really vague criterion, and many different diseases could fit in this definition.

And then there's the 1994 Fukuda criteria, which was somewhat better, required, again, a minimum of six months of symptoms. It also, however, did not require post-exertional malaise. You had to have any four of these symptoms that I'm listing here. But post-exertional malaise could be one of these, but it didn't have to be one of these. So, I think as a result of this, you can also include illnesses on that list, known chronic illnesses that would fit the Fukuda criteria that I don't believe should be called ME/CFS. The controversy over what should be called ME or ME/CFS has gone on for quite a long time.

And I just show a few examples here of people opposing vague definitions. So, I'm not going to read everything here. People can read this quicker than I can say it. But I will say the introduction of chronic fatigue syndrome to designate ME does nothing to indicate the unique epidemiological, geographical, clinical, and laboratory findings in ME, and can only add to the confusion. That was 1988 by a major ME researcher.

And then Melvin Ramsey, who is famous as the person who presided over the 1955 outbreak of ME, says that "This post-viral fatigue syndrome, it covers conditions such as post-influenza debility or the more severe post-infectious mononucleosis fatigue state. And these are in contrast to the three cardinal features of ME, which is a unique form of muscle fatigue ability, whereby even after a minor degree of physical effort, three or more days lapse before muscle power is restored, the extraordinary variability or fluctuation of symptoms, and the alarming chronicity."

So, this controversy has gone on for a long time, and I think that a very valuable exercise that was done somewhat similar to this roadmap exercise was done in 2015. A year's worth of study resulted in the IOM ME/CFS diagnostic criteria. And these IOM criteria for ME/CFS, what we now call ME/CFS as a compromised name, is now requires post-exertional malaise, unrefreshing sleep, and new fatigue of more than six months, and either cognitive impairment or orthostatic intolerance.

And I think using a definition like this is -- as well as the Canadian Consensus Criteria or the International Consensus Criteria that all require post-exertional malaise will give us a much better definition of a more uniform illness that we can then study. So, I want to give an example of the importance of a consistent criteria for selection of subjects because finding molecular biomarkers are going to be very difficult when multiple post-acute infectious syndrome cases are combined. And one issue is that a number of ME/CFS clinicians are likely to see patients who have a variety of chronic infections or post-acute infection syndromes. And now to this list we're adding long COVID.

But this is an interesting table that was in a letter to editor, published by John Chia and his son in which they looked at 200 patients that showed up with what apparently seemed to be chronic

fatigue syndrome. Well, a full 18 of those patients turned out to have a chlamydia pneumoniae infection that was treatable. So, you know, that's very important. And there were some patients with herpesvirus infections here that were also treatable. But what was quite fascinating is that by far, the majority of the people who showed up with the chronic fatigue syndrome symptoms had very high levels of antibodies to enteroviruses. And there were, of course, some that he couldn't identify their cause as well.

But this really shows that if you took this batch of patients and you tried to find a molecular marker that was common to all of these 200 people who could be defined as having chronic fatigue syndrome by either Fukuda or the Oxford criteria, what are your chances of finding something that will really define that group? This is really the importance of the consistent criteria for selection of subjects. There are other sources of diversity that you can't do anything about, for example, especially the genome of the subjects, there's no way you can control for that.

People are going to have co-infections with various herpesviruses, potentially with HIV and other persistent infections. Of course, sex is a variable and the gut microbiome. There's also the environment, the diet, and the drugs. So, with all those other aspects of diversity, we should at least have a defined illness that doesn't encompass any post-acute infection syndrome.

So, all of these pathogens are known to cause post-acute infection syndromes that likely don't fulfill the IOM or other PEM-requiring definitions. Although some of them have actually not been investigated carefully to see if they do. But many of these, there are patients who do not fulfill the IOM criteria. And I believe that then these should not be described as chronic fatigue syndrome nor as ME/CFS. Instead, they should be, for example, described as chronic Q fever or chronic toxoplasmosis or chronic SARS-CoV-2, in other words, long COVID.

So, once -- a very famous study is the Dubbo study from Australia. And this has convinced many that multiple pathogens can cause chronic fatigue syndrome. But I would like to point out that this study did not require PEM in the subjects. And they studied three infections. They had 253 patients with -- who had acute onset of mononucleosis, Q fever or Ross River virus. And at 12 months, 9 percent of these patients met the Fukuda criteria for chronic fatigue syndrome. But one of their viruses might have stifferly arrived during that time, especially the patients who had EBV. And I'm going to get to that point later.

But I don't believe that this study should be used to say that any of these pathogens can cause chronic fatigue syndrome because it depends -- unless you have a very loose definition of what chronic fatigue syndrome is. So, if all pre-2020 ME/CFS is caused by an acute infection, then why do only about two thirds of patients mention a preceding infection? Well, the fact is, a large proportion of enteroviral infections are asymptomatic. And I think one benefit of information

that's coming out of the SARS-CoV-2 pandemic is just how often infections with RNA viruses can be asymptomatic.

It's the advantage of the virus to not cause a symptomatic infection because then you go around and eat out in a restaurant, go to a conference and infect other people because you don't even know you were ill.

Now, when people search for an explanation, they might suspect an event that searched -- occurred around the time of falling ill. Now, if they don't have an acute illness around the time of falling ill, they have to come up with some explanation. So, many people will say, "Oh, I had a car accident. I had some mental trauma. I was exposed to some terrible chemicals or some mold or some other environmental exposure." Now, the fact is that these could factor into why someone has acquired ME/CFS, because these are all known to affect your immune system. But it's quite possible that people had asymptomatic infections with something, an enterovirus or something else, and that is why they acquired ME/CFS. In my opinion, all ME/CFS is going to turn out to be post-viral.

Now, a striking number of patients mention a herpesvirus infection, especially EBV, after which they weren't well. But here's the problem. What asymptomatic or even symptomatic viruses might have affected them either shortly before or after they became ill with mononucleosis? Now, this is especially an issue given the protracted recovery period that occurs following EBV infection in some people, even ones who get completely well. So, I think that we're going to hear more about herpesviruses. But I also think that we don't have information that's sufficient for us to conclude that EBV or other herpesviruses can be, by themselves, the viruses that incite ME/CFS.

A major point that I'd like to make is that post-acute infection syndromes that result in post-exertional malaise cannot be caused by any pathogen. There are pathogens that cause chronic infections that do not cause post-exertion malaise. So, which pathogens are known to cause post-acute infection syndromes that do include post-exertional malaise? Right now, we've got enteroviruses SARS-CoV-2 and maybe herpesviruses. We'll hear more in the next talk. And this list may not be complete. There may be other viruses that we don't know about that indeed are also causing post-exertional malaise and a post-acute infection syndrome that we would call ME/CFS. But right now, these are the only ones that we can really conclude fit the ME/CFS definitions that include PEM.

So, this is something I like to bring up because we've heard the abundant funding activity that's going on right now in long COVID studies. So, can study of pre-2020 ME/CFS be replaced by studies of long COVID that fulfill the 2015 IOM criteria? And researchers such as Michael Peluso, in fact are aware of the difference between people with past and long COVID to fulfill

the criteria. So, I agree with him completely that the similarity in symptoms suggest that some of the same molecular, biochemical, and physiological pathways are disrupted in both syndromes. And that new treatments that provide symptomatic relief of long COVID may benefit patients with pre-2020 ME/CFS.

However, if pre-2020 ME/CFS persists because of chronic infection, drugs that treat SARS-Co-2 chronic infection will not treat pre-2020 ME/CFS unless those drugs affect all RNA viruses. And there are a small number of drugs that do affect multiple RNA viruses. One of these is remdesivir. This is a nucleotide analog that confuses the RNA virus when it's replicating. So, it would work also on enteroviruses, but whether this would work on a chronic and enteroviral infection, we don't know. I would also argue that if pre-2020 ME/CFS persistence involves molecular mimicry to enteroviruses, then the auto antigens will likely differ from the autoimmunity induced by SARS-CoV-2. And SARS-CoV-2 coronavirus doesn't share sequence similarity with enteroviruses.

So, we really do need to know something about pre-2020 ME/CFS. I would also say that there could be some fundamental pathway disruption that differs between pre-2020 ME/CFS and long COVID. We are currently dismissing the differences at molecular level or in symptom constellation as a result of the more recent development of long COVID in patients, but we don't have supporting evidence for this. It's very difficult to get that supporting evidence. But, nevertheless, we can't assume that these differences are solely due to the difference in the length of time that people are ill. And something that is alarming that I'd like to point out is that long COVID that fulfills the pre-2020 ME/CFS with the diagnostic criteria, can't be distinguished by symptoms alone. With exceptions such as loss of sense of smell, we don't see that in ME/CFS.

What this means that cases and outbreaks arising from pre-2020 ME/CFS -- I see there's some typos in this sentence here -- but if there -- a virus that causes pre-2020 ME/CFS, they are still out there, and they can still cause cases and outbreaks. But we wouldn't know that this was happening because it would be assumed that it was actually SARS-CoV-2 causing long COVID. So, we really need to be able to distinguish pre-2020 ME/CFS and long COVID because there could be a new outbreak of me ME/CFS that we won't even know is happening.

So, how can we detect chronic enteroviral infections? One can do it by detection of viral nucleic acid, detection of viral protein products, or transmission of virus or disease to susceptible cells or hosts. And this list, of course, is the same as the list you would give for a persistent SARS-CoV-2 infection. Now, ordinary serological tests can sometimes be indicated if repeatedly showing high titers of anti-EV antibodies. And in that table, I showed from John Chia, he was able to see that a lot of his chronic fatigue syndrome patients, someone who had been ill would certainly more than that six months still were having high antibody titers. And these tests are especially useful at the time of an outbreak, but unfortunately were not usually used during the outbreaks.

But a caveat on this use of serological tests is the fact that it's estimated by the CDC that 15 to 30 million EV infections occur in the U.S. each year so that many healthy individuals will have high anti-EV antibodies from a recent acute infection. So, it's difficult to have perfectly negative controls in such an assay given the frequency of EV infections. So, I think that it's really critical to have knowledge of the inciting pathogen if you want to search for evidence of chronic infection in ME/CFS.

So, what is the evidence that enteroviruses can cause ME/CFS? One thing is that the non-paralytic enteroviruses, polio being a paralytic enterovirus. The first Coxsackievirus was actually not discovered until 1947. Now, Melvin Ramsey who is credited with coming up with the name myalgic encephalomyelitis, was aware of Coxsackievirus at the time of the 1955 Royal Free Hospital outbreak, but the technology then was not adequate to investigate non-polio enteroviruses.

So, the most evidence for enteroviruses as causes of ME/CFS outbreaks and sporadic cases comes from studies that were done at the time in the many outbreaks in the 1980s and the early 1990s. And interestingly enough, most of this evidence comes from Scotland and England that I'm going to tell you about because this was actually not very well, in fact, hardly at all, investigated in the US. And one question you might ask is, were these earlier cases ones that are of what's now called ME/CFS?

And so, from Melon Ramsey's article, he says that "Abnormal muscular fatigue ability is the dominant clinical feature." I think it's rather prescient for this colleague of his to say there is a prolonged metabolic disorder in many patients which may be affecting cellular energy systems, which is something that people are now investigating. And another paper in that era is their symptoms were overwhelming fatigue made worse by exercise -- it's one of the papers I'll be mentioning -- and extreme prolonged muscle fatigue after exertion. So, this is definitely ME/CFS with post-exertional malaise.

Now, before talking about these historical papers, I have to set the stage of what was our capability at that time? When did DNA sequencing of enteroviruses become possible? So, just to remind people, Sanger sequencing was developed in 1977. By the early 1980s, many academic and government labs could perform DNA sequencing. My lab was able to do this, but it was a cottage industry. You had to do it in your own lab. You couldn't go to some facility. And then PCR was invented in 1983. And although it was invented then, it really became feasible to do in individual labs when commercial PCR machines became available in 1990.

Now, because these centralized facilities for sequencing, they were rare in the late eighties and early nineties, most researchers used either Northern blots to detect enteroviral RNA or did RT-

PCR to amplify viral cDNA, which was then visualized on gels but not often sequenced. So, many of these old papers that I've been reading to prepare this talk did not do sequencing. And here's an example of this paper from Cunningham. This is a 1991 RNA slot blot. Slot blots are still used, but just for those who don't know what they are, you put, you know, your RNA in a device that has slots, and then the device will allow you to put an imprint of that RNA onto your filter paper. And then you have it on the filter paper, and you can hybridize with a probe. And in this case, they hybridized with positive strand probe for enterovirus, generic probe to a conserved region and a minus strand.

And these are all the subjects that they probed here. And this is, again, muscle RNA. And you can see that subject 1, 2, 5, 7, 8, 14, 15, all had both positive and negative strand, indication of enterovirus, number seven in particular. And there was a positive control of a known enterovirus put on this blot. Well, another thing that's interesting here is that the positive and negative strand amounts are approximately the same, which is not typical during an acute infection. An acute infection, you expect a lot more positive strand.

And here's another interesting paper again from muscle biopsy RNA by Gow et al. And to explain this, when you would do a gel like this, you would do PCR of a nuclear DNA as a control to make sure that your reverse transcriptase and PCR enzymes are working. And then this is showing the CFS -- I'm going to use CFS if that's what the person referred to the disease. So, these are CFS muscle biopsies here that were positive for PCR bands. And this is a positive enteroviral control.

Now, somebody can say, "Oh, that's just a band on a gel. That's not really enteroviral." So, one thing short of sequencing that people did at the time is that they would then take those bands, put them on a slot blot, and then hybridize with an EV probe. So, you could demonstrate that there was actually sequence of enterovirus by hybridizing with an EV probe. And this is again, a positive control. So, all of these samples here, definitely those bands actually were enteroviral PCR products.

Now, the other thing, of course, is that there were enteroviral antibodies detected in many reports of ME/CFS cases or outbreaks between 1970 and 1995. And a review of these papers can be found in a review article that we wrote in 2021. And I'd like to discuss a selection of reports of detection of EV sequences in ME/CFS subjects. Now there's three times as many as what I'm showing here. I'm just picking out a few to point out. And again, this is Scotland and England for the most part. So, I'm going to start with -- so, there are three reports here in which people found sequences in serum, and I've got five reports in muscle. I want to talk about this Galbraith one because this was unusual in that they actually not only detected EV sequence, but they actually were able to sequence them.

So, these are serum samples collected at a Scotland clinic between '92 and '94. And as I remember -- mentioned, there's a lot of outbreaks in the eighties and early nineties. So, they were able to see a signal in 44 of 238 cases and three of 130 controls. But they also sequenced a number of the positive cases and then made this phylogenetic tree. And you can see that the CFS patients here are -- most of them are on one side of this phylo tree. And this paper included this interesting figure here that shows the sample taken from serum and compared it to a throat swab at the same time. And there were some small differences, but largely, this PCR product in the serum and the throat swab are similar. But what was also interesting, a serum sample taken 10 months later had the same viral sequence in it.

But back then we didn't have the amazing tools that NIH has developed. So, we didn't have a GenBank online. We didn't have BLAST. So, you had to actually, if you wanted to find out what that was, your sequence, you actually had to acquire a batch of CDs and then load them on your computer and use special software. But now I copy that sequence, put it into BLAST, and five minutes later, I got this information that this sequence in fact corresponds to coxsackievirus A9. It's 94 percent identity. So, even though they knew it was an enterovirus, they didn't know which one.

I'm also now going to talk about a very interesting muscle study, this one by Lane. And they found enteroviral sequences in RT-PCR products from muscle biopsies and again, sequenced it. And they had 10 of 48 patient muscle biopsies, PCR are positive for virus and none of 29 controls. But what was particularly interesting about this paper is that they actually had these people exercise and check them for how much lactate they produced. And they had nine out of the 10 people who had positive biopsies for enterovirus, actually had an abnormally high lactate after a 15-minute exercise.

And this is actually a vertical drawing of the comparison of these enteroviruses that were found to a reference enterovirus sequence here. They were comparing this to Coxsackievirus B3 strain Nancy. Now, this was isolated in 1949 in Connecticut from a woman. I have a suspicion what that woman's name might have been. So, each one of these sequences here, the differences between those sequences and the reference virus are shown.

What I think is interesting about this study and the previous one is that you can't say this is due to contamination, that you contaminated your lab, and your lab was full of enteroviruses. Because most of these are different. There's only two of the patients who had the same virus. So, I think you can't explain this away as just the laboratory was contaminated.

So, how do enteroviruses persist in human cells after the acute infection? And there's actually a lot more known about this than there is about SARS-CoV-2. And I wouldn't be surprised if there's going to be some interesting parallels between what happens to SARS-CoV-2 as its

residents and what happens in enterovirus. So, persistence of enterovirus has been studied a great deal in heart, pancreas, brain, and cell lines because it has a connection with type 1 diabetes and cardiomyopathy and post-polio syndrome. In all of those illnesses, there's evidence that enterovirus is involved in inciting and can still be resident in individuals' tissues with those illnesses.

And with regard to our first presentation, what's interesting to me is that type 1 diabetes is associated with enteroviruses as is Hashimoto's thyroiditis. And so, one wonders if an enterovirus was involved in the testimony that we heard at the beginning. So, in steady-state infection, there are many cells that are infected, but there's a low viral replication. This results in a non-lytic phenotype. If there was a high viral replication, the cell would be likely to die and wouldn't be persisting. And you can also end up with double-stranded viral RNA due to equal presence of positive and negative strands that was shown in that slot blot that I showed earlier.

And it's been by a number of studies of these people, hearts and pancreas, that's shown its -- and also cell cultures, mice -- have shown that steady-state infections with enterovirus results from defective viruses, and especially 5 front terminal deletions in this untranslated region that then affect replication. And if you're interested to know more about this, there's some recent papers showing these deletions in acute -- people with acute myocarditis and cardiomyopathy, as well as, you know, many other illnesses.

So, my feeling is that we need to analyze tissues. And this is something that Michael Peluso also said in the connection with long COVID. We need to be analyzing tissues to determine whether the incidence -- what is the incidence of chronic enteroviral infection in present day samples? Now, I've been presenting the idea that chronic enteroviral infection is involved in ME/CFS, but we don't know if it's involved in every case. We don't know if, for example, an enterovirus infected someone, did some damage that we don't know about or made some changes, and those changes are what is causing ME/CFS. Or whether in fact, it is the continuing presence of enteroviruses.

So, studies of other diseases have used tissue extracts, and these tissue extracts especially have been heart, gastrointestinal tract, the pancreas, and the central nervous system. And we heard again in our first talk that gastrointestinal tract is being investigated in looking at long COVID. Now, there was a paper that appeared in 2023, doing a viral of analysis of blood, feces, and saliva. And enteroviruses in this paper were not found in plasma of 285 ME/CFS cases, nor in controlled individuals.

Now what's interesting about that is that this is a different result than those early nineties papers that I showed earlier in which they were able to find virus in serum. I have no way of -- I have no reason to doubt that this recent study isn't correct. And I have no other reason to doubt that

the old study isn't correct. I don't know how to rationalize these two disparate findings. But one possibility, of course, is that the viruses that were infecting people in the early nineties, again, those individuals have probably not been as sick as long as most of the individuals in this study. So, I have no explanation for this. And it's something that needs to be examined further.

It's evident also that PBMCs or feces are also not a great thing to use to look for chronic antiviral infection. I suspect that if you were to look at people with dilated cardiomyopathy, you might not find any enteroviruses in PBMCs or in feces. In saliva, there was one case and one control that had rhinovirus, and rhinoviruses are a member of the enterovirus. So, at least that sort of was a positive control that this technology could identify enteroviruses. But this study, to my mind, brings home the fact that we need tissue analyses in ME/CFS to find out whether there are still a proportion of individuals who have both -- who have chronic enteroviral infection.

Now stomach tissue is likely a suitable sample. And this was mentioned by Michael Peluso, the papers of John Chia. And just to mention one of his papers briefly, he had 165 consecutive CFS cases and 22 normal controls who underwent biopsies. And then immunohistochemistry was performed with an anti-EV monoclonal antibody. And 135 of the 165 CFS samples, as well as some of the controls were positive for the presence of a viral protein. And in addition, there were some samples that were studied and were found to have enteroviral RNA as well.

And this study here that I will also briefly mention, is one in which three patients who arrived with an acute enteroviral infection later had evidence of persistent infection and in fact developed ME/CFS. And so, here's a stomach biopsy reacted with an anti-VP1 antibody, the viral capsid protein. And you can see that there's a lot present in that stomach biopsy, the brown here. And a control with an anti-CMV antibody in which you don't see this.

So, what indirect evidence can be used to suspect chronic infection in ME/CFS? And that is what you can do is look at -- if you don't have direct evidence, what we and others have done, is look at disturbances in the immune system function that has characteristic chronic infection. And all of these items on this list have been seen in ME/CFS. And in fact, the reduced cytotoxicity of natural killer cells is something that was one of the earliest abnormal immune findings in ME/CFS. And if you want to, I'm not going to go into this, if you want to learn more about that, please see my talk in the Immune System Roadmap webinar. And also, Jessica Maya's talk in the Metabolism Roadmap webinar which talks more about these.

So, there are some corollaries of the enteroviral hypothesis. One is that failure to clear the enteroviruses could result in continued attempts by immune system to respond and therefore continued inflammation. And this, just like what Michael was showing for SARS-CoV-2, this could have a downstream effect, leading to a variety of phenomena in metabolism. Different enteroviruses have different preferences for where they go in your body. There are neurotropic

ones. There are ones that prefer muscles. But ones that are in the brain and nerves, these could be causing ongoing damage and disruption in the nervous system function.

The taxing of the immune system to continuous immune stimulation may result in the escape of previously controlled human herpesvirus infections. And this could also perhaps cause susceptibility to new infections with herpesviruses. I also do think that chronic EV infection and uncontrolled herpesviruses or endogenous retroviruses, which we're going to be hearing about later, may collaborate in producing symptoms and then prevent recovery. And so, they are very important in the illness.

I'd like to end by just mentioning that we're pursuing studies of the molecular basis of ME/CFS under the auspices of funding from NIH and NIH-ME/CFS Center. We also have funding from the AMAR and WE&ME Foundation private donors and Simmaron Research. And we have a collaboration with the Hospital for Special Surgery in Manhattan, New York. And we are going to be getting muscle biopsies from subjects there. And if you are interested, please send inquiries to carl.franconi@cornell.edu.

As far as research priorities -- this is my last slide -- these are what I think we need to know. We need to know what the incidence of chronic enteroviral infection is. Maybe it's low. Maybe it's the majority of patients who really don't know. We need molecular markers to distinguish pre-2020 ME/CFS cases from long COVID cases that have, as Michael, was referring to COVID related ME. We need to find out whether an acute enteroviral infection, even if it doesn't become chronic, may lead to ME/CFS. Does it cause some damage?

And given that enteroviruses are not only associated with ME/CFS but other terrible diseases like type 1 diabetes, cardiomyopathy, and acute flaccid myelitis, we need vaccines and drug treatments for both acute and chronic EV infections. We need to know whether or not herpesviruses and endogenous retroviruses are contributing to susceptibility too, or maintenance of chronic EV infection. And I also think that we need guidance from medical professionals about diagnosis of post-acute infection syndromes. Because it'd be a very bad shame that some people who think they have incurable ME/CFS actually have treatable infections, especially people with certain bacterial infections. Thank you.

Vicky Whittemore: Thank you, Maureen. So, there is a question in the Q&A that asks about swabbing skin surfaces to identify which viruses and bacteria live on the skin and back of the throat. You talked about a study that compared that. Perhaps persistence of ME/CFS symptoms are caused by infectious pathogens, viral bacterial, or fungal that live on this human skin that some people are genetically susceptible to. The person says that a similar study was recently done, they think on fibromyalgia in which they found a possible bacterial cause. What are your thoughts about that?

Maureen Hanson: I have seen a few studies in which people did think it could be a pathogen on your skin that could be involved. And certainly, pathogens can be anywhere that might be involved in ME/CFS. The throat swab is particularly, I think, important because respiratory viruses can often be found in throat swabs. That's -- as we well know when we were being swabbed for SARS-CoV-2. So, I think throat swabs are particularly important for analysis for respiratory pathogens. But I don't think we can eliminate any location in the body as potentially involved in having a pathogen that could be affecting ME/CFS.

Vicky Whittemore: Yeah. There's a comment here from Tobias that one study from their group in Stockholm found reactivation of latent herpesviruses, EBV HHV-6 and endogenous retroviruses HERV-K in an oral mucosa, but not in plasma, which was very interesting. Study was done on 95 individuals with ME/CFS and 110 healthy controls and provides the reference here.

Maureen Hanson: Yeah. Thanks for that reference. Maybe we'll be hearing about that from Tony Komaroff as well.

Vicky Whittemore: Yeah. So, someone says that OPKO and Merck are working on a vaccine for EBV. Is it even realistic for people sick so long, say, 10 years, middle and middle-aged, to think they may be candidates for the vaccine?

Maureen Hanson: I guess the question is whether that vaccine will be able to help people who are already infected --

Vicky Whittemore: Right.

Maureen Hanson: -- or whether this would prevent infection. So, we don't really know. There have been some people with long COVID who claim that the vaccine actually made them better, vaccine for SARS-CoV-2, and, you know, we can't really predict.

Vicky Whittemore: Right. So, those are all the questions that are in the chat for you. Maybe there's another one here. So, the question is really to us at NIH. How is NIH working with medical school research departments to get out presentations such as Dr. Hanson's? Yeah. We're making -- the videos are available online, and we'll make them available once we have the full research report that will also be made available.

Maureen Hanson: Yes. And of course, we also have a collection of videos done by not only myself, but other people in our group on our website that I showed. So, if you want to go to neuroimmune.cornell.edu and you'll see a whole collection of talks given by people associated

with our center.

Vicky Whittemore: So, question, are there any treatments for EV infections?

Maureen Hanson: Well, that's actually a big problem. There really aren't good drugs. There are some studies of people trying to develop good drugs. Some people have had success treating individuals with interferons. There's a preparation of Chinese herbs that's been used. And of course, remdesivir and the other RNA virus-affecting drugs. But we don't have a good drug for an enteroviral infection, for an acute enteroviral infection. And we really need them. And so, right now, if you were to find out that you have an acute enteroviral infection, there's not much we can do. But if we do find that, you know, suppose half the people who have ME/CFS have an enteroviral infection, I would certainly hope that that would stimulate much more interest in developing drugs that can overcome such an infection.

The other thing I may not have brought out is that I'm not saying that a single enterovirus is causing these infections. If you read the old literature and the descriptions of the disease, the outbreak, the outbreaks don't sound identical. There are some different features of the patient symptoms. I mean, they all had this post-exertional malaise. They all have terrible fatigue, muscle pain, but there's some differences that make you think that's may not be the same enterovirus. And the fact is that enteroviruses are actually far more variable than coronaviruses. They do not have a proofreading ability like coronaviruses are. So, there's far more different types of enteroviruses than coronaviruses. And look how many new coronaviruses we have had developed in the last three years. So, I do suspect that there are going to, if -- when we look at what might be present in people with ME/CFS, it's not going to be the same virus.

Vicky Whittemore: So, last question for you that came in via email. Does the research you described encompass bacterial infections as well as viruses that have triggered a dysregulated immune response leading to ME/CFS? And do you know who or where this research is currently being done if there's any research?

Maureen Hanson: Yeah. So, as I mentioned, whether you call, for example, post-Lyme or post-chlamydia pneumoniae, whether you call that ME/CFS is up for debate. I actually think it should be called post-Lyme. I think it should be called post-chlamydia or post-Ross River virus. And I do think all of these syndromes should be studied. All the victims of these terrible post-acute infection disease syndromes should have some recourse, some treatments. But I think like -- but we just can't lump them all together and expect that a treatment for a bacterial infection is going to work on someone who has ME/CFS because of a viral infection. But this is actually something that I'm hoping that long COVID will bring out that we should not be neglecting post-infectious disease syndromes, that there are many of them and they shouldn't be dismissed and swept under the rug.

Vicky Whittemore: Absolutely agree with that. So, we're at time, and it's time for us to take a break. So, we'll return at 1:15 p.m., for the next speaker. So, thank you very much, Maureen. That was really excellent.

Vicky Whittemore: And I'd like to welcome everyone back from the break and turn it over to you, Maureen, to introduce the next speaker. Maureen, back with us? Yes. There you are.

Maureen Hanson: Okay. It's my pleasure to introduce Dr. Komaroff now for the next talk. He is distinguished professor of Medicine at Harvard Medical School. And he's well-known to most ME/CFS researchers and patients due to his long career in the field, as he was one of the first clinicians to realize that he was seeing patients with a new syndrome in the early eighties. And he recognized the importance of and studied the Incline Village outbreak that occurred in the mid-eighties. He's published over 270 research articles. He was editor-in-chief of the Harvard Medical School Health Publications and wrote a syndicated newspaper column for the layperson that was called "Ask Dr. K." And at the recent IACFS/ME meeting, he received a lifetime achievement award. And he's well-known for his excellent reviews of ME/CFS. And I'm eager to hear what he has to say about herpesviruses.

Anthony Komaroff: Thanks so much, Maureen. Thanks for inviting me to participate today and to talk on this subject. The herpesviruses there, whoops, I wanted to start, preface the comments by saying I've designed this talk for a general audience, including I know people who are neither health professionals nor biologists. So, I'll be showing a minimum amount of actual data, but rather summarizing what many replicated studies have concluded from the data.

I'm going to make some general comments about different types of viruses and different responses of the body to different types of viruses. I'm going to address the questions we've all been asked to address, what do we know? What don't we know? What do we need to know? And among the things that we need to know, how would we prioritize the importance of getting answers?

Let me begin by saying I think it's extremely unlikely that ME/CFS is caused by a single, novel infectious agent. Always have thought that, and if anything, the last four years have only reinforced that judgment. Instead, I think there's increasing evidence that ME/CFS involves a dysfunctional immune and metabolic response to infection with any of several infectious agents, as Dr. Hanson has described. And also, it may -- that same dysfunctional response also may occur following physical trauma, major physical trauma. I'm not going to discuss that today, but there's evidence for that.

No virus, including the herpesviruses, has been proven to trigger or perpetuate ME/CFS, particularly to perpetuate it. And the herpesviruses that I think have been most closely linked to ME/CFS, EBV and human herpesvirus-6A and-6B, those clearly can trigger the illness, but whether they perpetuate the illness remains uncertain. And if it's true, it's only true in some people with ME/CFS.

So, a few comments about the different kinds of viruses that infect us. Some of them multiply but then are fully eradicated by the immune response. Others multiply but remain latent in some of our cells. Of those that multiply but then are fully eradicated, as Dr. Peluso has showed so nicely for SARS-CoV-2, even if the virus itself, living, replicating virus, has been eliminated, some of its nucleic acids and antigens can persist in the body in harbors where they defy eradication. And in those harbors, they can be triggering an ongoing chronic immune response. Among the viruses that multiply but then become latent, those viruses can periodically reactivate and multiply and thereby trigger an immune response.

I also want to introduce a concept that may be a tangent in this context but may prove relevant to ME/CFS to a handful of cases. Most of us recognize viruses as something we catch from other people. They're transmitted to us at some point in our lives from other people, but some viruses are inherited. Viruses have genes that are made either of DNA or RNA. These genes control how the virus works, how it infects our cells and reproduces.

And it turns out that thousands of years ago, some viruses inserted their genes into the DNA of a human egg or sperm cell. When that happened, the person who was born from that egg or a sperm cell had the viral DNA present in every cell of their body. And they were able to pass that viral DNA on to their offspring. We'll come back to that in a little more detail shortly. There are two examples of this. One is the endogenous retroviruses that you'll be hearing about next, and the other is human herpesvirus 6. The human herpesvirus family consists of nine viruses. There are eight numbers because these viruses, human herpesvirus 6 A and B, are really two distinct viruses. I think the evidence linking any of them with ME/CFS is strongest for Epstein-Barr virus, EBV, and HHV-6A and B and that's what I'll be discussing.

So, first, some general comments about all three of these viruses. They infect nearly everyone. Nearly 90 percent of all humans are infected with these viruses. And because, as I said, they're viruses that remain latent in the body, once infected, one is permanently infected, and starting from a young age. Another feature of all of these viruses is that they're neurotropic. They infect several types of brain cells. And they're immunotropic. They infect several types of immune cells. As I said, infection with these viruses is ineradicable and permanent. The immune system can recognize them, can try to eliminate them, but never succeeds. They remain.

The initial primary infection with EBV causes mononucleosis. Indeed, it's by far the most common, if not the exclusive cause of mononucleosis. HHV-6, particularly HHV-6B, is the cause of a childhood disease roseola. But with both of these viruses, with all three of these viruses, that primary infection often is silent, doesn't produce any symptoms or disease. Reactivated infection with any of these three viruses has been linked to several diseases, not a link that's absolutely proven, but pretty strong. And -- but despite that, most of us carry around these viruses which periodically reactivate without causing us any problems.

So, let's turn to a few comments about EBV and what we know about its relationship to ME/CFS. After mononucleosis, which is commonly caused by EBV, with mononucleosis, and for the weeks after, there's often severe fatigue. It's a debilitating part of the illness. While the textbooks say, and it's probably true, that the most -- most cases, the illness including the fatigue, are gone by eight weeks. It is now clear that it can last much longer in some people. And only since longer-term follow-up studies of people with mono have been done has that become clear.

Chronic fatigue syndrome, according to one study that Dr. Hanson mentioned, can occur in up to 9 to 10 percent of patients after mono, but rarely occurs, if at all, after other kinds of upper respiratory infections. People who develop ME/CFS and fatigue syndromes in general after mono are not more likely to have had a past psychiatric disorder. I say that because when this was all first being discussed and studied in the 1980s and 1990s, there were many people who said, the only thing that distinguishes a person who gets mono and then goes on to ME/CFS from one who gets mono and returns to normal health, is that the one who goes on to ME/CFS had some pre-existing psychiatric problem that led them to have ME/CFS. I think we can now say categorically, from scientific evidence, that's not the case.

Post-mononucleosis ME/CFS appears most likely to occur in people with severe fatigue, disruption or dysfunction of the autonomic nervous system or low-grade inflammation during the acute mono. And you notice some parallels there with what Dr. Peluso was describing for acute SARS-CoV-2 and long COVID. By six months post-mono, those who develop ME/CFS have elevated pro-inflammatory cytokine levels still six months after acute mono, reduced anti-inflammatory cytokine levels, which only makes the effect of the pro-inflammatory cytokine levels more potent. And they have lower ACTH levels when compared with those people who get mono but do not go on to ME/CFS.

There is one interesting protein, one mechanism perhaps, the EBV protein dUTPase, as Dr. Klimas and her group and others have studied, induces neuroinflammation and lethargy in animal models. And some people with ME/CFS have elevated antibodies against EBV dUTPase. So, it remains a plausible hypothesis that one mechanism by which EBV may produce fatigue and lingering fatigue syndromes is through this particular EBV-related protein.

So, new primary infection with EBV can trigger ME/CFS for sure. I think that's been well-established. Can reactivation of EBV then be explaining the recurrent cycles of symptoms in ME/CFS in the months and years thereafter? Serum antibody levels in people with ME/CFS often but not always indicate that the virus is reactivated, by which I mean, there are elevated levels of antibody to the early antigens of the virus that are associated with reactivation, with the lytic phase of the virus. And IgM antibodies or very high IgG antibodies to the virus' capsid antigen are seen more often in ME/CFS than in healthy control subjects.

What about HHV-6A and B? As I said, this produces a lifelong infection in most of us and persists in our brain and in some of our immune system cells for the rest of our lives. One of the remarkable things about these two viruses is their tropism. Most viruses target one or two types of cells in the body, and that's it. They look for those cells. They try to infect those cells. Those are the only cells they're capable of infecting.

HHV-6A and B, however, are able to infect a remarkably broad number of very different types of cells in our body. Many types of immune system cells, T-cells, B-cells, Natural Killer cells, monocytes, macrophages, myeloid cells, virtually all of them. And they're also able to infect many different brain, central nervous system cells, not just the neurons, but more prominently the immune system cells of the brain, the microglia, the astrocytes, the oligodendrocytes. They also are able to infect other than immune system or brain cells, fibroblasts, salivary gland cells, other cells elsewhere in the body.

So, any virus that can infect a lot of different cells, tissues, and organs raises questions about whether it can cause disease of those organs. What disease associations are there now solidly for HHV-6A and B? Well, as I said, the childhood disease roseola, or exanthem subitum, two different names for the same disease, are clearly caused by these viruses, particularly B. Febrile seizures, seizures in children when they have fevers are very common, and these are clearly caused by HHV-6A and B. In fact, they're -- those viruses are the most common causes of febrile seizures in young kids.

Encephalitis and pneumonia in people who are immunocompromised are caused by these viruses. And there I would argue, in my opinion, pretty strong evidence, there is pretty strong evidence that the viruses are linked to temporal lobe epilepsy and to multiple sclerosis as is EBV. And indeed, there's evidence that both viruses together, EBV and the Human Herpesvirus 6 viruses might have synergy in producing these illnesses. There's -- if anyone's interested in the details behind that broad assertion, there's a review article at the bottom of this slide that goes into it in agonizing detail.

What do we know about the relationship of HHV-6A and B to ME/CFS? As I said earlier, because it's such a common virus, because so many of us have it, all of us have some level of antibodies against it. So, finding that someone with ME/CFS has antibodies to these two viruses doesn't tell you anything.

However, if you do antibody and other kinds of studies to try to identify people not just who have infection, since all of us do practically, but people who have activated or reactivated infection, the studies do find a strong association with ME/CFS. And that includes studies using PCR of either the serum or plasma to look for viral DNA, studies of IgM antibodies to the early

antigens of the virus which are indicative of reactivated infection, and even includes some studies of primary cell culture from the lymphocytes of people with ME/CFS in which a characteristic cytopathic effect has been demonstrated and confirmed by monoclonal antisera specific per HHV-6 and confirmed by in situ nucleic acid hybridization studies in those lymphocytes and confirmed by electron microscopy showing the virus budding from the surface of those lymphocytes. And all of the studies that I summarized were the full bullet or some. The more important ones were summarized in that slide.

Might an unusual feature of Human Herpesviruses 6A and 6B affect a person's vulnerability to getting ME/CFS? As I said earlier, there is a very interesting thing that happens with these viruses, and that is that some people inherit them. How does that happen? Well, the viruses, these two viruses have a very unusual way, unusual for herpesviruses certainly, of achieving latency inside a cell. That unusual way of achieving latency, which I'm about to describe, has caused a remarkable phenomenon that I will also describe that could theoretically influence a person's vulnerability to developing ME/CFS. And in fact, that could have even broader implications for human health.

Most Human Herpesviruses like Epstein-Barr Virus, for instance, the viral DNA enters a person's cell, enters the nucleus of the cell, and then it curls up into a little ball, a circular -- a circle of DNA. As a circle, it can't reproduce itself, but it can remain sitting there, latent, quiet, ready if something happens to it to reproduce itself. With Epstein-Barr Virus, a typical infected cell has about 30 to 60 of these circular copies of the viral DNA inside the nucleus of the cell.

Sometimes that circle pops open. It becomes no longer a circle. It becomes linear. And when it's linear, then it can reproduce itself and produce a lot of copy viruses. With Human Herpesvirus six, its means of achieving latency is different. As the virus enters the cell and then enters the nucleus, it attaches -- it doesn't curl up into a circle, it attaches itself to the chromosome, to the host cell, the human DNA in the nucleus.

I'll come back to what -- how that affects the possibility of vulnerability to ME/CFS. So, I'm making a distinction here between what happens to nearly all of us in our lifetimes, which is that the HHV-6 virus has been transmitted to us from some other person, typically early in our lives, and that virus then infects a tiny fraction of our cells for the rest of our lives from this inherited condition. These somatic cells -- may be cells of our immune system, cells of our brain -- remain latent in us, but over the last 350,000 years or so of human history and hominin history, our predecessor species, on multiple different occasions, HHV-6A or B inserted its genome into the telomere of a human germ cell, an egg or a sperm cell.

And as I described earlier, as a result, the person born from that germ cell had the viral DNA in every cell in their body and was able to pass the viral DNA on to their offspring who were able

to pass it on to their offspring et cetera, throughout human history.

As a result of that, about 1 percent to 2 percent of the human race inherits HHV-6 DNA from a parent and has the viral DNA present in every cell in their body. And not only is the genome present in every cell of the body, but it has been shown that it is capable in many cell tissues, if not all, of reactivating, producing new virus and eliciting an immune response. So, what might be the health consequences of that inheritance? And in particular, could it increase or decrease even our vulnerability to getting ME/CFS? We just don't know. The number of studies so far is small, but there are underway, quite large studies that I hope will offer some clarity on that possibility in the next year or two.

Okay. So, herpesviruses and long COVID. We've talked up until now about herpesviruses in ME/CFS. What do we know about herpesviruses in Long COVID? I'm only going to very briefly summarize this, and Dr. Peluso and Dr. Hanson described part of this already. It is true that ME/CFS and long COVID share very many symptoms. There are some differences, and Dr. Hanson mentioned that the loss of smell and taste are the most obvious ones. Those almost certainly are symptoms that are unique to the SARS-CoV-2 virus, to its effect on the cells of the nose and the nervous system in the nose. But what is becoming clearer with time is that the two conditions also share many underlying pathophysiology -- share much underlying pathophysiology. This is outlined in a recent review article that's at the bottom of the slide.

It is also possible that not only the symptom similarity and the similarity in pathophysiology that ME/CFS shares with long COVID are also shared by other post-acute infection syndromes, as Dr. Hanson described. That, I think, as I will say in conclusion, is a very important thing for us to be pursuing right now.

Reactivation of EBV may also be associated with long COVID. I'll talk a little bit about that next. Reactivation of EBV during acute COVID, viremia during acute COVID turns out to be a strong predictor of the development of long COVID and is also correlated with increased percentages of CD4+ cells and cytotoxic CD8+ cells, as well as exhausted CD8+ cells. In other words, there's more of them, and they're getting more and more tired doing battle against presumably SARS-CoV-2.

There's elevated IgG against lytic antigens of EBV indicating that there's EBV reactivation, and that the increased reactivation of this virus in people with long COVID is different from what you see in people who got acute COVID but now have recovered fully and don't have long COVID. And these findings are summarized in the two papers at the bottom of the slide.

So, what don't we know and what do we need to know about the relationship of these three viruses to ME/CFS? I would say that my overview of the evidence is that while there's strong

evidence that the viruses are reactivated more often in people with ME/CFS than in healthy control groups, what we don't know is, is reactivation of these viruses specific to ME/CFS, or is it also seen in other post-acute infection syndromes?

We know it's seen in long COVID which presumably is one post-acute infection syndrome. But I think it's worth inquiring as to whether it's also seen in other illnesses that we think of as non-infectious that are characterized by chronic debilitating fatigue. And I'm thinking of multiple sclerosis, lupus, Sjogren's syndrome, major depression, and quite a number of others.

Do the reactivated viruses, which I think for which, at least with ME/CFS and long COVID, there's good evidence, do these reactivated viruses though contribute to producing the symptoms, or are they just an epiphenomenon? They're there. You can measure them. They are indicators that something's gone wrong with the immune system, that immunity is dysregulated, but they're not actually causing the symptoms.

We need to get a better handle, a better answer to that question. And if we find out the answer is yes, they do seem to be contributing to causing symptoms, then what are the mechanisms by which reactivation of these viruses causes these symptoms? For example, does the dUTPase that I mentioned that all three of these viruses have, does it -- when the viruses are in the brain, produce low-grade neuroinflammation that leads to the symptoms? That's a studyable hypothesis and a plausible one in my view.

Is reactivation of these viruses a feature of other fatiguing illnesses? I now put this as a high-research priority. As I said a minute ago, it's one of the important unanswered questions. I put it near the top of my list. Do reactivated viruses contribute to producing the symptoms, or are they just an epiphenomenon? That would be my number two priority. And it's important because if they're contributing to producing the symptoms, then you have a target for treatment.

If you could intersect the way these viruses -- the reactivation of them generates the symptoms, you could eliminate the symptoms. If the reactivation of the viruses does contribute to the symptoms, what are those mechanisms? And if there's a strong correlation found between reactivation of latent virus and flares of symptoms, then clinical trials could test whether antiviral therapy at times of symptom flares might offer symptom relief.

Such a trial would require you to be monitoring people with ME/CFS regularly for evidence with tests that provide evidence of reactivation of the virus during a time of a flare. And if you found diagnostic test evidence of reactivation, then treatment at the moment of reactivation might plausibly intersect the flare of symptoms. Worth pursuing.

So, in summary, ME/CFS is definitely not caused by a single novel infectious agent, certainly

including any of these three Human Herpesviruses. Instead, I think there's growing and quite persuasive evidence that ME/CFS involves a dysfunctional immune and metabolic response to infection with any of multiple infectious agents, but I would also echo what Dr. Hanson said, not with all infectious agents.

There is something about the infectious agents that have been linked to ME/CFS that they share in common. And I would propose, as Dr. Hanson did, that two things that are important are the fact that they're able to persist in the body or pieces of them at least are able to persist in the body, is one thing they share. Another thing they share is that they can infect the brain and the immune system -- and/or the immune system.

However, new primary infection with EBV, and I think there's some, not as good evidence with HHV-6A and B, can trigger the onset of ME/CFS. And whether these two viruses, when they are reactivated, whether that reactivation produces a flare of symptoms in ME/CFS still remains an open and very important question to pursue.

So, finally, I think the role of these herpesviruses, particularly since most of them are infections we acquire early in life, but then remain with us and reactivate periodically, whether this phenomenon that we've known for 50 years exists is not an issue. Whether the reactivation of these viruses produces diseases of any sort remains an increasingly plausible hypothesis and one that I think is very important to pursue more broadly, both in ME/CFS and in long COVID. So, I'll stop there and wait for the questions.

Vicky Whittemore: Thank you very much, Tony. That was really a wonderful review. So, there's a question, and I think you did address this, but I'll ask it. You mentioned incorporation of viral genome into human sex cells in the distant past. Can and does that event happen in recent times with newer viruses? Is there evidence of that?

Anthony Komaroff: There's some evidence that it continues to occur with Human Herpesviruses 6A and B. Whether it's occurring with other viruses currently is more debatable. There have been people who argued that it can occur occasionally with viruses called Borna viruses, for example. The evidence for that is not -- in my opinion, is not very strong. And if it happens, it's not very frequent. But it is -- you know, it does occur in 1 to 3 percent of the human race. And that's a lot of people, and it's a lot of potential disease associations that fortunately are currently being actively pursued.

Vicky Whittemore: Thank you. David, you have a question?

David Holcomb: Yes. I was curious, when a person has reactivated antibodies is it always the case where there's an increased viral load, or is it perhaps sometimes a misfiring of the immune

system caused by a different infection or something?

Anthony Komaroff: It's a good question, David, and I think it's not all -- to the extent it's been studied. I don't think, for these viruses, it's been studied very well. There isn't always a direct correlation between the viral load going up and the evidence of reactivation, the immunologic response to the viral reactivation going up, but they tend to correlate. But I think here the point that Dr. Hanson made is really important.

It's easy to study these viruses in the blood, which is why we do it in the blood. But if they're causing symptoms, they are doing it probably by what they're doing in the brain, and that's a much harder thing to study in a living human being than their blood is. So, I always take with a grain of salt any studies that draw inferences from blood studies of reactivation of these viruses to their link to a disease. Because the blood is the easiest compartment to sample, but it may not represent what's happening deeper inside the body where it's harder to do studies.

David Holcomb: Thank you.

Vicky Whittemore: Tony, do we understand -- I saw this question. Do we understand what causes a virus to reactivate?

Anthony Komaroff: No. I mean, the -- certainly, experimentally, in the lab, you can introduce into a cell culture with viruses in its certain chemicals that induce it to reactivate, but how those chemicals then change the switches in the viral genome to cause it to assume its replicative form is much less well-understood. Certainly, not well-understood by me, but it's also something that people have been pursuing for a long time. Some -- it's understood to some degree, but not in any depth.

Vicky Whittemore: Right. Are there any other questions? I don't see -- let me check the Q&A one more time. There's just a comment here that Jarred Younger uses sophisticated imaging that can look at inner brain inflammation and even very mild increased brain temperature, yeah, which is interesting as well. Are there any other questions from anyone on the panel? Okay, if not, thank you very much, Tony, and I'll turn it over to Maureen to introduce the next speaker.

Maureen Hanson: Okay. So, our next and last speaker is Simon Carding. He is professor and research leader at Norwich Medical School and member of the Norwich Institute for Healthy Aging and Gastroenterology and Gut Biology. He was a Howard Hughes fellow in the Immunobiology Group at Yale and on the faculty of the University of Pennsylvania before joining the faculty at Leeds University.

In 2008, he joined the University of East Anglia to help head the Gut Biology Research Program. His research concerns the mechanisms of intestinal microbial tolerance in the role that microbe host crosstalk plays in establishing and maintaining gut health. And he has worked on ME/CFS in this context. But today, he is going to be reviewing a different topic. So, Simon, go ahead.

Simon Carding: Thank you, Maureen, for the introduction. So, it'll come as probably no surprise, based on Maureen's introduction, that my interest in ME is in the human microbiome particular virome and how that may be a reservoir or source of microbial antigens or mediators that can cause or perpetuate ME. But as Maureen says, today, I'm going to talk about HERVs.

So, my talk -- I'm going to start off by describing what we know, and I'll give a little bit more background than probably the other speakers have because this was not only new to me, but I'm sure it's probably new to a lot of people in the audience. And these are the things that I'm going to cover eventually leading up to the small number of studies that have been carried out looking at HERVs in ME/CFS.

And I think in the context of virology and viruses in ME, the study of HERVs might be what Maureen will consider a cottage industry at this point. But at the end, I'm going to put up a hypothesis which I think will -- might link in very nicely to the previous speaker where we can maybe link HERVs to herpesviruses as a pathway in the development of ME/CFS.

So, this slide just includes some basics. So, HERVs are a type of retrotransposon, which is like jumping genes, so they can insert themselves, extricate themselves from various parts of the genome and insert themselves elsewhere. And the concept of transposon elements was discovered by Barbara McClintock who won the Nobel Prize for discovering them. HERVs are RNA viruses. So, to integrate into our genome, they first have to convert their RNA into DNA, a complimentary copy. And over human evolution, this infection integration has left us with about eight percent of our human genome being encoded or attributable to HERVs. And this has principally come about because as previous speaker mentioned, they can infect germ cells, sperm, and eggs, allowing them to be vertically inherited or transmitted to sustain their presence in the human genome.

And they can integrate randomly throughout the genome so they can influence global transcription, many different types, some that are critical to mammalian development. And

probably the best example of how important these are to us, and they're not -- shouldn't be considered half necessary, is that they play a critical role in development of the placenta. And some believe that the evolution of the placenta has been accelerated as a benefit to our evolution because of these retroviruses which encode syncytin, which is encoded by a particular type of retrovirus called ERVW-1, it's the envelope protein, and this plays an integral part, critical role in placenta development.

They can also influence other tissues, so, the salivary gland, the liver, gut, bile duct, and also adipose tissue. And they can also influence immune cell function. Interesting, they can change the major histocompatibility complex antigens which are important in enabling our immune system to react to foreign antigens, pathogens, but discriminate self from non-self. So, you can see that they could also be associated with autoimmune disease if they change the way our immune system reacts to our own cells.

The vast majority of HERVs are expressed in non-lymphoid tissue. They're in reproductive tissue. And I think that's important when we start to consider the studies that have been done so far on HERVs in ME/CFS research. Another important point is that HERVs sit at the crossroads between environmental factors that can cause changes in our epigenome or our genome that leads to change in turn to the regulation of our gene expression and in that regard, HERV activation.

They can also be activated as a consequence of changes in our own genome, DNA sequence variations, that leads to transcriptional regulation, activation of HERVs. So, they're at this crossroads of interactive environmental and genetic influences, and their ability to influence phenotype then makes them, if you like, quite sensitive sensors to our environment, but also our genome, and changes in that can result in their activation, which could be beneficial to health or detrimental to health.

So, they are relatively small. Their genomes comprise four functional genes shown here on this cartoon. And these genes are important for assembling virus particles. However, what's more interesting in terms of their influence on our own genetic makeup and expression of our genes are long-terminal repeats sequence that sit at the end of these functional genes, these called LTRs. And in fact, 90 percent of the -- these viruses are actually solo LTRs. So, they do not contain any of the functional genes. They're just LTR sequences.

And these, depending where they're integrated into our genome, can switch on or switch off our genes. So, they can influence a whole variety of functionality in our genome. And over time, the functional genes can acquire mutations and deletions, which essentially can make them non-infectious. So, the functional genes are much -- probably rare, if you like, in terms of trying to identify these viruses in the body. What's more indicative of their precedence and influence is

looking at these LTR sequences.

We can classify them according to structural and their genetic makeup. And there are three broad classes, the Gamma, Beta and Spumetroviruses, and then down at the family level that they're usually designated or distinguished by a single letter. And that letter, it relates to the amino acids that are close to the site where a transcription would occur in the virus genome. So, you can see we have, F, H, I, E, R et cetera, et cetera, and then some other unique number that sort of falls outside that category. So, a relatively small number of classes, but in total, there could be more than 500,000 different types of retroviruses, endogenous retroviruses in our genome.

They are associated with disease associations, and as I said, environmental lifestyle factors can impact on their regulation, their expression. Ageing is one of those things that changes the profile of HERV expression. They're implicated in various cancers and autoimmunity. So, for example, the gag and env genes of HERVs are biomarkers -- are used as biomarkers in kidney, prostate, lung, breast cancer.

How they work is not particularly clear at this point, but there are two probably mechanisms, an indirect one by inserting themselves into genome and disrupting genes that may regulate normal functions. And the other is to actually produce virus particles or virus antigens or transcripts, if you like, RNA, that then in of themselves can be harmful to cells. And it's interesting that the immune system is very reactive to HERV antigens. And in a large proportion of patients with myeloid malignancy, the T-cells -- 50 percent of the T cells have reactivity or specificity, if you like, to endogenous retroviruses. So, they are potent inducers of immune responses, and they can be used as an indicator of malignancy or the stage of malignancy.

In the -- regarding the brain, they're also associated with neurodegenerative diseases, amyotrophic lateral sclerosis, for example, the HERV-K env protein is neurotoxic, destroying neurons if they're expressed in neuronal cells and tissues. And we could detect the presence of virus particles and virus proteins in multiple sclerosis patients. And these proteins are potent inducers of inflammation. They can elicit the production of cytokines which are pro-inflammatory, Interferon Gamma, Interleukin 6 production by peripheral blood cells from MS patients.

And treatment with Interferon Beta as a therapy can reduce the viral load in the blood of MS patients. So, in fact, HERV-W and HERV-WE1, which is syncytin-1, their presence in the blood can be used as a risk factor and a biomarker for multiple sclerosis. And what's been proposed as a plausible mechanism or pathway to developing MS is that the reactivation of enteroviruses results in the production of viral antigens which are very good at stimulating lots of different types of immune cells. And that this overstimulation or chronic stimulation of the immune

system by these HERV-encoded antigens can then lead to the promotion of autoimmune disease, and in this case multiple sclerosis, but perhaps other autoimmune disease. And I'm going to return to this at the end of my talk as this may also apply to ME/CFS.

So, how is HERV expression normally regulated? So, as I've said, it's highly active in embryonic cells and in stem cells. And in most differentiated cell types, they remain silenced. And to be expressed, they rely on our host cell transcriptional machinery. So, they don't have the enzymes, the process to allow them to be transcribed into RNA and then into protein. So, they rely on the host cells machinery to achieve that.

Expression of HERV RNAs have been seen in lots of inflammatory conditions, in particular SARS-CoV-2 that we've heard quite a lot about already, autoimmune diseases like MS and others, and also in cancer. So, the way that the HERV virus -- that genes are repressed or held under control is through DNA methylation, which is classified under epigenetics, and also histone modification.

If the virus has escaped this DNA methylation mediated control and actually make transcripts or RNA, then cells have the ability to degrade that RNA before it's released or before it has the ability to produce protein and virus particles. So, those two steps, if you like, to prevent expression or release of virus particles, one at the level of transcription, pre-transcription, and one at post-transcription. And I'll just briefly talk about DNA methylation as this is one of the means by which this is studied in ME patients.

So, DNA methylation relates to the chemical modification of cytosine residues that are part of DNA molecules, depending on whether they're hypo or hypermethylation, determines the ability of machinery to access the genes to switch them on or switch them off. So, hypomethylation, for example, so that's less than normal levels of methylation of genes is related to cancer development because it increases instability of the chromosomes, allowing easy activation and more difficult to control activation transcription of these genes.

But on the other hand, hypermethylation can lead to the inactivation of our own tumor suppressor genes, which allows them mutations to accumulate in cells that can also lead to cancer development. So, the balance in hypo and hypermethylation is not just important in cancer, but also for regulating expression of viruses in other conditions and situations as well. So, methylation profiles, and particularly those that are associated with the LTR sequences of the viruses are an important way in which to gauge whether or not they may be expressed in various cells and tissues.

And methylation profiles can be identified by various methodologies. And I've identified some of them here. So, Bisulfite sequencing, which covers the whole genome generally. And then

there are other more restricting methods that look at certain regions of the genome of specific genes. A more direct method is just to sequence everything with shotgun genomic sequencing, using PCR as well to genome-wide sequencing. These all rely on bioinformatic tools to interpret the sequence data.

And there are a variety of software programs that have been evolved or have been generated to being able to look at this, in particular the methylation profiles. But these are still in their relative infancy, so there are issues about their performance variability. There are also microarrays, PCR. And then looking for proteins, we can use Western Blotting, ELISA, et cetera, from serum or tissues to visualize the prints of the proteins and maybe even the virus particles themselves. So, indirect and direct mechanisms for looking at -- measuring HERV expression.

So, this summarizes the studies that have been done so far in looking at global methylation patterns in ME/CFS. As you can see, there are eight studies here I think that have been published to date. All of them have, bar one, I think have relied -- no, all of them, sorry, have relied on the analysis of peripheral blood mononuclear cells, bulk preparations, or T-cell fractions of these. The diagnostic criteria, there's good consistent there in Fukuda or the Canadian consensus criteria.

Cohort size for all of these studies is quite small, particularly in terms of looking at ME patients and considering the heterogeneity. They are small cohorts. The application, the methodology is also somewhat variable. And also, this then translates into the number of methylated genes that can be identified. You can see there's a variation there in that column under analyze CpGs. Then looking at the differentially methylated sites. So, they could be hypo or hypermethylation. You can again see the different studies have identified anything from less to 100 to more than 17,000, so, a huge range across the different studies.

And then in terms of looking at the ratio of hypo to hypermethylation sites, again, the studies, there's no consistent finding. Some studies identify a higher abundance of hypomethylation sites. Other studies, it's the converse, they identify a higher abundance of hypermethylation. So, there's a lot of contradiction and conflict, if you like, in the findings. Interestingly, when the impact of these methylations has been looked up on gene ontology studies for trying to identify the cells and the systems that would be affected by these changes in methylation, one of the common features that comes out from this analysis is immune dysregulation.

So, a lot of these methylation changes are predicted to impact on immune cells and on immune cell function. So, that I think is sort of a consensus finding. But again, picking through the D-cells of this, there are discrepancies in the way the study's been carried out, the analysis. So, it's that is, I think, an interesting finding, but it needs to be substantiated.

This study I put up here as well because this takes methylation studies a step further and trying to combine them with a different approach to identify gene specific changes in ME/CFS patients. So, this is called recursive ensemble feature selection or REFS. And it was initially based on looking at messenger RNA signals that were distinct in ME/CFS patients, and these are from databases. So, they identified 23 genes, which distinguished ME patients from controls with over 90 percent accuracy.

But then they validated this against four methylation studies, which include those I mentioned on the previous slide. And from that, they identified 48 CpG methylation sites that were associated with marker genes that might predict disease status. And 10 of 23 of the encoded proteins these genes will produce are associated with immune function infection. Again, sort of emphasizing that this HERVs can impact on immune function and dysfunction, which is one of the key features I think of ME/CFS. And the cartoon on the right-hand side identifies some of the key immune functions that are affected by this.

So, I've talked about the major -- the compatibility complex cytokine receptors. There are also other ones which identify marker genes such with viral infection and oxidative stress and cellular metabolism. So, I think the message from this study is that by combining different data sets, we can increase the resolution and discriminatory power of an individual data set to have a little bit more certainty, leading hopefully to evidence-based biomarkers, which we're all hoping will come for ME/CFS. So, that, I think, is something that's very important, is combining data sets, integrating data sets.

Looking at specific gene methylation patterns. So, there are three studies here that I'm showing where they're just focused on specific genes, perforin gene, which is important for enabling T-cells to be cytotoxic, to kill virus-infected cells, for example, glucocorticoid receptor and the serotonin receptor for which these have been linked to ME/CFS in other studies. So, for perforin there were no differences in terms of methylation status. The glucocorticoid receptor, they identified a hypermethylation sequence that was a signature for MS patients. And the serotonin study also identified altered methylation, which they predicted would change the way these receptors function, again, linking to serotonin abnormalities in some ME/CFS patients. So, those are just gene-specific methylation patterns.

So, I want to now sort of also talk about how HERVs can be activated through virus infections. So, the list here shows RNA viruses as well as DNA viruses which were capable of eliciting or inducing HERV expression. So, HIV, for example, influenza virus, SARS-CoV-2 and then of course the herpes virus, cytomegaloviruses, these have all been linked to the induction or expression of specific types of human endogenous retroviruses. So, there is definitely a link there between enterovirus infection activation of endogenous retroviruses.

And then in terms of how these are expressed endogenous retroviruses impacts on the immune system, I've already sort of hinted at this in previous slides, but there are potentially four mechanisms. So, the promoters and enhancers, so the LTR sequences of endogenous retroviruses, as I said, can act as promoters or enhancers for antiviral genes. So, they can switch them off to leave cell -- the host more susceptible to virus infections.

The viruses themselves can encode RNA-DNA proteins which are very potent stimulators of the immune system. These are called microbe associated molecular patterns, MAMPs for example, that are bound by pattern recognition receptors on a variety of immune cells so they can stimulate lots of different immune cells. And then non-complimentary RNA and double stranded RNA that's produced on HERVs can also be another stimulus for the immune system. And then of course the particles themselves and the proteins that make up the particles also have the ability to elicit immune responses.

So, they're potent inducers of immune responses. Not surprisingly, they've been targeted as therapies. So, neutralizing antibodies to HERV-K, for example, have been used to reduce size of tumors. They've been used in multiple sclerosis, type one diabetes. There is an anti-HERV-W envelope specific antibody called Temelimab, which is currently in phase two trials with long COVID and multiple sclerosis. So, that would be something of interest to the ME community, I'm sure.

There can also be targeted bivaccines as well. And also, it's possible to engineer T-cells so that they are specific -- they can specifically react with particular HERV viruses. So, HERV-K for example, if you can engineer a patient's T-cells so that a large number of them have the capability of interacting with HERV-K virus, then these are proven to be effective in patients with melanoma and breast cancer in animal models as well.

And again, antibodies are T-cells that target HERV-K in PB, Peripheral Blood Mononuclear cells, can inhibit cancer cell growth. So, again, that links inappropriate expression or expression of particular types of enteroviruses to tumor genesis and cancer development. And of course, epigenetic modifying drugs as well to change back the suppressive nature of the epigenome to suppress activation of endogenous retroviruses is another target to knock down or control or suppress endogenous retroviruses and the various enzymes inhibitors that have been used -- starting to be used in preclinical studies at least.

So, in terms of HERV expression in ME, as I said at the beginning of my talk, it's a small number of studies. They're listed here, two -- five studies that have identified. All but one relies on the use of peripheral blood mononuclear cells, case definition consistency, small numbers of patients again, and small numbers of controls, some variation in the assays that are used,

antibody detection, in serum, RT-PCR, immunohistochemistry. And again, these are looking at specific not global HERV expression. It's specific types of viruses, specific families.

And the findings I've listed there, again, there's some variability in there from no difference when comparing ME patients to controls, to subsets of ME patients having high-levels or more prevalent expression profiles of viruses. So, again, there's a lot of variability and inconsistency in the results, but there again, this is only five studies in relatively small patient cohorts. That's probably not too surprising.

On this slide I'm just showing some exemplar data. The first one is from the only study that's been carried out so far on tissue from ME patients. And interestingly, this is on gastrointestinal tissue. And this is interesting in light of what we've been hearing from the earlier talks about the tissue burden and viruses hiding in tissue rather than in the blood. And so, this was a study dating to 2013 where they used antibodies to various HERV envelope proteins or gag proteins.

So, the top panel shows four different antibodies that are reactive, to some extent, in the majority of the samples they obtained from biopsies, eight of 12 patients, compared to healthy controls where there's a lack of reactivity. Using multiple different antibodies trying to identify the cells that are expressing the virus or which the virus proteins were present in, that's shown in the bottom panel. And they could colocalize expression of some of these viral proteins to CD303 cells, which are -- identify an important immune cell population called dendritic cells, which are really the conductors of the immune orchestra. They can initiate immune responses, and they can regulate immune responses. So, if a virus really wanted to have a maximum impact on the immune system, they would target dendritic cells.

And then the text at the bottom just shows that they substantiated or validated this data using more conventional sequence analysis and again, identified multiple contexts of known HERV genes in the same tissue and sample cells they extracted from the tissue. However, they were unable to identify the open reading from the proteins that encode the -- all the virus proteins to make virus particles. So, what they're looking at here, immunohistochemically, may be viral proteins and not virus particles.

The study on the right is an RT-PCR based study in a small cohort of severe or moderate ME patients and controls. All samples, to varying degrees, expressed, or had detectable expression of HERV-K and HERV-W as shown by the numbers at the top. But in looking at the levels of expression, they were able to distinguish moderately -- moderate ME/CFS patients from healthy control and severely affected ME/CFS patients. The moderate patient had higher levels of expression of both HERV-K and HERV-W. So, although the profile of expression virus is the same, some ME patients may have higher levels of -- certain types of HERV -- endogen HERV viruses.

And this was a very recent study. It's actually in a pre-print form from the group in Valencia where they've been looking at -- trying to see if there were HERV signatures which would segregate ME/CFS patients from those with fibromyalgia. And again, this is a relatively small study, small numbers of patients in the different groups. But what they were able to do from looking at peripheral blood cells and looking at specific HERV expression profiles is they could identify two clusters of ME patients, one cluster, which are 21 unique HERV families where they're upregulated. Cluster two had 12 unique HERV families which were silenced. And it was this group that correlated the closest to symptomology, disease severity in terms of comparing it to SF-36 scores.

So, when they then took these particular viruses and mapped them to another immune-based database to try and identify which immune cells might be affected by these particular viruses, they were able to demonstrate an association of the silencing of these families with lower levels of particular types of T-cells, gamma delta T-cells, memory T-cells, and plasma B cells. So, again, this links again the viruses to causing or related to change in immune profile, immune cell populations in ME/CFS patients.

Constraints of the study are like many of the other studies so far done on the HERVs, there's small cohorts of patients. We're extrapolating two unrelated databases here. So, there could be issues around that regarding the rigor of which that's done and the rigor in which the database is reassembled. Unfortunately, this study did not look at the immune cells in the patients from which they were doing the HERV analysis, so that would've been a nice validation on top of using the Cybersaw-X database. And interestingly, the association did identify the immune cells, didn't map to any of the known innate immune cell signatures which have -- which are quite common in ME patients and particularly natural killer cell defects.

So, I think what this reinforces, and it's been mentioned in several other talks, is we need better patient stratification for both research and C-teams to be able to tease apart which patients -- in which patients HERV activation and which HERVs in particular may be playing a more prominent role in the disease process.

So, I've listed on this slide some of the issues with HERV analysis. I won't go through all because you can read them. But I think number two is important because as I said, the highest levels of HERV expression are not in the cell immune cells. So, looking in PBMCs may not be the right place to look for evidence of HERV activation expression. We should perhaps be looking at non-lymphoid tissues. So, there's a bias there, I think.

There's also bias in methodologies. There are different methodologies used for this. All of them come with their caveats, their issues with sensitivity, reliability, et cetera. And of course,

looking for functionality, the question is what's more important? Is it the virus transcripts? Is it the proteins that they incurred, or is it the particles? Which is more important? Which is having the biggest impact in terms of impacting on the immune system and ME disease in -- of itself?

We've still got a lot to learn about understanding the silencing and activation of HERV expression. And the contributions to immunity, again, are only just starting to emerge. We're only just starting to get clues, but I think they're very intriguing clues. Then of course the key question is, is this a cause of the disease, or could it actually -- is it just simply an effect of another ongoing symptomology that's triggered the activation of these? So, lots of issues to address with HERV analysis in general.

And then for looking in ME, these are some of the obvious caveats, different types of methodologies I've already said. Differences among cohorts. So, case definition, there is relatively good consistency, but still there are different case definitions being used. Degree of severity, male versus female, disease duration, and infection history I think is very important here as well. And we need larger cohorts in order to achieve the relative statistical power to be able to discriminate what's ME-specific and which is not.

And we also need to consider environmental factors as well as epigenetic factors because HERVs are responsive to changes in an individual's environment, exposure to environmental insults, as well as the change that may occur in the genome of the patient. So, we need to take a little bit broader look perhaps at not just the patient's symptomology, but their environment, their lifestyle, their behavior.

And so, this is my summary and the questions that it raised. I think there's enough evidence to say that HERVs, be they LTRs, proteins and/or particles, are expressed in at least some ME patients and in their peripheral blood mononuclear cells. But we may be missing the real picture because we're not looking in the right cells in which HERVs would be more readily expressed or more abundantly expressed.

So, we need to address this further by looking at defined clinical subtypes. We need to correlate it with key physiological abnormalities, particularly the immune abnormalities correlate with disease severity. And it would be really important to have a careful and accurate disease infection history to really start to tease out the causality of this. And HERVs have the intriguing possibility of really being one of the drivers that could cause the immune dysfunction that's prevalent in ME/CFS patients. But again, there's a lack of uniformity, and the consensus at the moment, we need more studies to be able to tease out the noise from the genuine signal. And I think this is where combining different approaches, different data sets can help improve the resolution of that.

So, this is the hypothesis that I think is worthy of consideration and debate. Is the reactivation of HERV Super-Antigens, the antigens that the HERV virus encodes that stimulate lots of different type of immune cells, is that reactivation a consequence of an acute or chronic virus infection, particularly those associated with ME? So, could the reactivation of HERVs and the expression of these super-antigens be the missing link in the role to explain viral infections in ME/CFS disease etiology?

And with that, I will just leave you this slide, which is, in order to test this hypothesis, this is I think what we need to do, larger scale virus-epigenome-immune combined association studies that employ all of those things listed by the bullet points there. And in particular, the one I keep emphasizing is combinatorial data to improve the precision and accuracy, combining data sets. And in that way, we can get a better understanding of the virus genetic immune factors that may contribute to ME/CFS. Thank you. I'm happy to take any questions.

Vicky Whittemore: Thank you very much for that excellent overview, Simon. So, I'll start with a question from -- that came in here. Let me find it again. So, is there any evidence that exercise can activate HERVs?

Simon Carding: Well, if you consider that as a stress, and it certainly stresses your immune system as well as obviously muscle cell tissues, the hormonal system then potentially, yes. And I think maybe over-exercising where you're actually causing tissue injury might be a scenario that could lead to activation or repression of certain genes through HERVs. So, possibly yes, but I'm not aware of anybody that's even considered that for HERV expression ME. But's certainly a plausible hypothesis, I think.

Vicky Whittemore: Yeah. Thank you. So, another question. If HERV expression is dependent on host cell transcriptional machinery, wouldn't that link expression likelihood to major tissue damage that induces large tissue repair responses? Yet I think it was mentioned that physical damage has not been shown to have a strong association or to be seen in ME/CFS. What are your thoughts about that?

Simon Carding: So, I think the injury to cells and tissues could feasibly come from activation of HERVs which release to the production of these super-antigens, be they long -- be they transcripts, proteins, or virus particles. And that chronic overstimulation of the immune system will cause the inflammatory cascade. And if that's not effectively shut down, these antigens continue to get expressed. That, I think, then links -- can lead to systemic inflammation, all sorts of tissue cellular injury, which will be difficult to resolve once it's established a foothold.

Vicky Whittemore: So, another question. What is the most important resource that individuals with ME/CFS can direct their sometimes doubtful doctors to regarding the validity of

investigating chronic infection involvement in ME/CFS? And maybe that's a question for all the speakers.

Simon Carding: Yeah, I think so.

Maureen Hanson: I've already sent a couple references from Michael Peluso. And -- but I recently wrote an article in PLoS Pathogens that could also be referenced. I think if you google my name and PLoS Pathogens, you'll find that article.

Vicky Whittemore: Great, thank you. So, just a question I had from all of these discussions. What is the ideal tissue to be studying if not blood clearly, muscle I think is an interesting candidate, but what -- are there other tissues that we should be thinking about studying in ME/CFS.

Simon Carding: In terms of ease of access, I would say any mucosal tissue. So, you know, you can take samples from the upper/lower gastrointestinal tract, the nose, the respiratory, that could take a bowel sample, a vaginal swab, maybe even skin swabs. I think they're a good place to go. Otherwise, you're talking about more invasive procedures, taking biopsies from deeper tissues. So, I think mucosal tissues would be a good place to start.

Maureen Hanson: I agree, those are good places to start. The problem is we don't have a bank of cadaver tissue, which has really benefited the people working on long COVID, and it would be really valuable to have, you know, access to the central nervous system, to the brain that you really can't easily get from a living subject.

Simon Carding: Yes.

Vicky Whittemore: Absolutely. Are there other questions from -- I don't see any other questions in the chat. There's a specific question for you, Maureen, about when your study at the hospital for special surgery will be up and running?

Maureen Hanson: You can contact us now to get on the list of people to be contacted and screened. So, again, it's carl.franconi@cornell.edu. Just send an email and you'll be put on the list to be contacted.

Vicky Whittemore: Thank you. Any other questions or comments from anyone on the panel or any other -- in the Q&A? If not, I'll turn it over to you, Maureen, for any last words you might have.

Maureen Hanson: Okay. Well, I guess mainly my last words are to thank people for going to

the effort of doing this exercise because, especially Drs. Komaroff and Carding had to review information on work that they weren't themselves working on. And that's always more difficult than talking about your own work. So, I really appreciate it. I certainly really learned a lot about EBV, HHV, and HERVs and also endogenous retroviruses. And I certainly look forward to further research in these areas.

Vicky Whitemore: Right. With that, we'll call this webinar to a close, and I certainly thank all of you for attending and especially thank you -- excuse me -- thank you to all of the speakers who, as Maureen said, did a tremendous job. I'm sure it was a tremendous amount of work to review the literature. So, and very thoughtful information and thoughts about research priorities and future directions in research on ME/CFS in this particular area. So, I thank you and hope that you'll join us for the next webinar which will take place on December 8th. Thank you, everyone.