CURRENT INSIGHTS INTO COMPLEX POST-INFECTION FATIGUE SYNDROMES WITH UNKNOWN AETIOLOGY: THE CASE OF MYALGIC ENCEPHALOMYELITIS/CHRONIC FATIGUE SYNDROME AND BEYOND

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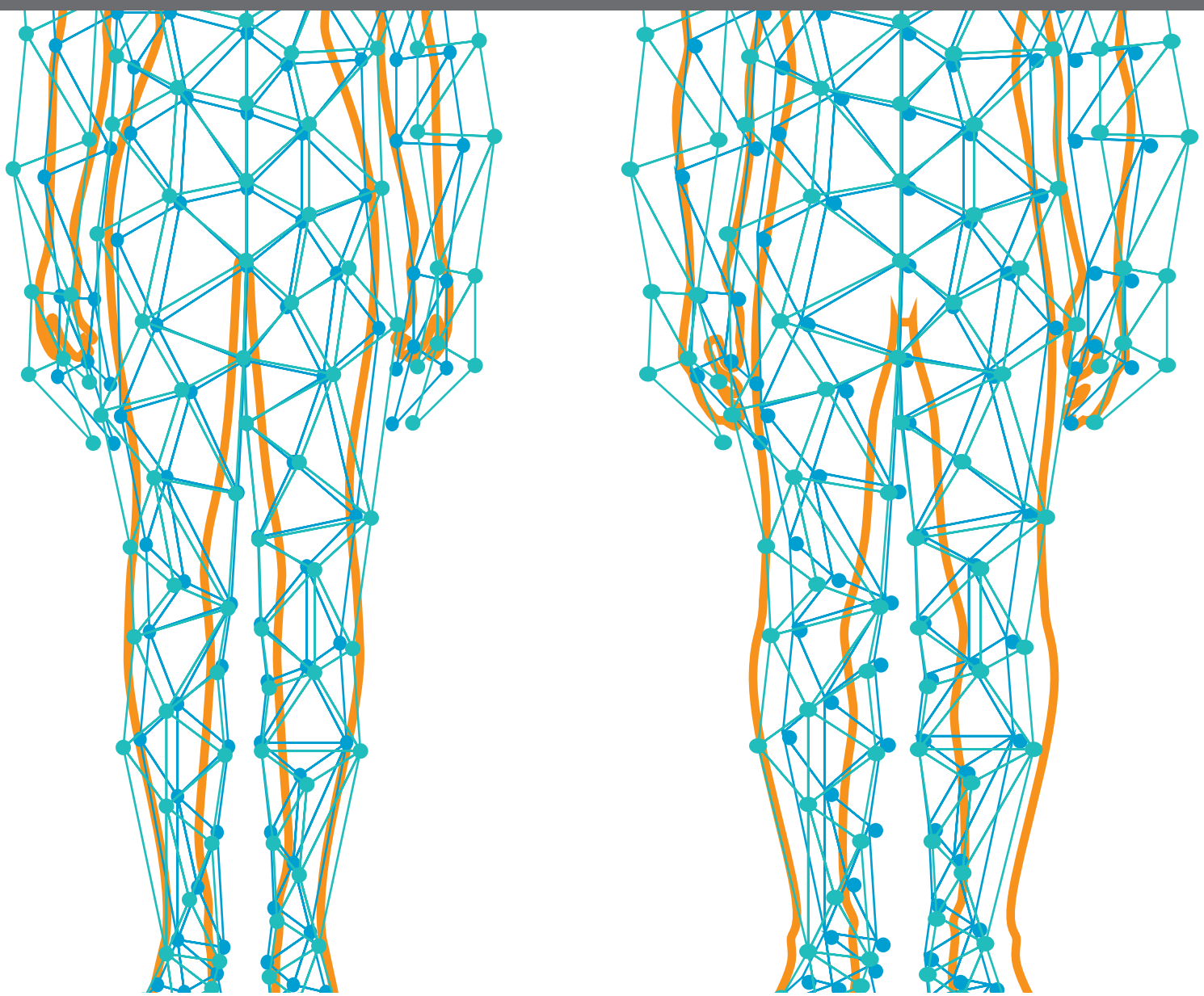

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EDITORIAL
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Editorial: Current Insights Into Complex Post-infection Fatigue Syndromes With Unknown Aetiology: The Case of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Beyond

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Editorial on the Research Topic

Current Insights Into Complex Post-infection Fatigue Syndromes With Unknown Aetiology: The Case of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and Beyond

INTRODUCTION

Black plague epidemics in Medieval Europe, the Spanish Flu pandemic during the first world war, and the pandemic of COVID-19 disease are just three devastating examples of the fragile co-existence between human beings and the microbial world. Remarkably, the human immune system with its innate and adaptive arms recognizes and clears the invading pathogens in most cases. However, like a scar after an injury, some people who had suffered from acute infections remain ill long after the clearance of the pathogen itself. These individuals develop complex fatigue-related syndromes whose pathological mechanisms remain poorly understood. A prime example of such syndromes is the Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) characterized by persistent fatigue and post-exertional malaise among other symptoms (1). Unfortunately, its diagnosis remains challenging due to the inexistence of objective biomarkers that could identify cases. However, researchers are gathering around multidisciplinary networks, such as the US ME/CFS Clinician Coalition and the European Network on ME/CFS, with the aim of fostering collaboration, standardizing research and clinical practices, while accelerating biomarker discovery (2–5). Less-known fatigue-related syndromes have been recently reported after the outbreaks of Ebola virus, Dengue virus, and Chikungunya virus in the Tropics (6–8). However, it is still unclear whether these syndromes constitute clinical entities beyond ME/CFS itself.

In this scenario, we invited the research community to contribute with studies on these complex fatigue-related syndromes. Our primary objective was to take the pulse of current data and hypotheses about how these syndromes are initiated and maintained over time. Our second objective was to understand how current insights can lead to successful treatments for patients. With the WHO notification of the COVID-19 as a pandemic on March 11, 2020, our third and final objective was to debate for the first time about ME/CFS as a sequela of post-SARS-CoV-2 infections. The graphical summary of all the contributions received is shown in **Figure 1**.

OLD AND NEW VIRAL TRIGGERS OF ME/CFS

Early on, it was immediately recognized the impact of herpesviruses on the pathology of ME/CFS (9–11). Follow-up studies made clear that other viruses could also elicit the disease (12). However, the respective pathological mechanisms remain to be uncovered. In this regard, O'Neal and Hanson offered a critical review about past research on enteroviruses as causative agents of ME/CFS. Another interesting review was conducted by Lidbury who discussed the immune evasion strategies of the Ross River virus, which is an arbovirus endemic to Australia, Papua New Guinea, and other islands in the South Pacific. We foresee this review to be useful for understanding post-infection fatigue syndromes due to other arboviruses, such as the Chikungunya, Dengue, and Zika. In this regard, it is a priority to study the burden of post-infection fatigue among Brazilian or Cape Verdean survivors who suffered from recent outbreaks of these arboviruses (13, 14). Finally, Lee et al. and Domingues et al. provided new research on herpesviruses in patients from the United Kingdom ME/CFS biobank. The first study is a rare longitudinal analysis of multiple herpesviruses in patients with ME/CFS; such studies should become standard given the natural fluctuations in disease dynamics. The second study concerns a re-analysis of published serological data using a stratification based on infection and non-infection triggers. The findings of this study clearly show the necessity of stratifying patients adequately, as suggested by Jason et al. (15).

With the onset of the COVID-19 pandemic, a new viral trigger of ME/CFS is currently spreading across the world: SARS-CoV-2. Past experience with the "original" SARS-CoV pandemic suggested this coronavirus as another trigger of ME/CFS (16). Before any mainstream discussion about "long-COVID" or "post-acute sequelae SARS-CoV-2 infection", Komaroff and Bateman on behalf of the US ME/CFS clinician coalition drafted a sort of memorandum alerting for the devastating long-term consequences in survivors of SARS-CoV-2 infections. In turn, Petracek et al. reported probably the first three ME/CFS cases after 6 months of SARS-CoV-2 infections. Other studies published elsewhere provide further evidence that some long-COVID patients suffer from ME/CFS (17, 18) and, as such, there is a window of opportunity to improve the understanding of both conditions.

NEW PERSPECTIVES ON DISEASE PATHOLOGY AND TREATMENT

A key challenge of investigating ME/CFS is that the disease is likely to be multifactorial and heterogeneous and, therefore, patients might show different pathological pathways that could explain their symptoms. To resolve this, many theoretical papers about possible disease mechanisms emerged in the literature over the years (19–23). In this Research Topic, Stanculescu et al. followed the footsteps of these early theoretical papers by paralleling the pathological mechanisms suggested for patients in an intensive care unit (ICU) and patients with ME/CFS. Their research premise is that the same "vicious circle" between inflammation, oxidative and nitrosative stress, and low thyroid hormone function is operating in both clinical populations. In a follow-up paper, Stanculescu et al. made a comprehensive review of available treatments for ICU patients with the idea of being repurposed to stop that "vicious circle" in patients with ME/CFS. Given the heterogeneous nature of ME/CFS, it is likely that the suggested parallelism might only hold true for some but not all the patients. In another theoretical paper, O'Boyle et al. provided a general discussion about treatment and case management using a previously proposed framework for the natural progression of the disease (24). These authors suggested that pre-disease and early disease call for rehabilitation strategies that could avoid long-term co-morbidity while the management of the established disease should be more holistic and tailored to the specific needs of each patient. The basic question is whether clinicians are able to estimate accurately at which disease stage a patient is.

As a follow-up from early clinical trials in Norwegian patients with ME/CFS (25, 26), Sørland et al. evaluated endothelial function in patients with ME/CFS at baseline and after a therapeutic intervention with cyclophosphamide, an immunosuppressive drug used in cancer. This evaluation was motivated by the growing evidence of vascular abnormalities in ME/CFS (27, 28). The authors also found endothelial dysfunction at baseline, which persisted after treatment irrespective of the clinical response of the patients. Interestingly, the authors also reported a significant correlation between high symmetric dimethylarginine (SDMA) levels and low flow-mediated dilation values. Thus, given that SDMA has been described to reduce the production of nitric oxide (NO) in endothelial cells (29), this study raises a new perspective to address endothelial dysfunction in ME/CFS by combining clinical and metabolic parameters. Endothelial dysfunction and inadequate regulation of blood flow resulting in hypoperfusion of the brain and muscles are considered as key pathological mechanisms in ME/CFS as further outlined in two recent papers (21, 30). There is increasing evidence that autoantibodies directed against vasoregulatory receptors contribute to the vascular dysregulation in ME/CFS (21, 30). These findings open perspectives for therapy. For example, one can target autoreactive B cells or autoantibodies, and preliminary studies provide evidence for clinical efficacy [reviewed in ref. (30)]. The use of drugs that help regulating vascular function is another possibility to treat patients with ME/CFS.

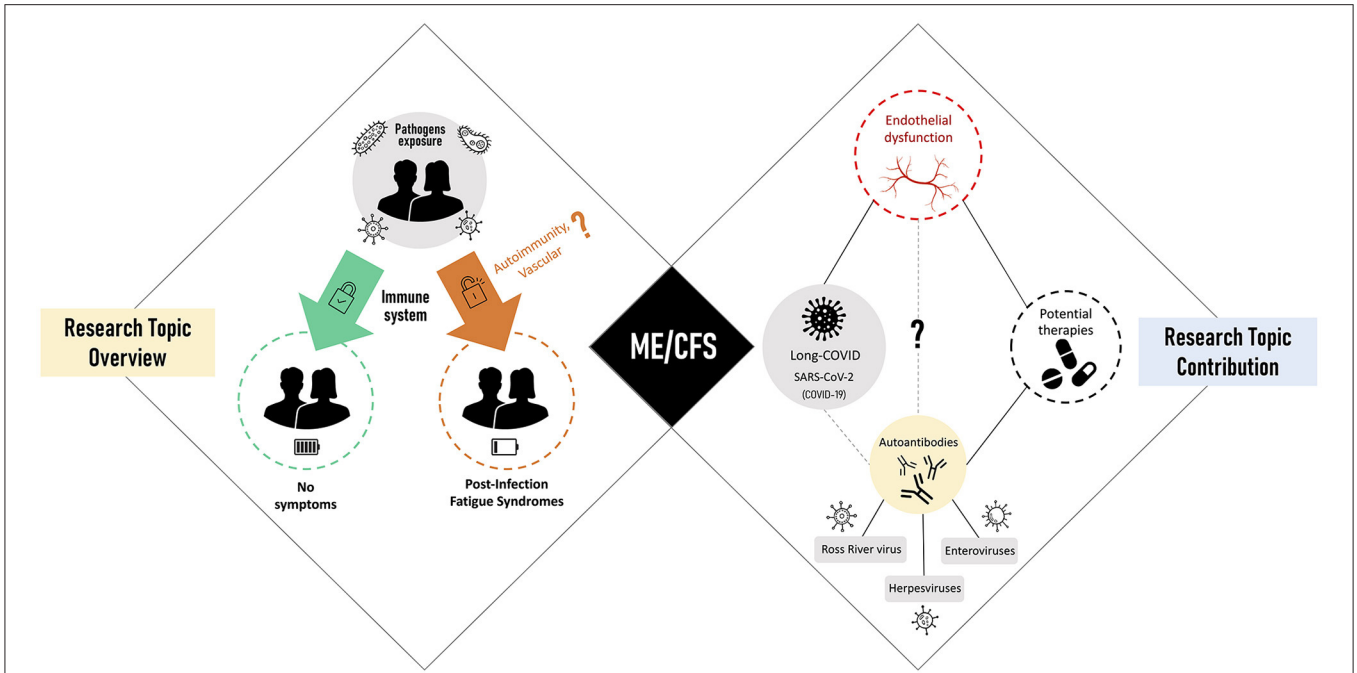

FIGURE 1 | Research Topic overview (left side) linked to post-infectious fatigue syndromes with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) as the main representative (center). Research Topic contribution (right) focused on viral triggers of ME/CFS, disease-related pathomechanisms, and potential therapies.

CONCLUSIONS

In conclusion, this Research Topic collects further pieces of evidence about how various viruses including SARS-CoV-2 can trigger ME/CFS. The neglect of research in ME/CFS during the last decades has left patients, carers, and clinicians alike adrift without a licensed drug to use in the disease. On the one hand, the COVID-19 pandemic will result in an unprecedented explosion of ME/CFS cases. At the same time, this pandemic is the perfect storm that can motivate different stakeholders, including funders and clinicians, to take the necessary steps to accelerate research on ME/CFS and other post-infectious syndromes. If taken, these steps will bring hope to all those outstanding patients who have been homebound or even bedridden for many years but neglected by national health authorities.

AUTHOR CONTRIBUTIONS

All authors contributed to this editorial and approved the final version.

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OPINION
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Will COVID-19 Lead to Myalgic Encephalomyelitis/Chronic Fatigue Syndrome?

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Keywords: COVID-19, SARS-CoV-2, post-infectious fatigue syndrome, post-viral fatigue syndrome, myalgic encephalomyelitis/chronic fatigue syndrome

INTRODUCTION

"Recovering" from COVID-19 does not guarantee a return to a person's usual state of health. For one thing, some people with multi-system injury—particularly to the brain, heart and kidneys—may develop permanent dysfunction of those organs.

In addition, a more subtle form of chronic illness may develop. For some people with COVID-19, even those who are only mildly affected at first, the ensuing weeks and months of "recovery" bring a surprise and a betrayal: they do not return to full health. Although nucleic acid tests no longer detect the virus, people still suffer from ongoing symptoms. They call themselves "long haulers," and the condition is being called "long COVID."

HOW COMMON IS A LINGERING POST-COVID-19 ILLNESS?

The Centers for Disease Control and Prevention (CDC) followed nearly 300 people who were PCR-positive for SARS-CoV-2 for several weeks. Three weeks after the positive test, nearly half of the patients still had symptoms, such as fatigue and cough—particularly people who were older or suffered from chronic diseases (1).

Italian investigators studied 143 confirmed COVID-19 patients after the most severe symptoms had ended. Sixty days after the onset of their illness, more than half of the patients continued to have multiple bothersome symptoms, and 41% reported a worsened quality of life (2).

Irish investigators studied 128 patients with PCR-documented SARS-CoV-2 infection and found that, at a median of 10 weeks after the initial COVID-19 symptoms, 52% reported persistent fatigue and 31% had not returned to work. Surprisingly, there was no association of post-COVID fatigue with the severity of the acute illness, nor with routine laboratory markers of inflammation and cell turnover (3).

Between December, 2019 and May, 2020, a group of patients conducted an online survey of patients who, by self-report, experienced symptoms consistent with COVID-19, in collaboration with University College, London; Weill Cornell Medicine, New York, NY; and Oregon Health and Science University, Portland, Oregon. The survey consisted of 257 questions, was translated from English to eight other languages, and was completed by 3,762 patients (age 18 or older) from 56 countries—predominantly white, middle-class, and English-speaking. Of the respondents, 8.4% reported being hospitalized, and 27% reported a laboratory-confirmed diagnosis. At 7 months after the onset of the illness, continue fatigue, post-exertional malaise and cognitive dysfunction (all core symptoms of ME/CFS) remained in 77.9, 71.2, and 56.8%, and 67.5% were unable to work

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or required a reduced work schedule compared to prior to the illness onset. Systematic physical examination and laboratory diagnostic panel was not performed (4). The data, as reported, don't allow a determination of how many of these people with possible COVID-19 met criteria for ME/CFS, but it is plausible that the majority did.

POST-INFECTIOUS FATIGUE SYNDROMES

It is not surprising that some people infected with the COVID-19 coronavirus (SARS-CoV-2) develop a debilitating chronic fatigue. Post-infectious fatigue syndromes follow in the wake of acute infections with several different types of infectious agents: viruses, such as SARS coronavirus (5), Epstein-Barr virus (6–8), Ross River virus (8), enteroviruses (9), human herpesvirus-6 (10), Ebola virus (11), West Nile virus (12), Dengue virus (13), and parvovirus (14); bacteria, such as *Borrelia burgdorferi* (15), *Coxiella burnetii* (16), and *Mycoplasma pneumoniae* (17); and even parasites, such as *Giardia lamblia* (18). The *acute* symptoms of these illnesses, and the organ damage they cause, can be very different. However, the lingering chronic fatiguing illness following each illness appears to be quite similar.

MYALGIC ENCEPHALOMYELITIS/ CHRONIC FATIGUE SYNDROME (ME/CFS)

People with post-infectious fatigue syndromes following these well-documented acute infections share a group of symptoms in common with people who have ME/CFS (originally called just "chronic fatigue syndrome"). Many, but not all, people with ME/CFS note that it began suddenly, with an apparently infectious illness characterized by respiratory symptoms, fever, adenopathy, myalgias, and other symptoms. Because such acute illnesses are common and typically resolve, often no attempt has been made to diagnose the inciting infectious agent. Yet the spectrum of symptoms in ME/CFS that follows an apparently infectious illness due to an undocumented infectious agent is very similar to the illness following a well-documented infectious agent.

Indeed, according to Dr. Anthony Fauci, the Director of the National Institute for Allergy and Infectious Diseases, patients post-COVID-19 can develop "a post-viral syndrome that's very strikingly similar to myalgic encephalomyelitis/chronic fatigue syndrome" (19).

A widely-used case definition of ME/CFS was proposed by the U.S. National Academies of Sciences, Engineering and Medicine (NASEM) (20). This case definition requires that the illness must have lasted for at least 6 months. Since most people who have developed COVID-19 in the U.S. have not yet been ill for 6 months, not enough time has elapsed to know how many will develop an illness that meets the case definition of ME/CFS. We think it is likely that some will.

What is the current burden from ME/CFS in the United States? CDC and NASEM estimate that between 836,000 and 2.5 million Americans suffer from ME/CFS; the direct and indirect economic costs of the illness to society are estimated to be between $17 and $24 billion each year (20).

HOW MANY ADDITIONAL CASES OF ME/CFS WILL BE CAUSED BY THE PANDEMIC?

One can only guess about the future, but we propose a few conservative estimates. As of late December 2020, nearly 20 million Americans have tested positive for SARS-CoV-2. Based on serological studies the CDC estimates that the true number of infections may be exponentially higher.

To estimate the number of people in the U.S. who may develop "long COVID" we make two conservative assumptions: (1) the introduction of effective vaccines in late 2020 and early 2021 will constrain the total number of people in the U.S. who become infected with SARS-CoV-2 to only 25 million Americans by the end of 2021; and (2) although over 50% of people with confirmed or suspected COVID-19 state that they remain with lingering symptoms at 3 months, we assume that only 10% will be left with an illness that meets the NASEM case definition for ME/CFS. This is consistent with a careful prospective study of the course of symptoms following three quite different acute infections (8). Over the course of 1 year, that would at least double the number of Americans suffering from ME/CFS. The annual incidence of the illness would equal or surpass the point prevalence—a remarkable event in the history of a chronic illness.

What might this mean globally? As of December 2020, COVID-19 has been documented in about 80 million people, globally. Using similar estimates to those we used for the U.S., that number would be predicted to increase to nearly 110 million during 2021, and to generate over 10 million new cases of ME/CFS, globally.

Of course, these number are all rough guesses. But they are informed by well-measured prior experience, and suggest that the U.S. and the world will see a substantial growth in the number of people with ME/CFS. How lasting that illness will be, we cannot know. Most long-term longitudinal studies of people with ME/CFS before the pandemic found that, in most patients, the illness had not abated after many years (21, 22), although the prognosis may be somewhat better in children with ME/CFS (23).

WHAT CAUSES POST-INFECTIOUS FATIGUE SYNDROMES AND ME/CFS?

Any acute infectious disease, like COVID-19, that damages multiple organ systems can cause chronic symptoms (along with objective physiological abnormalities) in some people. The symptom of chronic fatigue could be caused by impaired function of the heart, lung, or kidneys. It is too early in the COVID-19 pandemic to know how many will suffer permanent dysfunction of these organs, but it surely is possible. Therefore, in some people with persistent, debilitating fatigue following COVID-19, documentable damage of these organs

may be a sufficient explanation of their fatigue. Careful longitudinal studies assessing both symptoms and physiologic function will be necessary to know whether, and how often, this happens.

Experiencing a life-threatening illness, particularly when extreme measures, such as artificial ventilation are required, can lead to post-traumatic stress disorder (PTSD). And if a patient has not been able to return to pre-illness function due to chronic symptoms, the persistent symptoms may also trigger major depression. These psychiatric disorders also may lead to chronic fatigue and related symptoms.

Yet, many cases of post-infectious fatigue follow in the wake of acute infections that are not known to cause permanent damage to the heart, lungs or kidneys—and in people without comorbid PTSD or depression. In the typical case of ME/CFS, in particular, the inciting "infectious-like" illness most often appears to be a transient infection, or a primary infection that becomes permanent but does not typically produce chronic organ dysfunction (such as occurs with Epstein-Barr virus).

What might explain the fatigue and other symptoms if there is no documented heart, lung or kidney damage? Although uncertain, it is likely that the causes of all post-infectious fatigue syndromes share with each other and with ME/CFS many common elements (24). Longitudinal studies of people who develop COVID-19 may help reveal the biological underpinnings of many post-infectious fatigue syndromes.

In people with lingering fatigue post-COVID-19—and without chronic cardiac, pulmonary or renal dysfunction—one likely explanation for the chronic fatigue is a state of chronic low-grade neuroinflammation generated by the disease (25).

SARS-CoV-2 can infect the brain, causing neuroinflammation (26). Moreover, inflammation elsewhere in the body can activate the innate immune system in the brain via both humoral and retrograde neural signals, largely involving the vagus nerve (27, 28). As argued elsewhere (24), neuroinflammation can produce fatigue through the action of various cytokines, perhaps acting on a "fatigue nucleus"—a collection of neurons dedicated to diminishing energy-consuming activities ("sickness behavior"). Such energy-conserving behavior in an organism that is infected or injured would help to focus available energy stores on the process of healing (27, 29). In addition to activation of a "fatigue nucleus" by neuroinflammation, a state of chronic, severe fatigue and related symptoms could also be explained by other abnormalities identified in ME/CFS: impaired energy production (30), oxidative stress (31), ion channelopathies (32), and impaired cerebral perfusion (33).

The longitudinal studies that need to be conducted include repeatedly collecting information on the presence and severity of various symptoms—symptoms common in people with COVID-19 and symptoms that are part of case definitions of ME/CFS. Such studies also should include repeated laboratory studies of the immune system, metabolism, gene structure, and the transcriptome, as well as tests of thinking, sleep, and the functioning of the nervous system, heart, and cardiovascular system.

WHAT ARE THE IMPLICATIONS FOR PRACTICING PHYSICIANS?

Although there now is a literature of over 9,000 peer-reviewed studies of ME/CFS, as identified by NASEM (20), it is our experience that many practicing physicians know little about the illness. If a wave of new cases that doubles the number of Americans with ME/CFS is about to emerge, we need to increase efforts to prepare physicians to deal with this burden. A U.S. ME/CFS Clinician Coalition—physicians experienced in the care of people with ME/CFS—has created a website containing useful information[1]. CDC[2] and NIH[3] also provide online information.

CONCLUSION

The COVID-19 pandemic has been a tragedy. It has devastated the health and financial well-being of many people around the world. An unprecedented effort is underway to understand, prevent and treat the disease, including substantial recent funding to study post-COVID illnesses, in the U.S. and elsewhere.

We should not forget the importance of studying all people who become infected with SARS-CoV-2, even those with only mild initial illnesses, and to study the recovery period and the long-term health consequences of COVID-19. We need to know how to prevent and treat "long COVID." What we learn may apply to the prevention and treatment of ME/CFS, as well.

AUTHOR CONTRIBUTIONS

AK conceptualized the paper and wrote the original draft. AK and LB reviewed and finalized the paper. All authors contributed to the article and approved the submitted version.

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[1] MECFSClinicianCoalition.org
[2] https://www.cdc.gov/me-cfs
[3] https://www.nih.gov/mecfs/about-mecfs

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Hypothesis: Mechanisms That Prevent Recovery in Prolonged ICU Patients Also Underlie Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

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Here the hypothesis is advanced that maladaptive mechanisms that prevent recovery in some intensive care unit (ICU) patients may also underlie Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Specifically, these mechanisms are: (a) suppression of the pituitary gland's *pulsatile* secretion of tropic hormones, and (b) a "vicious circle" between inflammation, oxidative and nitrosative stress (O&NS), and low thyroid hormone *function.* This hypothesis should be investigated through collaborative research projects.

Keywords: myalgic encephalomyelitis, critical illness, non-thyroidal illness syndrome, low t-3 syndrome, pituitary, cytokines, oxidative and nitrosative stress, post-intensive care syndrome

INTRODUCTION

Critical illness refers to the physiological response to virtually any severe injury or infection, such as sepsis, liver disease, HIV infection, head injury, pancreatitis, burns, cardiac surgery, etc. (1). Researchers make a distinction between the *acute* phase of critical illness—in the first hours or days following severe trauma or infection; and the *chronic* or *prolonged* phase—in the case of patients that survive the *acute* phase but for unknown reasons do not start recovering and continue to require intensive care (i.e., "chronic ICU patients"). Independent of the nature of the critical illness, the *acute* phase is associated with an excessive response of pro-inflammatory cytokines (2) and is characterized by a uniform dysregulation of the endocrine axes (3). In *prolonged* critical illness, this dysregulation is maintained even once the initial inflammatory surge has settled (4). Regardless of the initial injury or infection, patients that suffer from *prolonged* critical illness experience profound muscular weakness, cognitive impairment, loss of lean body mass, pain, increased vulnerability to infection, skin breakdown, etc. (1, 5, 6). Whereas, the *acute* phase is considered to be an *adaptive* response to the severe stress of injury or infection (shifting energy and resources to essential organs and repair), the physiological mechanisms in the *prolonged* phase are now increasingly considered to be *maladaptive* responses to the stress of severe injury or infection, hindering recovery (7–10). Some have also suggested that the non-recovery from endocrine disturbances could explain the development of "post-intensive care syndrome" (PICS) (11); i.e., "the cognitive, psychiatric and/or physical disability after treatment in ICUs" (12, 13).

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating, multi-system disease of unclear etiology (14, 15). The most common peri-onset events reported by patients are infection-related episodes (64%), stressful incidents (39%), and exposure to environmental toxins (20%) (16). "Impaired function, post-exertional malaise (an exacerbation of some or all of an individual's ME/CFS symptoms after physical or cognitive exertion, or orthostatic stress that leads to a reduction in functional ability), and unrefreshing sleep" are considered to be core symptoms (14). The severity of the symptoms varies: "very severely affected patients experience profound weakness, almost constant pain, severe limitations to physical and mental activity, sensory hypersensitivity (light, touch, sound, smell, and certain foods), and hypersensitivity to medications" (17). We have listed a few hall mark symptoms that are often found in critically ill patients in chronic intensive care (ICU) patients and ME/CFS patients (**Table 1**).

Here the hypothesis is advanced that maladaptive mechanisms that prevent recovery in some ICU patients also underlie ME/CFS. Specifically, these mechanisms are: (a) suppression of the pituitary gland's *pulsatile* secretion of tropic hormones, and (b) a "vicious circle" between inflammation, oxidative and nitrosative stress (O&NS), and low thyroid hormone *function*. These mechanisms characterize *prolonged* critical illness regardless of the nature of the initial severe injury or infection (3, 8–10); similarly, we propose that these mechanisms could underlie the perpetuation of illness in ME/CFS regardless of the nature of the peri-onset event (i.e., infection, stressful incident, exposure to environmental toxins, or other). We provide an overview of these mechanisms in ICU patients and discuss their relevance for understanding ME/CFS. We also bring findings from fibromyalgia into the discussion here because ME/CFS and fibromyalgia are often jointly considered in the literature (20, 21); fibromyalgia is similarly a syndrome that is medically unexplained, often comorbid with ME/CFS, and "shares the core symptoms of fatigue, sleep problems and cognitive difficulties" (22). Additional research projects are required to investigate the validity of this hypothesis building on the findings from critical illness and ME/CFS summarized here.

This hypothesis may be particularly relevant in light of the current COVID-19 pandemic. Many COVID-19 patients continue to experience a variety of debilitating symptoms despite successfully defeating the virus—termed "post COVID-19 syndrome" or "long COVID-19"—that resemble ME/CFS (23–26).

Abbreviations: ACTH, Adrenocorticotropic hormone; ARV, Arginine vasopressin; CRH, Corticotrophin-releasing hormone; DHEA, Dehydroepiandrosterone; GH, Growth hormone; GHIH, Growth hormone inhibiting hormone; GHRH, Growth hormone releasing hormone; HPA, hypothalamus-pituitary-adrenal axis: "Adreno-cortical axis"; HPS, Hypothalamic-pituitary-somatotropic axis: "Somatropic axis"; HPT, Hypothalamic-pituitary-thyroid: "Thyrotropic axis"; ICU, Intensive Care Unit; IGF-1, Insulin like growth hormone-1; IGFBP, Insulin like growth hormone binding proteins; ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; NTIS, Non-thyroidal illness syndrome; O&NS, oxidative and nitrosative stress; PICS, Post-intensive care syndrome; POTS, Postural Orthostatic Tachycardia Syndrome; TRH, Thyrotropin-releasing hormone; TSH, Thyroid stimulating hormone.

SUPPRESSION OF *PULSATILE* PITUITARY SECRETIONS

Endocrine patterns observed during the initial *acute* phase of critical illness (in the first few hours or days) differ markedly from those observed during *prolonged* critical illness (after a few days) (27, 28). Indeed, the *acute* phase is characterized by *increased* release of pituitary hormones; the *prolonged* phase is characterized by *suppression* of the release of pituitary hormones. Simultaneously, hormone half-life and hormone up-take by the peripheral tissues differ markedly between these two phases (4, 29). This *biphasic* pattern of the endocrine system during critical illness, however, is not readily observable in single or average measurements of circulating tropic and non-tropic hormone concentrations—which are a function of both hormone release and elimination from the blood stream. This pattern was thus only discovered in the early 1990s with measurements of the *frequency* and *amplitude* of pituitary secretions (i.e., *pulsatility*) performed as often as every 10 min over 24 h on ICU patients (29). The *pulsatility* of tropic hormone secretion is part of the signaling to the peripheral glands and thus considered a determining factor of hormone *function* (i.e., impact on target glands or tissues), in addition to overall volume of hormone release (30, 31). The finding that *pulsatile* pituitary secretions are suppressed during *prolonged* critical illness was critical in understanding the physiology of the syndrome and the curious failure of patients to recover (32). We describe the biphasic endocrine patterns during *acute* and *prolonged* critical illness for each of the main endocrine axes in further detail below, as well as the implications for the autonomic nervous system, metabolism and the immune system. We also provide evidence suggesting that the endocrine patterns observed in *prolonged* critical illness also underlie ME/CFS.

The Adreno-Cortical Axis (HPA Axis)

The adreno-cortical axis—also called hypothalamic-pituitary-adrenal (HPA) axis—is the body's primary stress management system. The HPA axis responds to physical and mental challenges in part by controlling the body's glucocorticoids levels, notably cortisol (33). Cortisol in turn modulates inflammation response, cardiovascular function and glucose metabolism (34). An inability to deal with stress, proneness to exaggerated immune responses and weight loss are associated with hypocortolism or poor HPA axis function (35–38). The HPA axis also regulates mineralocorticoids that, in turn, regulate water and electrolyte balance (i.e., blood pressure). Low blood pressure and dizziness upon standing up are associated with a compromised HPA axis (35). Finally, the HPA axis (in addition to the gonadotropic axis not covered here) also contributes to the production of androgens, notably DHEA and testosterone, which are steroids that impact muscle mass, fat storage, pain, brain function and many other physiological traits. Low androgens are associated with muscle fatigue, joint pain, and noise intolerance (39–42).

In normal conditions, the adrenal gland secretes cortisol during the day in pulses, with the highest amounts in the early morning hours and lower amounts at night. The hypothalamus signals to the pituitary with corticotrophin-releasing hormone

TABLE 1 | Comparison of the typical clinical picture of ICU patients and patients with ME/CFS.

Type	Prolonged critical illness (5)	ME/CFS (18, 19)
Neuromuscular/Neurological	**"Profound weakness** attributed to myopathy, neuropathy, and alterations of body composition including loss of lean body mass, increased adiposity, and anasarca" **"Ventilator dependence"**	• Extreme fatigue or lack of energy • Persistent exhaustion • Post-exertional malaise (symptoms worsen after exertion) • Ataxia and muscle weakness • Pain (muscle and joint pains) • Orthostatic intolerance
Endocrine/Autonomic	**"Distinctive neuroendocrine changes** including loss of pulsatile secretion of anterior pituitary hormones, contributing to low target organ hormone levels and impaired anabolism"	• Temperature instability • Weight loss or gain • Sleep dysfunction & unrefreshing sleep • Postural Orthostatic Tachycardia Syndrome (POTS) • Light-headedness • Change in appetite • Nausea & irritable bowel syndrome
Immunological	**"Increased vulnerability to infection,** often with multi-resistant microbial organisms" **"Skin breakdown** associated with nutritional deficiencies, edema, incontinence, and *prolonged* immobility"	• Infection-immune like symptoms • Recurrent flu-like symptoms • Sweating/Fever • New sensitivities to food, medication, chemicals • Sore throat • Tender lymph nodes
Cognitive	**"Brain dysfunction** manifesting as coma or delirium that is protracted or permanent"	• Cognitive impairment • Brain Fog • Confusion and Disorientation • Difficulty concentrating • Short-term memory issues • Hypersensitivity to noise and light
Emotional	**"Distress** from symptoms including pain, thirst, dyspnea, depression, and anxiety"	• Emotional instability, anxiety, and depression

(CRH), and to a lesser extent, arginine vasopressin (AVP), to produce adrenocorticotropic hormone (ACTH). This is in turn signals the adrenals to release cortisol and other hormones. Most cortisol circulating in the blood is bound to carrier molecules (29, 43). Production of cortisol is regulated by an inhibitory feedback loop. When free circulating cortisol attaches to glucocorticoid receptors on the hypothalamus and pituitary, these glands reduce production of CRH and AVP, and ACTH, respectively. The number and affinity of glucocorticoid receptors is thus considered one of the most important determining factors in the regulation of the HPA axis (43)

In Critical Illness

During the *acute* phase of critical illness, plasma cortisol concentrations rise rapidly. Increased cortisol availability is considered a vital response that allows for fluid retention, increased cardiac output and blood pressure, and induces an appropriate immune response while protecting against excessive inflammation (29, 44, 45). Until recently believed to be the result of increased cortisol production by the adrenals, it is now known that high cortisol availability during this phase of critical illness is in fact largely driven by two peripheral mechanisms: a decrease in the abundance and affinity of the cortisol carrier molecules in circulation, and a slowing of cortisol breakdown in the liver and kidney (29, 34, 44, 46, 47). Via inhibitory feedback loops, these higher cortisol concentrations suppress the HPA axis at the central level: the secretions of CRH and AVP by the hypothalamus and of ACTH by the pituitary fall, leading to an eventual drop in plasma cortisol levels (48).

Whereas, in critically ill patients that begin to recover, the HPA axis essentially normalizes within 28 days of illness, in cases of *prolonged* critical illness ACTH levels (surprisingly) continue to be depressed despite dropping cortisol levels (49, 50). Why and how this central suppression of ACTH is maintained is not clear and continues to be debated. Pro-inflammatory cytokines and O&NS likely play a leading role. Cytokines can mediate tissue-specific changes in the abundance and affinity of glucocorticoid receptors—which are major factors determining the activity of the HPA axis (2, 44). Specifically, the cytokine IL-1β is known to modulate CRH release by the hypothalamus; TNF-α is known to impair ACTH release by the pituitary; and TNF-α is also known to impair cortisol production by the adrenal glands (2).

Without sufficient *pulsatile* stimulation by the tropic hormone ACTH, adrenal glands begin to atrophy and lose zonational structure. This is evidenced in the post-mortem dissection of patients that had been critically ill for a few weeks, but not in the patients that quickly died from their illness or trauma (34, 51). The weakening of adrenal glands not only compromises patients' ability to cope with external stressors but also permits excessive inflammatory responses. In sum, the initial beneficial increase in cortisol availability induced by peripheral mechanisms during the *acute* phase of critical illness leads to a suppression of the HPA axis at the central-level from which a subset of patients appears unable to escape (**Figure 1**).

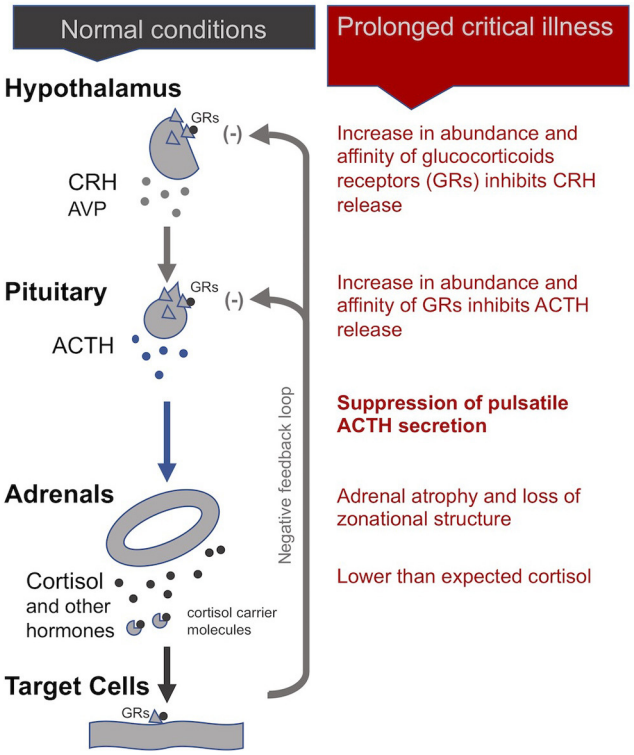

FIGURE 1 | The adreno-cortical axis (HPA axis) during normal conditions and *prolonged* critical illness.

In ME/CFS

Dysfunction of the HPA axis has been documented extensively in ME/CFS patients since the early 1980s (52–63). Researchers have observed decreased baseline cortisol levels, blunted HPA axis responses to physical and psychological stressors, reduced HPA axis responsivity to provocation tests (such as CRH and ACTH administration), and a heightened inhibitory feedback loop (consistent with a higher abundance and affinity of glucocorticoid receptors at the level of the pituitary and hypothalamus). Strikingly, the magnitude of HPA axis dysfunction becomes more pronounced with illness duration and is associated with symptom severity (43, 64). Very few have studied *pulsatility* of ACTH release: one study of 36 study-pairs found no statistically significant differences in ACTH *pulsatility* between ME/CFS and matched controls (65), while another found a differential pattern of ACTH release over 24-h periods (66). Variations in the study-participants' severity of illness—and methods used to control for these—may explain these apparently contradictory findings. Several studies have found the morning peak of ACTH is missing or weak in ME/CFS patients (43). A recent study assessing secretory events of cortisol found that CFS/ME patients have the same number of secretory events but secrete lower quantities in early morning hours (67). Significantly, a group of ME/CFS patients were found to have 50% smaller adrenals than controls (68), resembling adrenal atrophy in *prolonged* critical illness.

ME/CFS researchers have also proposed models to explain the persistence of a suppressed HPA axis (33, 69, 70). Essentially, a short stress (i.e., a burst of cortisol) will produce a small perturbation in the glucocorticoid receptor concentration on the central glands that quickly returns to normal levels. However, long, repeated stress—from which the system doesn't have time to recover—leads to a persistent high glucocorticoid receptor concentration, forcing the HPA axis to an alternate steady state. More recent models of the HPA axis have also included non-genomic feedback-controls (71), the endogenous effects of circadian rhythm (72), and interactions with the gonadotropic axis and the immune system (73, 74) to explain how HPA axis suppression is maintained even after the initial stress is gone.

HPA axis dysfunction is also present in the majority of fibromyalgia patients (75–77). Various mechanisms have been suggested, including depressed secretion of CRH by the hypothalamus, a deficiency of CRH receptors on the pituitary, and adrenal atrophy due to chronic under-stimulation by reduced ACTH levels (78).

Moreover, the dysfunction of the HPA axis in ME/CFS and fibromyalgia has also been associated with pro-inflammatory cytokines and O&NS (43, 55, 79, 80). A recent paper considering the bidirectional relationship between the function of the HPA axis and inflammation finds that immune-inflammatory and O&NS pathways *induce* HPA axis dysfunction in ME/CFS (81); the direction of causality is analogous to inflammatory pathways inducing endocrine dysfunctions in critical illness. Others have similarly theorized that local inflammation in the hypothalamus leads to a disturbed HPA axis in ME/CFS (82).

In sum, the HPA axis dysfunctions in ME/CFS are not unlike the dysfunctions in *prolonged* critical illness. However, to our knowledge a comprehensive study of the pituitary *pulsatile* secretions of ACTH in ME/CFS patients—which proved revelatory in understanding *prolonged* critical illness—does not yet exist. The relationship between the pituitary's *pulsatile* ACTH secretions, severity of illness, the integrity and function of adrenal glands and resulting physiological alterations in ME/CFS thus remains largely unexplored.

The Somatotropic Axis (HPS Axis)

The somatotropic axis—also called hypothalamic-pituitary-somatotropic (HPS) axis—plays important roles in growth and development of children, but also contributes to a variety of physiological pathways in adults, including balancing *catabolic* (i.e., the break-down of molecules and tissues) and *anabolic* activities (i.e., the building of molecules and tissue) (4). An HPS axis dysfunction is known to cause loss of muscle and bone mass, induces weakness (29), and impacts gut mucosa integrity as well as glucose and fat metabolism (83). Low energy, exhaustion, mental fatigue, weak muscle strength as well as poor recovery after physical activity are associated with an inhibited HPS axis function (42, 84, 85).

Uniquely, in the case of the HPS axis, the hypothalamus sends both stimulating (+) and inhibiting (-) signals to the pituitary for the production of growth hormone (GH): these are, respectively, the GH-releasing hormone (GHRH) and the GH-inhibiting hormone (GHIH, also called somatostatin) (4).

In addition, ghrelin, mostly produced by the gut, also stimulates GH production by the pituitary. In normal conditions, GH is released by the pituitary in a *pulsatile* fashion under the control of these three signals, with peaks of GH levels alternating with virtually undetectable valleys in 3- to 5-h intervals over the course of the day (29). GH in turn has direct effects on some tissues and also stimulates the production of insulin-like growth hormone-1 (IGF-1), mostly by the liver. Nearly all of the IGF-1 hormones in the plasma are bound to IGF-binding proteins (IGFBP). IGF-1 and GH exert inhibitory feedback on the hypothalamus and the pituitary to maintain homeostasis. The half-life of GH is only 10 to 20 min, whereas the half-life of IGF-1 is more than 12 h. Thus, IGF-1 plasma concentrations are regularly used as proxies for GH secretion in clinical settings. This, however, overlooks the function of the *pulsatile* secretion of GH on the balance of anabolic and catabolic activities in the body (4).

In Critical Illness

In the *acute* phase of critical illness, the pituitary produces more GH: higher peaks, lower valleys and increased pulse frequencies (86). The rapid onset of two main peripheral mechanisms explain this finding: First, under the influence of cytokines, the liver expresses fewer GH receptors (i.e., becomes resistant to GH) and thus produces less IGF-1. Second, alterations in IGF binding proteins results in IGF-1 being cleared out faster from the system (i.e., IGF-1 has a shorter half-life) (87). The lower IGF-1 concentrations resulting from these two peripheral mechanisms will—via the feedback loop inherent to the axis—spur more GH production (29). The resulting increase in *catabolic* activity during the *acute* phase of critical illness serves to mobilize amino acids derived from the breakdown of peripheral tissues, such as skeletal muscle and bone, for use by the central organs (4).

However, if a critically ill patient fails to recover within a few days, GH secretion becomes *erratic* and almost non-pulsatile. Experiments have demonstrated that this is largely due to a lack of stimulation of the hypothalamus and pituitary by the hormone ghrelin. There is also evidence for changes in the relative amounts of GHIH and GHRH signals from the hypothalamus (4). As for the peripheral hormone, IGF-1, its levels are low or normal in *prolonged* critical illness. The liver's resistance to GH (which previously suppressed IGF-1 production during the *acute* phase of critical illness) does not persist during *prolonged* critical illness (29, 87). However, without a concomitant pulsatile release of GH, the *anabolic* function of IGF-1 becomes inhibited (4).

In sum, although the increase in catabolic activity during the *acute* phase of critical illness may initially be beneficial because it serves to mobilize amino acids, the perpetuation of the imbalance in catabolic vs. anabolic activity (due in part to the loss of the pulsatile function of GH) during *prolonged* critical illness may be considered maladaptive (**Figure 2**). The imbalance in catabolic relative to anabolic activity in *prolonged* critical illness leads to protein break-down in skeletal muscle, liver, kidney and heart, reducing their cell mass and leading to impaired function (7). These processes are ultimately reflected in muscle and bone wasting typically present in *prolonged* critical illness (88, 89).

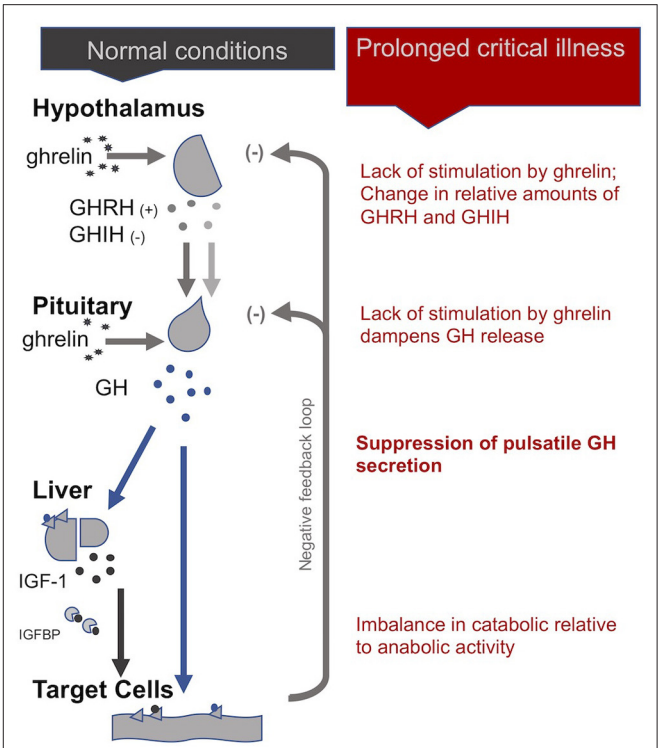

FIGURE 2 | The somatotropic axis (HPS axis) during normal conditions and *prolonged* critical illness.

In ME/CFS

GH regulation in ME/CFS has been studied since the 1990s. The findings are mixed, but almost none addresses the question of the *pulsatility* of GH release. Some described low nocturnal GH secretion (90, 91), while others have found normal levels of 24-h urinary GH excretion (92). Some have found reduced response to induced hypoglycemia (90, 91), while others describe normal GH responses to stimulation (93). One study describes unaffected diurnal patterns of GH release in ME/CFS, but it focused on assessing basal levels rather than the nature of secretory patterns (i.e., pulsatile vs. erratic) and may not have accounted for variations in the severity of illness of patients (66). In terms of IGF-1, there are no consistent differences between ME/CFS patients and controls (93, 94), which is consistent with findings from *prolonged* critical illness.

Studies in fibromyalgia show relative GH deficiency (76, 78, 95–99) and low or low-normal IGF-1 levels (95, 96, 100). Interestingly, some studies showed that fibromyalgia patients "failed to exhibit a GH response to exercise" (97, 101), consistent with a loss in *pulsatility* of GH release.

In sum, endocrine observations in ME/CFS are not unlike HPS axis dysfunctions found in *prolonged* critical illness. To our knowledge the pituitary *pulsatile* secretions of GH in ME/CFS patients has not been comprehensively studied. The relationship between the pituitary's *pulsatile* GH secretions, severity of illness and the balance between catabolic and anabolic activities in ME/CFS thus remains largely undiscovered.

The Thyrotropic Axis (HPT Axis)

The thyrotropic axis—also called hypothalamic-pituitary-thyroid (HPT) axis—regulates the basal rate of our metabolism. Dysfunctions of the HPT axis are associated with tiredness, stiffness, constipation, dry skin and weight gain, among a myriad of other hypothyroid-like symptoms (35, 42).

In normal conditions, an inhibitory feedback loop works to maintain stable circulating thyroid hormone concentrations according to a daily rhythm (102). When unbound circulating thyroid hormone concentrations dip below a certain threshold, the hypothalamus produces thyrotropin-releasing hormones (TRH) in order to signal the pituitary to produce thyroid stimulating hormone (TSH), which in turn signals the thyroid gland to produce more thyroid hormones.

In Critical Illness

Dysfunctions of the HPT axis during critical illness have been studied extensively. Starting in the early 1970s, clinicians working in ICUs observed that patients with a wide range of critical conditions had low plasma concentrations of the *active* form of thyroid hormones (T3) relative to plasma concentrations of *inactivated* thyroid hormones reverse T3 (rT3) (103–105). They gave this condition the name "non-thyroidal illness syndrome" (NTIS), also called "euthyroid sick syndrome" or "low T3 syndrome." While NTIS was initially considered to be beneficial in critical illness—i.e., a state of "protective" down-regulation of metabolism during times of duress (106) —it is increasingly seen as maladaptive and hampering the recovery of patients in the case of *prolonged* critical illness (9, 10, 29, 103, 104, 107, 108).

During *acute* and early stages of critical illness, peripheral mechanisms involving cytokines (notably IL-1β, IL-6, TNF-α) lead to the quick depression of thyroid hormone activity (104, 105, 109–111) to help conserve energy resources (48, 104). The mechanisms include the alterations in the amount and affinity of thyroid hormone binding globulines in the blood (112–114); modifications in the expression of the transporters that carry the thyroid hormone into the cells (115, 116); the down- and up-regulation of deiodinase enzymes that convert the thyroid hormone into active and inactive forms, respectively (113, 117); and the variation in the quantity and isoforms of cellular thyroid hormone receptors present (notably in the liver, adipose tissue and muscle) (118–120). An alteration in any of these steps—which determine thyroid hormone *function*—can lead to large time- and tissue-specific adjustments in cellular metabolism (121, 122)—even without, or with only minor, changes in the blood concentrations of thyroid hormones (121, 123, 124).

During *prolonged* critical illness these peripheral mechanisms are supplemented by central mechanisms that also depress thyroid hormone *function* (125, 126). Cytokines (notably IL-12 and IL-18), in association with other signaling factors (including leptin, glucocorticoids, etc.), are believed to up-regulate the deiodinase enzymes D1 and D2 in the hypothalamus resulting in higher local levels of T3 that inhibit TRH release irrespective of circulating thyroid hormone concentrations (10, 127, 128). Moreover, cytokines (notably IL-1b and TNF-α) also suppress the release of TSH by the pituitary (129, 130). Finally, by reducing iodine uptake and thyroid hormone excretion, cytokines (notably IL-1) also impact the activity of the thyroid gland itself (103, 113). Together, these mechanisms can alter the inhibitory feedback mechanisms of the HPT axis (i.e., its "set-point") during *prolonged* critical illness. Single measurements of circulating TSH, however, are ineffective in revealing such alterations in the set-point of the HPT axis.

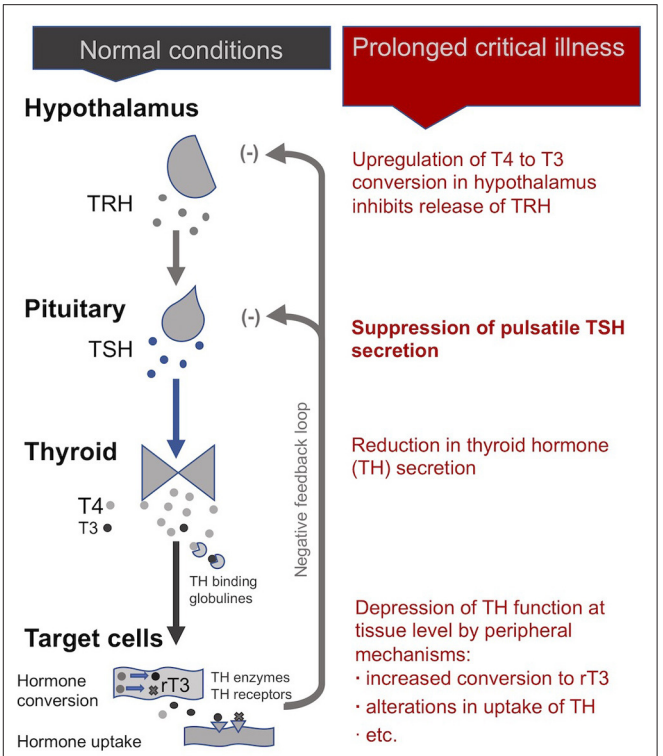

FIGURE 3 | The thyrotropic axis (HPT axis) during normal conditions and *prolonged* critical illness.

In sum, an initial beneficial alteration of thyroid hormone activity in the periphery during *acute* critical illness is followed by a cytokine-mediated central suppression of the HPT axis resulting in a virtual complete loss of *pulsatile* TSH secretion (29). Peripheral mechanisms (notably variations in the conversion and transport of thyroid hormones) may further modulate thyroid hormone *function* in time- and tissue-specific ways resulting in complex physiological alterations in these patients (**Figure 3**) —not readily observable in blood concentrations of thyroid hormones. How these alterations of the HPT axis persist as well as their broader implications on metabolism and the immune system are further described below (see section A "Vicious Circle" Perpetuating Illness).

In ME/CFS

Dysfunctions of the HPT axis have long been suspected to play a role in ME/CFS (77, 131–134) and fibromyalgia (135–140). A recent study showed that ME/CFS patients had similar TSH levels as controls, but lower Free T3, Total T4, and Total T3, which the authors suggest resembles NTIS (141)—the typical feature of critically ill patients in ICUs described above.

In sum, alterations of the HPT axis in ME/CFS resemble dysfunctions found in *prolonged* critical illness. However, there does not to our knowledge exist a thorough study of the *pulsatility* of pituitary TSH secretion events in ME/CFS patients, nor a study of the tissue-specific alterations in thyroid hormone *function*—which proved revelatory in understanding *prolonged* critical illness. The relationship between the TSH axis dysfunctions, severity of illness, hypometabolic state and organ/tissue specific symptoms in ME/CFS thus remains largely unexplored.

Intermediate Conclusions

The endocrine axes control many of the most fundamental physiological processes; their suppression is associated with a myriad of symptoms (see **Table 2**). Essentially, the suppression of *pulsatile* pituitary secretions of ACTH, GH, and TSH are central to *prolonged* critical illness. Inflammatory pathways play a role in inducing and maintaining this suppression irrespective of the nature of the original illness or trauma (see **Table 3**). The resulting endocrine patterns may be considered maladaptive and have wide ranging implications, including dysfunction of the balance between anabolic and catabolic processes, metabolism, and the regulation of the immune system. The physiological parallels between ME/CFS and *prolonged* critical illness would suggest that the suppression of *pulsatile* pituitary secretions of these tropic hormones might also underlie ME/CFS, and that the severity of ME/CFS might be a function of the strength of the mechanism; this however remains largely unstudied. In the next section we provide an overview of a model from critical illness that explains the perpetuation of these endocrine dysfunctions and we describe the relevance of the model for understanding ME/CFS.

A "VICIOUS CIRCLE" PERPETUATING ILLNESS

Based on nearly five decades of research, critical illness researchers have proposed a model that describes how NTIS is maintained by reciprocal relationships between inflammation (notably pro-inflammatory cytokines), O&NS and reduced thyroid hormone *function,* forming a "vicious circle" (9, 10) (**Figure 4**). This model can help to explain the perplexing failure to recover of some critically ill patients in ICUs that survive their initial severe illness or injury. We describe the main elements of this model in a simplified manner below, as well as the implications for metabolism and the immune system. We also provide evidence suggesting that the "vicious circle" observed in *prolonged* critical illness also underlies ME/CFS.

In Prolonged Critical Illness

The key elements of the suggested "vicious circle" in *prolonged* critical illness include the following mechanisms:

(a) Cytokines depress thyroid hormone function: As described above [see section The thyrotropic axis (HPT Axis) In Critical Illness], in *acute* and early stages of critical illness, various peripheral mechanisms involving cytokines lead to the quick depression of thyroid hormone activity in tissue-specific ways. In *prolonged* critical illness, cytokines in association with other signaling factors targeting the hypothalamus, as well as the pituitary and the thyroid glands, also inhibit thyroid hormone production. The relative sequence and importance of these various mechanisms in depressing the HPT axis and thyroid hormone *function* in different tissues and phases of critical illness are the subject of most NTIS publications (10, 104, 105). Notwithstanding the effect of other mechanisms, alterations in the activity of the deiodinase enzymes lead to a decrease in T3 and an increase in rT3 and thus a reduction in thyroid hormone *function* in peripheral tissues during *prolonged* critical illness [based on biopsies on ICU patients who died (142) and studies on mice (143, 144)]. Circulating thyroid hormone concentrations, however, only reveal the "tip of the iceberg" of the alterations occurring at the tissue level (141, 145), which thus are often missed altogether in clinical settings (146).

(b) Low thyroid hormone function contributes to oxidative and nitrosative stress: The relationship between thyroid hormone *function* and O&NS is complex, and both hyperthyroidism and hypothyroidism have been associated with oxidative stress (147). Nonetheless, it seems clear that depressed thyroid hormone *function* hinders tissue cells from maintaining a healthy O&NS balance. Mechanisms include alterations to the lipid concentration of the cell membranes that maintain the cell's O&NS balance (148), and reduced function of two proteins (Uncoupling Proteins-2 and -3) with anti-oxidant properties (149). Moreover, in low thyroid hormone *function* conditions, mitochondria damaged by O&NS are not cleared out of cells (9). In turn, it appears that oxidative stress depletes the glutathione required by the abovementioned deiodinase enzymes for the conversion of T4 into T3 (104). Similarly, competition for, and the resulting depletion of the trace mineral selenium—a component of both the deiodinase and the anti-oxidant enzymes (150) —may amplify the self-perpetuating link between increased oxidative stress and low thyroid hormone *function*.

(c) Oxidative and nitrosative stress stimulate the production of pro-inflammatory cytokines: The final mechanism which completes the "vicious circle" in *prolonged* critical illness is the link between O&NS and inflammation. O&NS stimulates the production of pro-inflammatory cytokines, notably leptin, resistin, TNF-α and IL-6 (151). In turn, pro-inflammatory cytokines (notably IL-6) further increase O&NS by triggering the production of superoxide radicals (104, 152). There is thus a tendency for O&NS and pro-inflammatory cytokines to perpetuate each other as well.

In sum, according to a model proposed by critical illness researchers, a "vicious circle" involving O&NS, pro-inflammatory cytokines, and low thyroid hormone *function*—as well as reciprocal relationships across these elements—can perpetuate a hypometabolic and inflammatory state, and thus help to explain why some critically ill patients fail to recover.

In ME/CFS

Similar patterns of O&NS, cytokines, and low thyroid hormone *function* have recently been documented in ME/CFS patients providing the elements for a similar "vicious circle." We briefly summarize the findings from ME/CFS research relevant to each of these elements.

TABLE 2 | Summary of endocrine axes and function of the main hormones in adults.

Name of Axis	Peripheral endocrine glands	Main hormones	Function	Symptoms of suppressed function
Adreno-cortical axis: "HPA Axis"	Adrenal glands	Glucocorticoids, notably cortisol	Stress response via changes in glucose metabolism. Regulation of immune system.	Inability to deal with stress. Proneness to exaggerated immune responses. Weight loss.
		Mineralocorticoids, notably aldosterone	Regulate water and electrolyte balance (blood pressure).	Low blood pressure. Dizzy on standing up.
		Androgens, notably DHEA (can also be derived from gonadotropic axis).	Function as steroids on muscle mass, fat storage, brain function, etc.	Muscle fatigue. Noise intolerance.
Somatotropic axis: "HPS Axis"	Liver (mostly)	Growth hormone (GH) (produced by the pituitary) and IGF-1 (by the liver).	Regulation of insulin sensitivity, protein building (anabolic activity) and gut mucosal function.	Low energy and weak muscle strength. Poor recovery after physical activity. Exhaustion. Anxiety.
Thyrotropic axis: "HPT Axis"	Thyroid gland	Thyroid hormones: T4, T3, T2, T1, and reverse T3 (rT3).	Regulate baseline level of metabolism.	"Hypothyroid-like" symptoms: tiredness, stiffness, constipation, dry skin, etc. Weight gain.

TABLE 3 | Summary of endocrine dysfunctions and mechanisms in critical illness and ME/CFS.

Name of Axis	Dysfunctions Prolonged critical illness and ME/CFS	Mechanisms Prolonged critical illness
HPA Axis	**Prolonged critical illness:** - Lower than expected cortisol levels - Loss of pulsatile ACTH release **ME/CFS:** - Lower cortisol baseline - Blunted HPA axis response to stressors - Increased negative feedback - Loss of morning ACTH peak	**Hypothalamus:** cytokine-mediated increase in abundance and affinity of glucocorticoids receptors (GRs) inhibits CRH release **Pituitary:** increase in abundance of GRs inhibits ACTH release **Adrenal gland:** adrenal atrophy (due to lack of pulsatile ACTH stimulation during acute phase)
HPS Axis	**Prolonged critical illness:** - Loss of pulsatile GH release - Low or normal IGF-1 **ME/CFS:** - Low nocturnal GH - Mixed response to stimulation - Failed response to exercise (fibromyalgia) - Low or normal IGF-1	**Hypothalamus:** lack of stimulation by ghrelin; change in relative amounts of hypothalamic stimulating/inhibiting hormones (GHRH/GHIH) dampens GH release **Pituitary**: lack of stimulation by ghrelin dampens GH release
HPT Axis	**Prolonged critical illness** - Loss of pulsatile TSH release - Lower T3 - (Lower T4) - Higher rT3:T3 **ME/CFS:** - (Lower Free T3) - Higher rT3:T3	**Hypothalamus:** cytokine-induced alteration in set-point for release of TRH (local upregulation of T4 to T3 conversion) **Pituitary:** cytokine-mediated suppression of TSH secretion **Thyroid gland:** cytokine-mediated reduction in T4 secretion by the thyroid gland. **Periphery:** upregulation of T3 to rT3 conversion (notably in liver), tissue specific alteration in T3 uptake (i.e., reception and transport), etc.

Reduced thyroid hormone function: An immune-mediated loss of thyroid hormone *function* in ME/CFS has long been suspected (132). As mentioned above [see section: The thyrotropic axis (HPT Axis) In ME/CFS], a recent study confirmed that CFS patients have lower circulating levels of Free T3, Total T4, and Total T3 than controls (141). Moreover, this study found a significantly higher ratio of rT3 to T3 hormones. These findings imply a depressed thyroid hormone *function* resembling NTIS. Given the possible tissue-specific alterations in thyroid hormone activity resulting from peripheral mechanisms, the authors suggest these circulating levels only reflect the "tip of the iceberg" of genuine T3 deficits in target tissues.

Oxidative & nitrosative stress: Numerous studies have found increased O&NS in ME/CFS and identified this as a factor in the observed metabolic dysfunction (153, 154). Indeed, Pall proposed a model that describes a "vicious circle" involving oxidative stress and cytokines in ME/CFS a decade ago (cf. the "NO/ONOO-Cycle") (155). Researchers also suggest that high lactate and low glutathione levels found in the brains of ME/CFS patients likely derive from similar mechanisms involving oxidative stress (156).

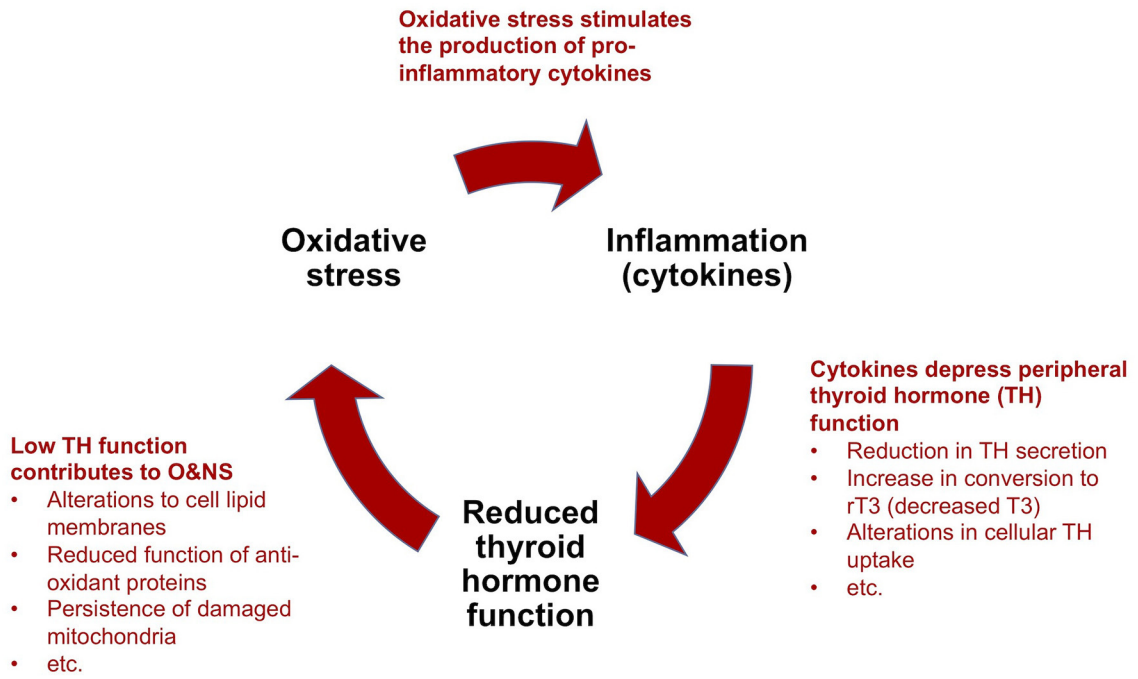

FIGURE 4 | Simplified model to explain the perpetuation of *prolonged* critical illness: a "vicious circle".

A recent study described the relationship between O&NS and immune-inflammatory pathways in ME/CFS (80).

Pro-inflammatory cytokines: Neuro-inflammation is central to ME/CFS, and many researchers have tried to develop diagnostic biomarkers for ME/CFS based on cytokine profiles of patients (157, 158). Montoya et al. found that some 17 cytokines were positively correlated with the severity of ME/CFS, of which 13 are pro-inflammatory. Similarly, circulatory levels of pro-inflammatory cytokines are altered in fibromyalgia patients (159). However, others have argued that given the innumerable sources of potential variance in the measurement of cytokines, it is "unlikely that a consistent and replicable diagnostic cytokine profile will ever be discovered" for ME/CFS (160). It may therefore be ineffectual to compare the cytokine profiles of ME/CFS and *prolonged* critical illness patients.

In sum, given the presence of reduced thyroid hormone *function*, O&NS and pro-inflammatory cytokines in ME/CFS, the "vicious circle" model proposed by critical illness researchers to explain *prolonged* critical illness may also help to understand why ME/CFS patients fail to recover.

Implications of the "Vicious Circle" and Its Elements

Reduced thyroid hormone *function*, increased O&NS and pro-inflammatory cytokines discovered in *prolonged* critical illness as well as in ME/CFS have important implications notably on metabolism, organ function, immune responses and the endocrine system. These are further described below:

Reduced thyroid hormone function: The *prolonged* down-regulation of thyroid hormone activity certainly has implications for the immune system. Authors describe the profound effects of circulating thyroid hormone levels on the activity of monocytes, lymphocytes macrophages, neutrophils, dendritic cells and natural killer cells; as well as cytokines (161–170). Notably, depressed thyroid levels appear to depress the activity of natural killer cells (171)—a signature finding in ME/CFS (172). Such immune dysfunctions might explain other pathologies, such as viral reactivation observed in ICU patients (173–175) and suspected in ME/CFS patients (176, 177). Experimenting on rats, researchers have shown that depressed thyroid hormone levels occur in a specific sequence, manifesting (from first to last) in the liver, kidney, brain, heart and adipose tissues (145). An implication of a tissue-specific down-regulation of thyroid hormone activity is differential impact on organ function. Some ME/CFS practitioners have argued that tissue-specific modulation of T3 can help explain the disparate and evolving symptoms in ME/CFS and fibromyalgia (133, 134, 138, 140). In aggregate, depressed thyroid hormone *function* would engender a general hypometabolic state. Finally, thyroid hormone *function* also impacts other endocrine axes as well (178, 179)—notably the HPA axis—setting the stage for further complex interactions between the various endocrine axes and the immune system.

Oxidative & nitrosative stress: The implications of chronic oxidative stress in the body are widely documented. In addition to inducing inflammation, oxidative stress causes cell damage and disrupts normal cellular transcription and signaling mechanisms (9). O&NS has been shown to cause mitochondrial damage during critical illness (180) and ME/CFS (153).

Pro-inflammatory cytokines: Researchers are finding that the more than 100 different cytokines play a part in determining

the function of hormones through both central and peripheral mechanisms (32). As described in the previous section, cytokines are likely culprits in the central (i.e., hypothalamic and pituitary) suppression of the HPA, HPS and HPT axes in *prolonged* critical illness (29). Pro-inflammatory cytokines and inflammation also hinder normal mitochondrial function during critical illness (181). The alterations in cytokines found in critical illness likely have many further implications that have yet to be fully understood (182) which is also the case for ME/CFS (183).

Intermediate Conclusions

In sum, critical illness researchers have proposed that the self-perpetuating relationships between inflammation (notably pro-inflammatory cytokines), O&NS and low thyroid hormone *function* explains the maintenance of illness in some ICU patients following severe injury or infection. Given that the same elements of such a "vicious circle" have also been documented in ME/CFS, we suggest that the model can also explain the failure of ME/CFS patients to recover. Moreover, these elements have been shown to have profound implications on metabolism, as well as on the function of the immune and endocrine systems—which in in turn could explain the myriad of symptoms in *prolonged* critical illness and ME/CFS.

RELATIONSHIP TO OTHER HYPOTHESES OF ME/CFS PATHOGENESIS

Our hypothesis that maladaptive mechanisms which prevent recovery in *prolonged* critical illness also underlie ME/CFS complements several other hypotheses of ME/CFS pathogenesis. In this section we provide an initial and non-exhaustive discussion of some of these complementarities.

Allostatic overload: Some researchers consider ME/CFS to be a maladaptive response to physical, infectious, and/or emotional stressors. They describe an "allostatic overload" (i.e., the cumulative effect of stressful situations exceeding a person's ability to cope) or a "'crash' in the stress system" (184, 185). Our hypothesis fits into this theoretical framework and offers an explanation for the possible underlying physiological mechanisms by drawing on the research from critical care medicine.

Hypothalamic endocrine suppression: Researchers have suggested that hypothalamic endocrine suppression could explain ME/CFS (132, 186) and fibromyalgia (187–189). Our thesis upholds this hypothesis and seeks to strengthen it by suggesting that the controversy around the existence of central endocrine suppression in ME/CFS may be resolved by studying the *pulsatile* secretions of the pituitary—rather than single or average measurements of circulating tropic and non-tropic hormone concentrations, which can fail to discern the dysfunctions of the endocrine axes.

Anomalies in thyroid hormone function: Numerous clinical practitioners and researchers believe that anomalies in thyroid hormone *function*—including changes in the conversion of thyroid hormones, a resistance of thyroid hormone receptors at cellular level, etc. —contribute to ME/CFS and fibromyalgia (133–141). Indeed, practitioners have written about their successes in treating ME/CFS patients with thyroid hormone supplements (42, 77, 188, 190–194); and patients have published books on their experiences (195–197). Our hypothesis complements this reasoning: we propose that both the central and peripheral mechanisms altering thyroid hormone *function* during critical illness (c.f. NTIS, euthyroid sick syndrome or "low T3 syndrome") also occur in ME/CFS. Moreover, by applying a model from critical illness, we suggest that low thyroid hormone *function* is one element of a "vicious circle" perpetuating illness in ME/CFS.

Viral Reactivation: It has long been suggested that viral reactivation plays a role in ME/CFS, particularly reactivation of Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (176, 177). Similarly, high incidences of viral reactivation have also been observed in ICU patients, notably in patients with sepsis and *prolonged* critical illness. ICU researchers propose that this viral reactivation is a result of immune suppression occurring during critical illness (173–175). Thus, critical illness research would suggest that viral reactivation is a secondary pathology in ME/CFS—except in cases in which the viral infection was the onset event.

Viral infection: Viral infection is recognized to be a leading onset event of ME/CFS (16, 198–201). This is particularly concerning in the context of the COVID-19 pandemic. Many COVID-19 patients continue to experience a variety of debilitating symptoms after defeating the virus that resemble ME/CFS. Building on our hypothesis, we would suggest that post COVID-19 syndrome is evidence of a maladaptive response to the stress of infection akin to that experienced in *prolonged* critical illness and ME/CFS.

Chronic inflammation: Researchers have found that chronic inflammation—auto-immune, allergic or bacterial/viral—underlies ME/CFS (194, 202, 203). Others also ascribe the perpetuation of ME/CFS to the relationship between inflammation and O&NS (80, 155). Our hypothesis is largely complementary to these findings and associated theories. Indeed, following a cytokine surge during the *acute* phase of critical illness, inflammation is believed to persist in the case of *prolonged* critical illness (4). Moreover, pro-inflammatory cytokines and O&NS are elements in the "vicious circle" model of *prolonged* critical illness, which we propose also serves to understand the perpetuation of illness in ME/CFS patients.

Neuroinflammation of the brain: ME/CFS is associated with inflammation of the brain (hence the name myalgic encephalomyelitis) (204, 205). Some have specifically proposed that inflammation of the hypothalamus underlies ME/CFS (81, 82). Similarly, alterations of the endocrine axes through mechanisms mediated by pro-inflammatory cytokines which impact the hypothalamus and pituitary are central to *prolonged* critical illness (see section Suppression of Pulsatile Pituitary Secretions).

Energy metabolic defect: Researchers have found impairment in energy production (205, 206), reduced mitochondrial activity (207–209) and irregularities in the metabolites of ME/CFS patients (210, 211) —suggesting that they experience a hypometabolic or "dauer" state (212). Our hypothesis is

compatible with analyses that emphasize metabolic defects in ME/CFS. Indeed, the suppression of pituitary secretions, depressed thyroid hormone *function,* O&NS and immune system dysfunction—hallmarks of *prolonged* critical illness—have severe impacts on metabolism, including on glucose utilization and mitochondrial activity (see section A "Vicious Circle" Perpetuating Illness). Certainly, *prolonged* critical illness resembles a hypometabolic "dauer" state as well.

Genetic predisposition: Research also suggests there may a genetic element in the pathogenesis of ME/CFS (213–216). Our hypothesis is compatible with a possible genetic predisposition for ME/CFS. Indeed, it is not known why some critically ill patients succumb to *prolonged* critical illness while others begin recovery (217, 218); genetics may play a role. The findings from the field of ME/CFS in the area of genetics might inform the field of critical illness in this regard.

In sum, our hypothesis is largely complementary to hypotheses that emphasize metabolic, hormonal and/or immune dysfunctions in the pathogenesis of ME/CFS. Our hypothesis—drawing from research on critical illness—integrates these dysfunctions into a single framework and provides arguments for the direction of causality between them.

CONCLUSION

Decades of research in the field of critical medicine have demonstrated that in response to the stress of severe infection or injury, endocrine axes experience profound alterations. An assessment of the pituitary's *pulsatile* secretions reveals that in the subset of patients which survive their severe infection or injury but do not begin recovery (i.e., *prolonged* critically ill patients), the suppression of endocrine axes is maintained irrespective of the initial severe infection or injury. Recent pathological models propose that mechanisms involving pro-inflammatory cytokines, O&NS and low thyroid hormone *function* explain the perpetuation of these endocrine dysfunctions (i.e., a "vicious circle").

The symptoms, physiological abnormalities and endocrine patterns observed in severe ME/CFS are not unlike those of *prolonged* critical illness. Moreover, the same elements of a "vicious circle" also exist in ME/CFS. However, unlike in critical illness, the pituitary's *pulsatile* secretion and its relationships to metabolic and immune functions remain largely unstudied in ME/CFS.

Without excluding possible predisposing genetic or environmental factors, we propose the hypothesis that the maladaptive mechanisms that prevent recovery of *prolonged* critically ill patients also underlie ME/CFS. The severity of ME/CFS illness may be a function of the strength of these mechanisms; very severe ME/CFS most resembles *prolonged* critical illness. We further argue that this hypothesis should be investigated through collaborative research projects building on the findings from critical illness and ME/CFS. If this hypothesis is validated, past trials to break the "vicious circle" that perpetuates critical illness, and the early successes to reactivate the *pulsatile* secretion of the pituitary in ICU patients, may provide avenues for a cure for ME/CFS—including cases onset by infections. Certainly, given the similarities described above, active collaboration between critical illness and ME/CFS researchers could lead to improved outcomes for both conditions.

Finally, we suggest that immediate collaborative efforts should be sought among the researcher community in order to conduct longitudinal studies with the aim of identifying similarities and differences across *prolonged* critical illness, post-ICU syndrome, ME/CFS, fibromyalgia and long-COVID in relation to the hormonal axes, O&NS and pro-inflammatory response with the objective of discovering diagnostic and therapeutic targets mitigating the functional disability that these conditions induce.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Reduced Endothelial Function in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome–Results From Open-Label Cyclophosphamide Intervention Study

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Introduction: Patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) present with a range of symptoms including post-exertional malaise (PEM), orthostatic intolerance, and autonomic dysfunction. Dysfunction of the blood vessel endothelium could be an underlying biological mechanism, resulting in inability to fine-tune regulation of blood flow according to the metabolic demands of tissues. The objectives of the present study were to investigate endothelial function in ME/CFS patients compared to healthy individuals, and assess possible changes in endothelial function after intervention with IV cyclophosphamide.

Methods: This substudy to the open-label phase II trial "Cyclophosphamide in ME/CFS" included 40 patients with mild-moderate to severe ME/CFS according to Canadian consensus criteria, aged 18–65 years. Endothelial function was measured by Flow-mediated dilation (FMD) and Post-occlusive reactive hyperemia (PORH) at baseline and repeated after 12 months. Endothelial function at baseline was compared with two cohorts of healthy controls ($N = 66$ and $N = 30$) from previous studies. Changes in endothelial function after 12 months were assessed and correlated with clinical response to cyclophosphamide. Biological markers for endothelial function were measured in serum at baseline and compared with healthy controls ($N = 30$).

Results: Baseline FMD was significantly reduced in patients (median FMD 5.9%, range 0.5–13.1, $n = 35$) compared to healthy individuals (median FMD 7.7%, range 0.7–21, $n = 66$) ($p = 0.005$), as was PORH with patient score median 1,331 p.u. (range 343–4,334) vs. healthy individuals 1,886 p.u. (range 808–8,158) ($p = 0.003$). No significant associations were found between clinical response to cyclophosphamide intervention (reported in 55% of patients) and changes in FMD/PORH from baseline to 12 months. Serum levels of metabolites associated with endothelial dysfunction showed no significant differences between ME/CFS patients and healthy controls.

Conclusions: Patients with ME/CFS had reduced endothelial function affecting both large and small vessels compared to healthy controls. Changes in endothelial function did not follow clinical responses during follow-up after cyclophosphamide IV intervention.

Keywords: myalgic encephalomyelitis, chronic fatigue syndrome, ME/CFS, endothelial function, flow-mediated dilation, post-occlusive reactive hyperemia, cyclophosphamide

INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease that affects both children and adults and is associated with very low health-related quality of life (1, 2). Patients present with hallmark symptoms such as post-exertional malaise (PEM), fatigue and lack of adequate restitution from rest or sleep, accompanied by pain, cognitive symptoms and sensory hypersensitivity (3, 4). Prevalence of ME/CFS as diagnosed by the Canadian consensus criteria (4) is estimated in the United Kingdom in primary health care at 0.1 to 0.2 per cent of the population (5). A report commissioned by the US Institute of Medicine concluded that ME/CFS is a serious, systemic disease with no known cause or cure, and that there is a great need for research to further understanding of the disease (6). There is a scarcity of research evidence and the disease mechanisms are poorly understood (7).

Symptoms frequently present following an event such as infection, physical trauma or exposure to environmental factors (4), and there is evidence of a genetic predisposition (8, 9). Research suggests the involvement of the immune system (10, 11), an impaired energy metabolism (12–14) and alterations in the gut microbiome (15). Orthostatic intolerance and autonomic dysfunction are also frequently reported, and can present with symptoms such as light-headedness, nausea, concentration difficulties, sweating, palpitations, dyspnea, and chest pain after prolonged sitting or standing (6).

It has been suggested that a dysfunction of the blood vessel endothelium could be a contributing mechanism, possibly associated with inadequate fine-tuned regulation of blood flow according to the metabolic demands of tissues (16, 17). One possible explanation for such endothelial dysfunction could be a reduced bioavailability of nitric oxide (NO) derived from endothelial cells. NO is a messenger molecule and neurotransmitter with important effects on vasodilation, thus contributing to the regulation of blood flow to tissues. NO is involved in many biologic processes, including effects on cognitive function, smooth muscle tone in the gastrointestinal and urogenital tracts, cardiac contractility, skeletal muscle and mitochondrial function (18, 19). Non-invasive measures for endothelial function include *flow-mediated dilation* (FMD) of the large arteries, which is believed to reflect the release of NO from endothelial cells caused by shear stress in vessel walls (20–22). As part of an FMD investigation, it is recommended to also measure endothelium-independent vasodilation, reflecting smooth muscle function, through the administration of sublingual nitroglycerin (20, 21). In the microcirculation, the measure *post-occlusive reactive hyperemia* (PORH) is understood to represent a more complex response involving nervous and myogenic responses as well as several vasodilators including NO (23).

There is growing evidence for endothelial dysfunction in autoimmune diseases (20, 24, 25) and fibromyalgia (26). However, research into blood vessel function in ME/CFS is limited so far. A study by Newton and colleagues (16) showed reduced FMD and PORH in a group of ME/CFS patients compared to healthy controls. This finding was confirmed by Scherbakov et al. who reported peripheral endothelial dysfunction in 18 of 35 (51%) ME/CFS patients compared to 4 of 20 (20%) healthy controls. This study also indicated a correlation between endothelial dysfunction and disease severity (17). In contrast, another study of 24 ME/CFS patients and 24 sedentary controls, using a different assessment method, found no significant difference in peripheral endothelial function at rest or after exercise (27).

In 2014, we performed a small pilot study at Haukeland University Hospital and found very low values of FMD in ME/CFS patients (unpublished). Following the pilot experience, studies of endothelial function were performed as substudies to two intervention drug trials for patients with ME/CFS at Haukeland University Hospital: a phase III trial of rituximab vs. placebo (28) and a phase II trial of cyclophosphamide (29). This article reports the results from the endothelial function substudy of the cyclophosphamide trial.

The purpose of this study was to explore: (1) endothelial function, measured by flow-mediated dilation (FMD) and post-occlusive reactive hyperemia (PORH), in a Norwegian cohort of ME/CFS patients compared with healthy individuals, (2) changes in FMD and PORH from baseline to 12 months, and possible associations between clinical response to cyclophosphamide and changes in FMD/PORH over time.

METHODS

Design

This study was performed as a substudy to the open-label phase II trial "Cyclophosphamide in myalgic encephalomyelitis/chronic fatigue syndrome" (29). The study has a cross-sectional design, comparing endothelial function in patients at baseline with healthy individuals. In addition, the study has a longitudinal element, which explores associations between clinical response after cyclophosphamide intervention and changes in endothelial function for the patient group over time.

Setting

Patients were recruited from March to December 2015 at Haukeland University Hospital in Bergen, Norway. Baseline measurements were performed successively after inclusion in

the clinical trial (March 2015–January 2016), followed by intervention with six infusions of cyclophosphamide IV 4 weeks apart. Measurements were repeated 12 months after inclusion in the trial (March 2016–January 2017).

Patient Inclusion

Patient Group

The 40 patients included in this study were all enrolled in the open-label phase II clinical trial *Cyclophosphamide in ME/CFS*. Inclusion criteria were age 18 to 65, a confirmed ME/CFS diagnosis according to Canadian consensus criteria (4), disease duration of minimum 2 years, disease severity from mild-moderate to severe (excluding mild and very severe ME/CFS) and signed informed consent. Following the publication of previous intervention trials (30, 31), we received referrals from general practitioners and applications directly from patients, requesting evaluation for any future clinical trials. After a preliminary assessment of eligibility based on records of medical history and current disease severity, we performed a random selection among candidates who met the inclusion criteria. Candidates were invited to receive further information on the trial and this substudy, and subject to informed consent, they were screened according to protocol (29). Fifteen patients were recruited among previous rituximab trial participants (non-responders or responders with full or partial relapse) (30, 31).

Healthy Individuals

Reference baseline values for FMD and PORH for healthy individuals were obtained from two other studies performed by the authors and using the same protocols for measurement of endothelial function as those employed in this study. The reference group for FMD consisted of 66 healthy controls participating in a study of endothelial function in women with pre-eclampsia performed by MKS (32), and the reference group for PORH consisted of 30 healthy volunteers examined by KS for an endothelial function substudy to the multi-center RCT *RituxME* (28). The FMD reference group was all-female, while the gender distribution of the PORH reference group was similar to that of the patient group. There were no significant differences between the patient and reference groups with regards to age, BMI, resting blood pressure or heart rate.

Intervention

Patients were scheduled to receive medical intervention with six intravenous infusions of cyclophosphamide (initial dosage of 600 mg/m^2, increased to 700 mg/m^2 for the following five infusions conditional on acceptable hematological toxicity). Nine patients deviated from the treatment protocol and received from 3 to 5 infusions. During the trial, 22 of 40 patients met the criteria for clinical response, defined as Fatigue Score $\geq$4.5 for a minimum of 6 consecutive weeks. SF-36 Physical Function scores among the 22 responders increased from mean 35.0 to 69.5 points (scale 0–100). The median response duration in 18 months follow-up was 44 weeks, and among responders the majority had prolonged remission for years. See Rekeland et al. (29) for details on the trial schedule and results.

Outcomes

Main outcomes were measures of endothelial function—flow-mediated dilation and post-occlusive reactive hyperemia—at baseline and at 12 months after inclusion in the trial. Variables included in the statistical analyses included clinical response status, disease severity and other clinical variables, as well as biological markers of endothelial function.

Measurement of FMD and PORH

Measurements were performed and reported according to a standardized protocol following guidelines from the International Brachial Artery Reactivity Task Force (21). Subject preparations and measurement procedures are described in detail in the study protocol (**Supplementary Material**). Flow-mediated dilation (FMD) of the brachial artery was measured using a GE Vingmed Vivid E9 ultrasound system (GE Vingmed Ultrasound, Horten, Norway) with a multifrequency linear probe, 6–13 mHz (M12L). Participants were instructed to fast with regards to food, fluids (except water), tobacco and medicines for 8 h before assessment. The assessments took place in a quiet, dark, and temperature-controlled room, where participants relaxed on the examination table for at least 10 min pre-assessment. The brachial artery was imaged in the longitudinal plane above the antecubital fossa and a baseline rest image was acquired. A blood pressure cuff, positioned on the forearm, was inflated to 200 mmHg or at least 50 mmHg above above systolic pressure, for 5 min. Following deflation of the cuff, images were recorded continuously from the same area of the artery during the next 5 min. All measurements were performed during end diastole. FMD is expressed as diameter increase in per cent from baseline to time of maximum dilation. After rest, a dose of nitroglycerine spray (0.4 mg) was administered sublingually, and images were recorded continuously for another 5 min. The maximal diameter was measured to express endothelium independent vasodilation.

During the same procedure, measurements of microvascular function (post-occlusive reactive hyperemia, PORH) were performed using a Periflux 5000 laser Doppler unit (Perimed, Stockholm, Sweden). A temperature-controlled probe was placed on clean, intact forearm skin and microvascular perfusion was recorded before and after occlusion. PORH is expressed in perfusion units (PUs) as the difference (area under the curve) between circulation in the skin during 2 min at baseline and the first 2 min after cuff deflation.

FMD and PORH measurements were performed by KS. All ultrasound images were analyzed by KS. In addition, M.S. analyzed a randomly selected 10 % of the ultrasound measurements for FMD ($n = 10$), one of which had questionable image quality and was excluded from analyses. Inter-observer variability for the remaining measurements was computed using a two-way mixed effects, absolute agreement, single measures model (33, 34). The intraclass correlation coefficient was excellent for baseline measurements of artery diameter (ICC = 0.99) and good for FMD (ICC = 0.77).

Clinical Response

Every 2 weeks during 18 months follow-up, patients were requested to complete a self-report form to capture the relative change in symptoms from baseline to the time of recording.

The scale for symptom change was adapted from the Clinical Global Impression Scale which had previously been used in studies of ME/CFS (35), and has been employed in the follow-up of patients in previous clinical trials (28, 30, 31, 36). The scale for each symptom was 0 to 6, where 0 denoted major worsening, and 6 major improvement of the symptom compared to baseline. The variable Fatigue Score was calculated as the mean change score for four fatigue-related items: "Fatigue," "Post-exertional malaise," "Need for rest" and "Daily functioning." Clinical response was defined as Fatigue Score $\geq$4.5 for at least 6 consecutive weeks (29).

Clinical and Sociodemographic Variables

Before inclusion in the cyclophosphamide trial, participants were subject to a clinical examination/interview. Clinical variables reported are age, sex, disease severity and duration, BMI, resting blood pressure, and heart rate. Disease severity was categorized into six categories: mild, mild-moderate, moderate, moderate-severe, severe and very severe, based on the including physician's evaluation and patients' self-reported function level from 0 to 100% on a standardized form with scoring examples. Patients with mild or very severe ME/CFS were excluded from participation. Physical function was measured by the Short Form-36 Physical Function subscale (37, 38), and mean steps per 24 h were measured using a SenseWear® armband (BodyMedia Inc., Pittsburgh, PA, USA) in a home setting for 5–7 consecutive days (39). Demographic data (family, educational and employment status) were collected from a modified DePaul questionnaire (40) completed at baseline.

Biological Markers of Endothelial Dysfunction

From baseline serum samples we measured serum concentrations of amino acids and derivatives which are associated with endothelial function (41–43). These analyses were performed as part of a comprehensive metabolic profiling of participants in three ME/CFS trials (12). Potential risk markers for endothelial dysfunction and cardiovascular disease include low levels of arginine (Arg) and homoarginine (hArg) (43), and elevated levels of asymmetric dimethylarginine (ADMA) (41), symmetric dimethylarginine (SDMA) and high-sensitivity C-reactive protein (hs-CRP) (42). Arg, hArg, ADMA, and SDMA were analyzed by liquid chromatography-tandem mass spectrometry with within and between day CVs of 3–12% (44), and hs-CRP was measured by immunoMALDI mass spectrometry (45) (Bevital, Bergen, Norway).

Statistical Analyses

Continuous variables were described with median and range, categorical with counts and percentages. Due to a limited sample size and skewed distribution, statistical comparisons were performed using non-parametric methods. Comparisons between groups (patient and reference groups) regarding baseline FMD and PORH were made using a Mann-Whitney test for independent samples. Possible associations between FMD and PORH and ME/CFS illness duration and severity and steps per 24 h were assessed with Kruskal–Wallis test. When analyzing the patients only, paired Wilcoxon signed ranks tests were used to compare FMD and PORH results at baseline and at 12 months. The difference between groups (responders and non-responders) in change of FMD/PORH values from baseline to 12 months was analyzed using a General Linear Model (GLM) of repeated measures with clinical response as a between-subjects factor. The correlation between FMD and PORH values at baseline was computed using Spearman non-parametric correlation. The result is expressed as the correlation coefficient rho. $P < 0.05$ were considered statistically significant and all tests were two-sided. We consider our study exploratory and no correction for multiple testing was performed. All analyses were done using SPSS Statistics ver. 25 (IBM Corp., Armonk, NY) and Graphpad Prism ver. 8 (Graphpad Software, La Jolla, CA).

Ethics

The study "Cyclophosphamide in ME/CFS" including this substudy was approved by the Regional Committee for Medical and Health Research Ethics (2014/1672) in Norway. Candidates received information about the study in writing, in individual consultations with investigators (ØF, OM, IR), and in follow-up telephone consultations with the study nurse (KS). Participation was subject to signed informed consent. Ultrasound images were stored on CDs in a locked cabinet, and data registered in a secure electronic case report system (Viedoc, PCG Clinical Services, Uppsala, Sweden). All data were de-identified and the scrambling key stored in a dedicated area of the hospital's research server.

RESULTS

Participant Characteristics

Fifty individuals were invited to receive information about the clinical trial. Three did not wish to participate and seven did not meet the inclusion criteria. All 40 trial participants consented to participation in the endothelial function substudy. A majority (78%) was female and young to middle aged, with a median age at baseline of 42.4 (range 21.5–61.1). All participants had an established ME/CFS diagnosis according to the Canadian diagnostic criteria. ME/CFS severity based on clinical assessment ranged from mild-moderate to severe, and self-reported physical function ranged from 5 to 40%, with a median value of 16% (range 0–100%). Half the participants reported a disease duration of more than 10 years. The median score for Short Form-36 Physical Function was 35 (raw score, range 0–100). Two patients had a medical history of cardiovascular disease (one case of myocardial infarction, one of hypertension), and 16 out of 40 reported allergies to food or other allergens. Further details concerning the demographics and clinical characteristics of the included participants are listed in **Table 1**.

Endothelial Function Measurements

The entire cohort of 40 trial participants attended endothelial function tests at baseline, but not all measurements were available for all participants. Individuals with missing data at one or both time points ($n = 6$ for PORH, $n = 13$ for FMD), due to withdrawals, failure to comply with test preparations or inadequate image quality, were not included in the analyses for change from baseline to 12 months. Participants with

TABLE 1 | Demographics and baseline clinical characteristics of study participants.

N = 40	*N*	%
Female sex	31	78
Marital status/family		
Single, widow/er, divorced	15	37.5
Married, registered partner, co-habiting	25	62.5
Have children	24	60
Highest level of completed education		
Primary or secondary education	17	42.5
University, college, or higher university degree	23	57.5
Employment status		
Work part time, homemaker	7	17.5
Work assessment allowance, disability	33	82.5
ME/CFS severity		
Mild-moderate	14	35
Moderate	13	32.5
Moderate-severe	7	17.5
Severe	6	15
ME/CFS duration		
2–5 yrs	7	17.5
5–10 yrs	13	32.5
10–15 yrs	9	22.5
>15 yrs	11	27.5
Comorbidity[a]		
Known history of cardiovascular disease	2	5
Fibromyalgia	3	7.5
Hypothyroidism	4	10
Allergy	16	40
History of depression	4	10
	Median	**Range**
Age, years	42.4	21.5–61.1
Body mass index, kg/m^2	23.4	17.1–33.1
Mean steps per 24 h	2,944	568–9,637
Short form-36 physical function[b]	35	0–65
Self-reported function level, per cent[c]	16	5–40
Systolic blood pressure, mmHg	120	88–160
Diastolic blood pressure, mmHg	77	55–96
Resting heart rate, bpm	68	42–113

[a]*Self-reported at baseline.* [b]*Raw score; range 0–100.* [c]*Self-reported; range 0–100%. ME/CFS, Myalgic encephalomyelitis/chronic fatigue syndrome.*

TABLE 2 | Measurements of endothelial function at baseline.

FMD baseline median (range)	**Healthy controls, *n* = 66**	**ME/CFS, *n* = 35**	***P*-value**
FMD, per cent	7.7 (0.7–21.0)	5.9 (0.5–13.1)	*0.005*
Arterial diameter at rest, mm	3.01 (1.61–3.80)	3.0 (2.29–4.61)	0.34
Absolute increase in mm	0.23 (0.02–0.53)	0.20 (0.02–0.33)	*0.01*
Arterial diameter after nitroglycerin[a], mm	3.65 (2.03–4.82)	3.75 (2.90–5.80)	0.06
Increase in diameter after nitroglycerin[b], per cent	21.2 (8.4–40.1)	25.3 (11.2–42.4)	*0.02*
FMD/nitro ratio[c]	0.37 (0.04–1.03)	0.23 (0.02–0.51)	*<0.001*
PORH baseline median (range)	**Healthy controls, *n* = 30**	**ME/CFS, *n* = 39**	***P*-value**
PORH, perfusion units	1,886 (808–8,158)	1,331 (343–4,334)	*0.003*

ME/CFS group vs. healthy control group. Mann–Whitney U-test for independent samples. [a]*Maximum dilation after sublingual administration of nitroglycerin, absolute value.* [b]*Maximum dilation after sublingual administration of nitroglycerin, increase in percent compared to baseline.* [c]*Ratio of FMD (in percent) by maximum dilation after nitroglycerin (in per cent). Missing data: One patient failed to complete measurements at baseline. For FMD, a further 4 cases were excluded from analyses due to inadequate image quality. ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; FMD, Flow-mediated dilation; PORH, Post-occlusive reactive hyperemia. Italics denote statistically significant values.*

TABLE 3 | Measurements of endothelial function, ME/CFS group at baseline vs. at 12 months.

FMD baseline vs. 12 months median (range).	**ME/CFS baseline, *n* = 27**	**ME/CFS 12 months, *n* = 27**	***P*-value**
FMD, per cent	5.7 (0.5–13.1)	5.3 (0.2–15.4)	0.9
Arterial diameter at rest, mm	2.98 (2.29–4.47)	3.14 (2.38–4.61)	*0.02*
Absolute increase in mm	0.19 (0.02–0.33)	0.18 (0.01–0.48)	0.85
Arterial diameter after nitroglycerin[a], mm	3.70 (2.90–5.30)	3.77 (2.91–5.38)	0.07
Increase in diameter after nitroglycerin[b], per cent	25.3 (11.2–42.4)	23.5 (11.6–35.6)	0.75
FMD/nitro ratio[c]	0.23 (0.02–0.51)	0.22 (0.2–0.46)	0.75
PORH baseline vs. 12 months median (range)	**ME/CFS baseline, *n* = 34**	**ME/CFS 12 months, *n* = 34**	***P*-value**
PORH, perfusion units	1,323 (343–4,334)	1,428 (387–4,335)	0.18

Only patients with values for both timepoints included, and analyzed by Wilcoxon signed ranks test.
[a]*Maximum dilation after sublingual administration of nitroglycerin, absolute value.* [b]*Maximum dilation after sublingual administration of nitroglycerin, increase in percent compared to baseline.* [c]*Ratio of FMD (in percent) by maximum dilation after nitroglycerin (in per cent). Missing data: 2 patients withdrew from study before 12 months, one of whom also failed to complete measurements at baseline. Four patients failed to complete measurements at 12 months, due to intercurrent illness or non-compliance with preparations. For FMD, a further 7 cases were excluded from comparative analyses due to inadequate FMD image quality at either time point. ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; FMD, Flow-mediated dilation; PORH, Post-occlusive reactive hyperemia. Italics denote statistically significant values.*

missing data did not differ significantly with regards to age, sex, disease duration, or severity from those with complete data. See **Tables 2**, **3** for details.

Flow-Mediated Dilation

At baseline, median FMD was significantly lower for ME patients compared to healthy women; 5.9 vs. 7.7%, $p = 0.005$ (**Table 2**, **Figure 1A**). FMD <5% was present in 14 (40%) of the ME patients compared to 14 (21%) of the reference group. After administration of sublingual glyceryl nitrate, maximum dilation compared to diameter at rest was higher in the ME group (25.3%) than the reference group (21.2%) ($p = 0.02$), showing intact ability to dilate vessels adequately.

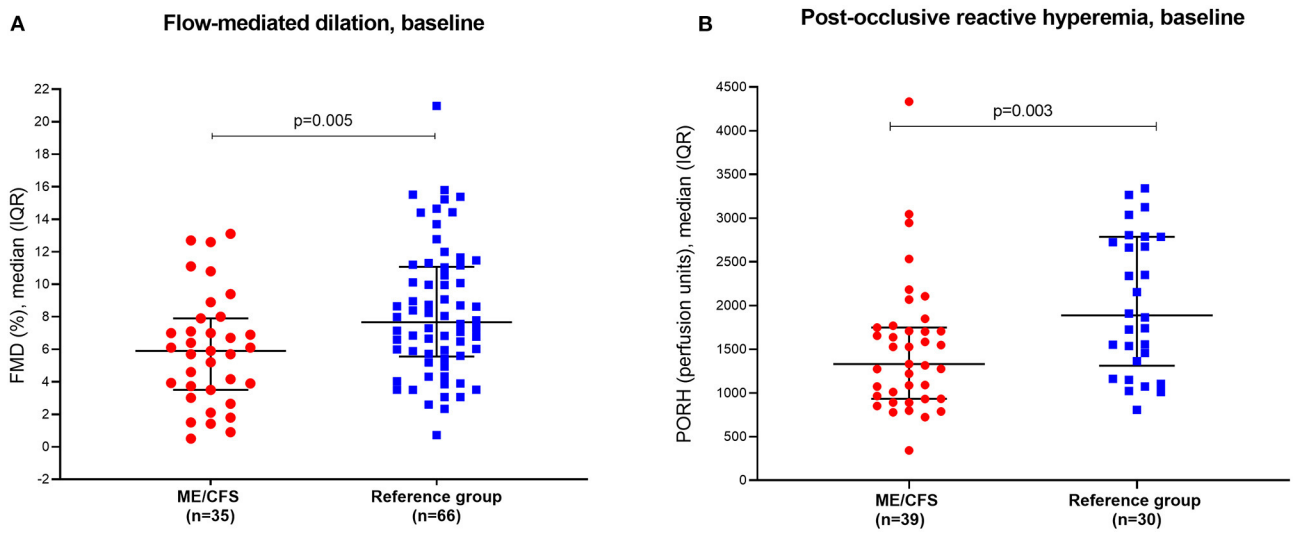

FIGURE 1 | (A) Baseline values for flow-mediated dilation (FMD) in patients and reference group of healthy women. FMD is expressed as dilation in per cent in response to 5 min occlusion. **(B)** Baseline values for post-occlusive reactive hyperemia (PORH) in patients and reference group of healthy controls. PORH is expressed as increase in perfusion units during the first 2 min after cuff release. One healthy control outlier at 8,158 p.u. is not shown, but is included in the analysis.

TABLE 4 | Correlations between vascular measures and laboratory and clinical parameters.

		FMD[a]	PORH[b]
		Spearman's rho	Spearman's rho
Clinical parameters	Age, years	−0.289	−0.380
	Body mass index, kg/m^2	−0.139	−0.098
	Total function level, per cent[c]	0.009	0.128
	Short Form-36 Physical Function score[d]	−0.076	0.259
	Steps per 24 h[e]	−0.09	0.21
Laboratory parameters	Arginine, μM	0.048	0.253
	Asymmetric dimethylarginine, μM	0.022	0.050
	Symmetrical dimethylarginine, μM	−0.470*	0.255
	Homoarginine, μM	−0.017	0.272
	High-sensitivity C-reactive protein, μM	−0.089	0.073

*[a]Flow-mediated dilation at baseline. [b]Post-occlusive reactive hyperemia at baseline. *p < 0.05. [c]Self-reported; range 0–100%. [d]Raw score; range 0–100. [e]Measured by Sensewear® armband for 5–7 consecutive days.*

Post-Occlusive Reactive Hyperemia

Median PORH at baseline was significantly lower for patients compared to healthy individuals; 1,331 vs. 1,886 perfusion units, $p = 0.003$ (**Table 2**, **Figure 1B**). PORH <1,000 p.u. was present in 11 (28%) of the ME group and only in one individual in the reference group (3.5%).

Relation to ME/CFS Severity and Duration

There were no statistically significant associations between FMD or PORH and age, BMI or ME/CFS severity measured by clinical assessment, self-reported function level or SF-36 Physical Function, disease duration, or number of steps per 24 h (**Table 4**).

Correlation Between FMD and PORH

There was no significant correlation between measured FMD and PORH in the patient group at baseline ($r = -0.12$, $p = 0.47$) or between changes in FMD and PORH from baseline to 12 months ($r = 0.12$, $p = 0.53$).

Changes in Endothelial Function and Association With Symptom Change

Although 22 of the 40 patients reported clinical response after cyclophosphamide treatment (29), our data did not reveal any statistically significant changes in endothelial function (FMD or PORH) from baseline to 12 months (Only patients with values for both timepoints included, and analyzed by Wilcoxon signed ranks test) (**Table 3**, **Figures 2A,B**). Furthermore, GLM repeated measures analyses showed no significant differences in changes of endothelial function (FMD or PORH) from baseline to 12 months, between responders and non-responders after cyclophosphamide treatment, i.e., there were no significant differences assessed by the interaction term response group-by-time (**Figures 2C,D**).

Biological Markers of Endothelial Dysfunction

There was no statistically significant difference between ME/CFS patients and healthy controls for arginine, homoarginine, ADMA, or hs-CRP. For SDMA, which is considered a risk marker for endothelial dysfunction and cardiovascular disease, the ME/CFS group had a significantly lower mean serum level than healthy controls (0.54 vs. 0.63, $p = 0.014$; **Table 5**). However, correlation analyses showed a significant inverse correlation between SDMA levels and FMD values, indicating a relationship between high SDMA levels and low FMD (**Table 4**).

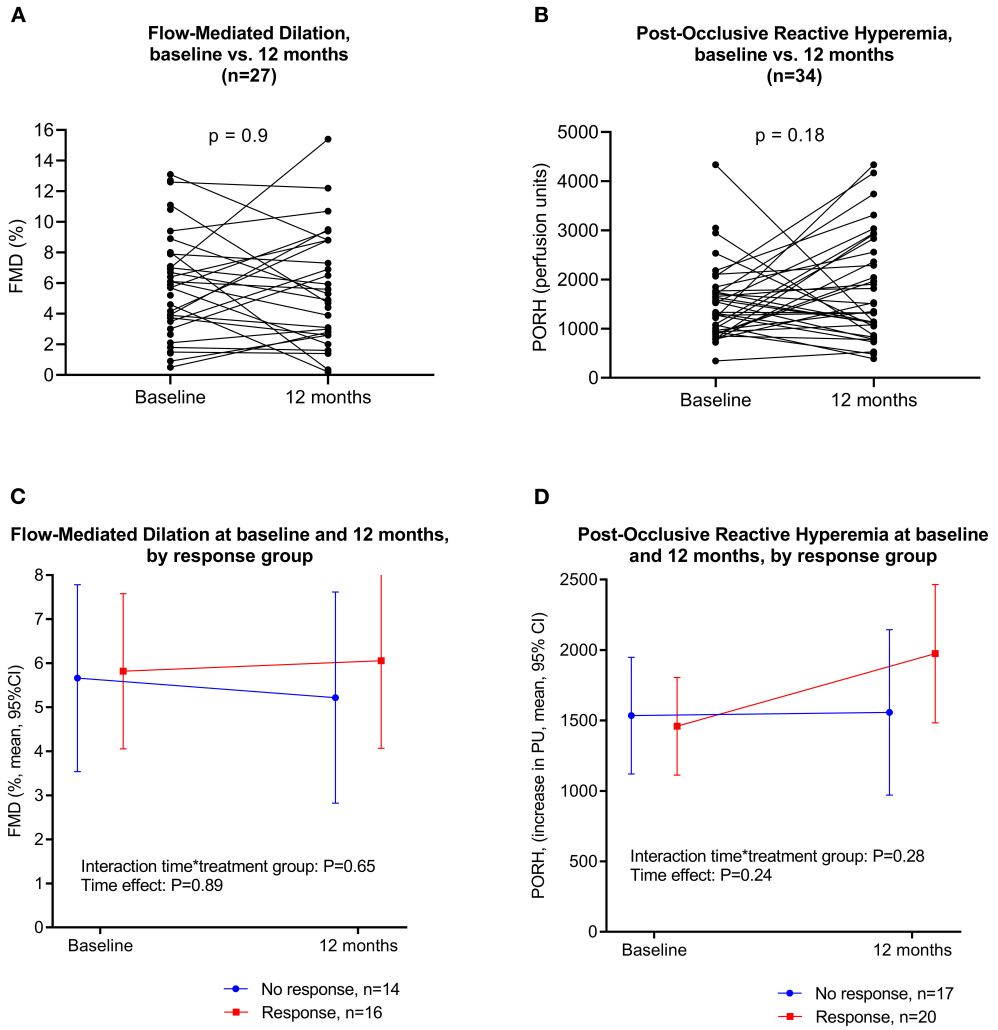

FIGURE 2 | (A) Flow-mediated dilation at baseline and at 12 months. **(B)** Post-occlusive reactive hyperemia at baseline and at 12 months. **(C)** Flow-mediated dilation at baseline and at 12 months, by response group. GLM repeated measures with clinical response as between-subjects factor. **(D)** Post-occlusive reactive hyperemia at baseline and at 12 months, by response group. GLM repeated measures with clinical response as between-subjects factor.

DISCUSSION

This study investigated endothelial function in ME/CFS, and was the first to describe the changes in endothelial function before and after therapeutic intervention with IV cyclophosphamide in ME/CFS patients. Endothelial function measurements at baseline indicated that at the group level, the patients had significantly reduced large and small vessel endothelial function compared to healthy individuals, in line with results from previous studies (16, 17). Although more than half of the patients met the criteria for clinical response during the study, we were unable to detect any significant associations between clinical response and changes in endothelial function from baseline to 12 months follow-up.

A strength of this study was the inclusion and rigorous follow-up of well-characterized ME/CFS patients who fulfilled the Canadian consensus criteria. We used two complementary methods to measure different aspects of endothelial function. Flow-mediated dilation (FMD) is the "gold-standard" method for large vessel endothelial function assessment (21), and the measurements have followed a well-established protocol. However, FMD is a challenging and operator-dependent technique, which is subject to interpretive error. After images of suboptimal quality were excluded from analyses, inter-observer variability in this study was good to excellent. The measurements of post-occlusive reactive hyperemia (PORH) by Doppler laser are less operator-dependent, but studies show varying reproducibility (46, 47). Assessments were well-tolerated by the patients.

One weakness of the study was a possible inclusion bias. The participants were recruited for the primary purpose of a clinical, open-label drug trial, which excluded patients with either mild or very severe disease. The sample size was relatively small, but comparable to other studies using these methods. Due to the exploratory nature of the study, no power calculation or sample

TABLE 5 | Serum levels of metabolites that may affect endothelial function, in ME/CFS patients and healthy controls.

Biological markers of endothelial function. μM, median (range)	ME/CFS[a] $n = 40$	HC[b] $n = 30$	*P*-value[c]
Arginine	80.83 (51.88–129.81)	85.63 (52.38–136.40)	0.20
Asymmetric dimethylarginine	0.55 (0.31–0.78)	0.50 (0.34–0.69)	0.11
Symmetrical dimethylarginine	0.54 (0.38–0.78)	0.63 (0.27–0.79)	0.014*
Homoarginine	1.76 (0.64–3.53)	1.94 (0.90–4.24)	0.19
High-sensitivity C-reactive protein	0.35 (0.00–4.51)	0.53 (0.00–6.20)	0.38

[a]*ME/CFS patients, non-fasting.* [b]*Healthy controls, non-fasting.* [c]*P-values from Mann–Whitney tests; *p < 0.05.*

size assessment was performed for the purpose of the endothelial function analyses, and the study may be underpowered to detect significant correlations. The study did not include a designated control group, but relied on endothelial function data from healthy individuals included as control groups for two other studies, one of which consisted of women only. However, the median FMD values for men and women in the ME group were identical. Severity and response evaluations were largely based on physicians' assessments and patient self-report. Although patient-reported outcome measures are vital in order to capture the patient perspective, the study could have benefited from more objective parameters. By way of objective functional assessments, we performed activity monitoring using a Sensewear armband for 5–7 days at three designated time points, and we found that the patient-reported parameters correlated well with changes in activity level.

The level of physical activity of the healthy individuals was not recorded, so we have not been able to control for the effects of a sedentary or active lifestyle. A 2017 meta-analysis of the effects of exercise training on brachial artery FMD concluded that exercise training, particularly in patients with established cardiovascular disease, overweight/obesity and hypertension, contributed to a significant increase in FMD (48). However, patient inactivity alone is not a plausible main mechanism for the observed reduced endothelial dysfunction. For example, studies on vascular function in patients with rheumatoid arthritis suggested that the peripheral vasculature was adversely affected by sedentary behavior in these patients, while sedentary behavior was not predictive of endothelial dysfunction of the large vessels (49). Furthermore, we found no significant association between endothelial dysfunction and disease severity, SF-36 Physical Function scores or physical activity measured by steps per 24 h.

In our study, there was no correlation between microvascular and brachial artery endothelial function. This finding was expected, as a poor or absent correlation between FMD and PORH has also been reported in other diseases (47, 49) and in healthy individuals (46, 50). This could be explained by the different mechanisms behind the two measures. While the vasodilator nitric oxide (NO) is the principal mediator of FMD (21, 22), PORH represents a more complex response which is believed to involve metabolic vasodilators, endothelial vasodilators, sensory nerves and myogenic response to shear stress (23, 51, 52).

While endothelial function measurements are of interest on a group level, in our data there was a broad overlap between patients and controls, as well as a lack of correlation with symptom severity. Further studies and validation would certainly be required if FMD or PORH testing of ME/CFS patients were to be applied in a clinical or diagnostic setting. The lack of correlation between symptom improvement and changes in endothelial function could imply that reduced endothelial function, although present in a subgroup of ME/CFS patients, does not relate directly to the underlying pathomechanism of the disease. It is, however, also conceivable that the sample size is insufficient to detect correlations with symptom severity and improvement, such as reported by Scherbakov et al. (17).

One might speculate that endothelial dysfunction in ME/CFS could be associated with inadequate autoregulation of blood flow according to the demands of tissues, with resulting local hypoxia and lactate accumulation upon limited exertion. The clinical symptoms of ME/CFS suggest inadequate regulation of autonomic functions including blood flow. In a recent study of invasive cardiopulmonary exercise testing in upright position in ME/CFS patients, two types of peripheral neurovascular dysregulation were demonstrated; reduced cardiac output due to impaired venous return with low ventricular filling pressure ("preload failure"), and arterio-venous shunting with impaired peripheral oxygen extraction (53). These physiological changes are plausible contributors to several hallmark symptoms of ME/CFS, such as post-exertional malaise, and are associated with microcirculatory dysregulation, possibly related to small-fiber neuropathy (53).

Measures of known metabolites associated with endothelial function in cardiovascular diseases (Arg, hArg, ADMA, and SDMA) showed no significant differences between ME/CFS patients and healthy controls. This may argue for a different mechanism underlying the observed endothelial dysfunction in ME/CFS. We speculate that among ME/CFS patients, many of whom are relatively young women, the endothelial dysfunction could be related to an initial abnormal immune response, rather than atherosclerosis.

Further research is required in order to reach firm conclusions on any possible associations between ME/CFS symptoms and endothelial function. Future studies should aim to integrate objective activity measures as a supplement to validated patient-reported outcome measures, in order to control for the effect of physical activity or inactivity.

In conclusion, this study showed an association between ME/CFS and reduced endothelial function, both in large vessels assessed by FMD, and in small vessels by PORH. In this relatively small study, there were no significant associations between clinical response after cyclophosphamide and changes in FMD or PORH. Continued research efforts are warranted to further understand the possible circulatory disturbances involved in ME/CFS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was reviewed and approved by Regional Committee for Medical and Health Research Ethics of North Norway. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KS, ØF, OM, and MKS: conception and design of study. KS, ØF, OM, and IR: inclusion of patients and acquisition of data. KS, MS, ØF, LR, and MCS: analysis and interpretation of data. KS: drafting of manuscript. All authors: critical revision for important intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.642710/full#supplementary-material

Supplementary Material | Study protocol.

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Ross River Virus Immune Evasion Strategies and the Relevance to Post-viral Fatigue, and Myalgic Encephalomyelitis Onset

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Ross River virus (RRV) is an endemic Australian arbovirus, and member of the Alphavirus family that also includes Chikungunya virus (CHIK). RRV is responsible for the highest prevalence of human disease cases associated with mosquito-borne transmission in Australia, and has long been a leading suspect in cases of post-viral fatigue syndromes, with extrapolation of this link to Myalgic Encephalomyelitis (ME). Research into RRV pathogenesis has revealed a number of immune evasion strategies, impressive for a virus with a genome size of 12 kb (plus strand RNA), which resonate with insights into viral pathogenesis broadly. Drawing from observations on RRV immune evasion, mechanisms of relevance to long term idiopathic fatigue are featured as a perspective on infection and eventual ME symptoms, which include considerations of; (1) selective pro-inflammatory gene suppression post antibody-dependent enhancement (ADE) of RRV infection, (2) Evidence from other virus families of immune disruption and evasion post-ADE, and (3) how virally-driven immune evasion may impact on mitochondrial function via target of rapamycin (TOR) complexes. In light of these RRV measures to counter the host immune - inflammatory responses, links to recent discoveries explaining cellular, immune and metabolomic markers of ME will be explored and discussed, with the implications for long-COVID post SARS-CoV-2 also considered. Compelling issues on the connections between virally-induced alterations in cytokine expression, for example, will be of particular interest in light of energy pathways, and how these perturbations manifest clinically.

Keywords: myalgic encephalomyelitis, fatigue, virus, inflammation, immunity, cytokines, mitochondria

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INTRODUCTION

The history of Myalgic Encephalomyelitis (ME) features outbreaks as defining events, suggesting an infectious etiology behind the cluster of symptoms experienced by affected individuals (1, 2). Outbreak examples include Akureyri disease (Iceland - 1940s), Royal-Free Hospital London (UK - 1950s), "Tapanui Flu" (New Zealand - 1980s), and Lake Tahoe (USA - 1980s), with the description of "flu-like" symptoms common among patients (3, 4). While respiratory viruses were

obvious candidates given this history, other virus families have been linked to ME (5), as well as the intra-cellular bacterial agent of Q-Fever, *Coxiella burnetti* (6, 7).

Viruses rely upon the host-cell machinery to replicate, and knowledge of these processes for diverse virus families is well-established (8). Appreciation of the manipulation of host-immunity by viruses has emerged during the previous three decades (9, 10), with early insights gained from large genome DNA viruses, for example poxviruses and herpesviruses, which often involved cytokine ligand or receptor mimicry to evade host-immune responses (11–15). RNA viruses also have evolved ingenious strategies for immune evasion (16, 17), with examples including members of the flavivirus and coronavirus families (18–21) that are responsible for disease on a global scale. From an evolutionary perspective, the author has previously theorized that RNA viruses rely upon "erroneous replication" strategies to avoid destruction by the host, but at the potential cost to the virus of killing the host and impeding the propagation of viral progeny (16).

The perspective presented here centers on the endemic Australian virus, Ross River (RRV), a positive-strand RNA (12 kb) Old World Alphavirus. RRV is transmitted by mosquitos and responsible for Ross River virus disease (RRVD), which is defined by lethargy, myalgia, rash and polyarthritis, as well as post-viral syndrome (22, 23). RRV is suspected of being a microbial precursor to Australian ME cases, along with Epstein-Barr Virus (EBV) and *C. burnetti* (7).

How infection leads to ME is the subject of the perspective presented herein, with RRV as the primary ME-linked virus example. Of particular interest are the observations on the dysregulation of cytokine expression in macrophages post RRV-ADE (antibody-dependent enhancement) infection, and thus inflammatory responses by the host, as well as the impact on mitochondrial function. A perspective on how these interacting features result in the ultimate clinical manifestations of long-term fatigue, post-exertional malaise and other symptom clusters (2) is presented herein.

Consideration of these questions has contemporary urgency due to the emergence of "long-COVID," which for some patients resembles ME once recovered from the acute SARS-CoV-2 infection (24).

Perspective Context

The concepts investigated to formulate the perspective presented are of particular contemporary importance, since at the time of writing, the world is confronting the SARS-CoV-2 (COVID-19) pandemic that has raised issues related to:

(a) Vaccine safety in the context of antibody-dependent enhancement (ADE) of virus infection, and
(b) The emergence of cases of "long-(haul)-COVID," which in some present symptoms identical to ME.

Vaccines are not the primary focus here, but understanding ADE as an avenue of immune evasion, and thereafter manipulation of host immune - inflammatory responses by the virus, raise pertinent questions linked to the eventual development of idiopathic fatigue in some individuals post-acute virus infection. The concept of "cytokine storm" is well-recognized for COVID-19 and other diseases, but in other cases impacts of immune manipulation are maybe subtler? And what do these events mean for the regulation mitochondrial function and energy production if the virus is assisted by ADE?

Antibody-Dependent Enhancement

Hawkes first reported ADE of virus infection in 1964, observed for members of the *Togavirus* Family as classified at the time (25). The studies focussed on Class A and B Togaviruses, with Getah (Togavirus - *Alphaviridae*) Murray Valley Encephalitis, West Nile and "Japanese Encephalitis viruses" (Flaviruses - *Flaviridae*) displaying up to 12-fold growth enhancement in chick embryo fibroblast (CEF) cultures, and on chorioallantoic membranes, with the effect only seen with antibodies raised in "domestic fowls," not from other species. Further investigations revealed that the enhancing properties were specific to the IgG fraction of the anti-serum that enhanced virus growth (26).

Presciently, once antibody was identified as the enhancing factor, the authors stated; *Another possibility that should be considered is that the enhancing antibody is taken into the cell along with the virus and influences subsequent intracellular events. These problems can only be answered by further studies of the interactions between complexes of virus and antibody and susceptible cells* (26).

Subsequent studies conducted over the 1970s—80s established *in vitro* ADE for the global pathogen Dengue virus (DEN), as well as identified the role for Fc-Receptor (Fc-R) engagement in ADE via studies with other flaviviruses (27–29). By the 1990s, ADE was recognized as a factor in severe DEN disease (Haemorrhagic Fever, Shock Syndrome), on subsequent infection with a DEN serotype different to the original case (30), which has also frustrated attempts to develop a DEN vaccine (31). Many virus families have been observed as displaying enhanced *in vitro* growth post-infection due to ADE, but the impact *in vivo* and on disease manifestation are not currently well-understood (32–34).

While ADE was observed for the close RRV relative, Getah virus, during Hawkes's original ADE observations in 1964, RRV-ADE was not reported until the 1990s (35). As found for earlier

Abbreviations: AAF, IFN-α-activated factor; ADE, Antibody-Dependent Enhancement; ATG5, Autophagy-related 5; COVID, Coronavirus Disease; CR, Complement Receptor; DEN, Dengue virus; EBV, Epstein-Barr virus; FcR, Fc Receptor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, Interleukin; IP-10, IFN-inducible protein 10; IRF, IFN-regulatory factor; ISGF3, IFN-stimulated gene factor 3; MIP-1, macrophage inflammatory protein-1; MCP-1, Monocyte chemoattractant protein-1; ME, Myalgic Encephalomyelitis; NF-κB, nuclear factor kappa B; NOS2, nitric-oxide synthase 2- inducible NOS; p.i., post-infection; PIP_2, Phosphatidylinositol 4,5-biphosphate; PIP_3, Phosphatidylinositol 3,4,5-triphosphate; PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; PTKs, protein tyrosine kinases; RIG-1, Retinoic acid Inducible Gene-I; RLRs, RIG-I-Like Receptors; RLR-MAVS, RNA-triggered RLR-mitochondrial antiviral signaling protein; RRV, Ross River virus; SARS-CoV, Severe Acute Respiratory Syndrome-Coronavirus; SOCS3, Suppressor of Cytokine Signaling 3; Sp1, Specificity protein 1; STAT, Signal Transducer and Activator of Transcription; Syk, Spleen Tyrosine Kinase; TOR, Target of Rapamycin; mTOR, mechanistic TOR; TORC, TOR Complex; TNF, Tumor Necrosis Factor; TGF, transforming growth factor.

examples, RRV-ADE was demonstrated *in vitro* for monocytes and macrophages, including the macrophage cell line RAW 264.7, which was later central to the elucidation of molecular mechanisms post-ADE entry.

The Disruption of Macrophage Pro-inflammatory Responses as an RRV-ADE Mechanism

While enhanced virus uptake helped explain ADE mechanism (28), the "… *subsequent intracellular events* …" suggested by Hawkes and Lafferty were eventually revealed by *in vitro* models of RRV infection using RAW 264.7 macrophages. The important intracellular events were linked to the regulation of pro- and anti-inflammatory cytokines, at the level of the transcriptional machinery. While the impact on early virus growth is clear, it must be assumed that the dysregulation of cytokines during an innate response also has implications for longer term immunity, and perhaps the vigor of subsequent inflammation that follows infection.

The RRV *in vitro* models showed a clear disruption of expression for TNF, NOS2, IFN-β, IP-10 that involved the temporary post-ADE ablation of STAT-1 (ISGF3 and AAF), IRF-1 and NK-κB transcriptional complexes, which occurred at the same time as enhanced RRV growth. As well as the downregulation of proinflammatory - antiviral gene expression, IL-10 expression was significantly increased, as was the transcription factor Sp1 (36, 37). Similar patterns were also detected in a flavivirus (DEN) (38–42) and an arterivirus (PRRSV) (43–46). Post-ADE IL-12 and IFN-γ suppression was also observed for DEN, as well as concomitant impacts on the associated transcription factors (e.g., STAT, IRF), while IL-10 expression was similarly unaffected or increased (**Table 1**).

Knowledge of intracellular events post-ADE has been assisted through understanding the biology of Fc-gamma-Receptors (FcγR), including the identity of FcγR classes, the affinity of IgG (or complex) receptor binding and linked intracellular activating (e.g., FcγRIIa ➢ ITAM) or inhibitory (FcγRIIb ➢ ITIM) pathways (55). For the inhibitory action of FcγRIIb, phosphatases are recruited to the ITIM domain post receptor cross-linking (SRC family kinases), and ultimately leads from PIP_3 to PIP_2 conversion via hydrolysis. PIP_3 is a cell surface receptor linked second messenger synthesized from PI3K isoforms, involved in a range of cellular functions post ligand engagement. Of potential relevance to the role of ADE-mediated suppression of inflammatory pathways after FcγRIIb interaction, mouse models of disease have demonstrated that the inhibition of PI3Ks diminishes the severity of inflammation (56).

DEN has provided a strong focus into the intracellular consequences post-ADE infection, describing other intracellular mechanisms beyond those originally identified by RRV. These extend to type I IFN restriction via autophagy, SOCS3 and *Syk*-regulated pathways (**Table 1**). DEN-ADE and cytokine expression changes have been also reported for mast cells (48). Ebola virus ADE showed interesting FcγRIIa signaling pathways, without details on cytokine expression (49–51), although Ebola has been shown to alter cytokine expression without ADE via secreted viral glycoprotein (57).

SARS-CoV is an ADE virus, but with a difference (**Table 1**). While ADE for FcR bearing cells was observed, longer term infection was abortive, and the post-ADE mRNA expression profiles for IFN-α/β, MCP-1, IP-10, TNF, MIP-1 were not altered (52–54). Whether ADE is a factor in SARS-CoV pathology, or poses a threat post-vaccination, are currently being debated and assessed (58, 59). However, there is broad consensus that "cytokine storm," which can be understood as a gross dysregulation of appropriate cytokine responses leading to hyperinflammation, is a factor in disease (e.g., acute respiratory distress symptom). The link of SARS-CoV to ME is the recognition of "long-COVID," which shares symptoms such as long-term unexplained fatigue, "brain fog," pain and so on (24, 60), and as such provides a connection between an acute virus infection and long-term sequelae, as has been suspected in ME for decades. Definitive cytokine profiles for long-COVID have not been determined as yet, and drawing from the ME experience of establishing cytokine profiles for long term illness is not helpful due to the lack of consistency and validation by larger studies, although TGF-β has attracted some interest (61, 62).

Linking Inflammation, Cytokine Expression, and Mitochondrial Function

The core ME symptoms of long-term fatigue and post-exertional malaise (PEM) logically point to bioenergetic pathways, metabolomics and mitochondrial function, which have attracted biomedical research attention over the previous 10–15 years (63–66). Very recently, studies by Missailidis et al. (67, 68) have investigated mitochondrial function in immortalized *ex vivo* lymphoblasts collected from ME patients. Among a number of observations, Complex V rate of ATP synthesis was significantly reduced compared to healthy control lymphoblasts, with a statistical difference also found for lymphocyte death rate, and with chronic TORC I (TOR-Complex I) hyperactivation in ME lymphoblasts observed (suggesting compensatory activity via the upregulation of proteins required for oxidative phosphorylation and general mitochondrial function). TORC signaling is critical for stress sensing, cell growth and energetics, hence important to *homeostasis* and life span in general, with implications for disease if altered (69, 70).

Infection and the virally-induced disruption of cytokine signaling impacts homeostasis, and evidence exists to support mTOR - STAT signaling interactions in the context of immunity, including IL-10 expression (71). Therefore, chronically upregulated TORC signaling, as recently observed (67), may be linked to upsets in STAT, or vice versa. Inflammation impacts TOR function, and of relevance to the disruption to cytokine expression in macrophages post RRV-ADE, mTORC2 regulation is necessary for IFN-stimulated genes (ISGs) (37, 71).

The disturbance of homeostasis is an obvious result of infection, with the advent in some patients of severe illness due to cytokine storm, which represents a serious disequilibrium outcome due to the compromise of normal inflammation regulation processes. In discussing pathology, it is often forgotten

TABLE 1 | Examples of ADE-mediated virus infections and the impact on subsequent cellular pathways and cytokine expression.

Virus family (*Genus*)	Examples (*Disease*)	Cell examples - intracellular events post-ADE: cytokines impacted	References
Togaviridae (*Alphaviridae*)	Ross River (RRV) - (*Lethargy, Myalgia, Polyarthritis*)	Monocyte, Macrophage (RAW 264) - Post ADE suppression of TNF, NOS2, IFN-β, IP-10 expression via IRF-1, NF-κB, STAT-1 complex (ISGF3, AAF) ablation; Increased IL-10 expression (mRNA, Protein); Sp1 elevated	(37) (36)
Flaviviridae (*Flavivirus*)	Dengue - (*Fever, Shock, Myalgia, Hemorrhage*)	Monocyte, Macrophage - Post ADE suppression of IL-12, IFN-γ TNF; Increased IL-6, IL-10 (0-5 days p.i) with pSTAT-1, IRF-1 impacted; Increased IL-10 expression SOCS3, Syk-regulated; Early NOS2 via RLR-MAVS (without IFN); Autophagy role (ATG5) in IFN restriction; Early Syk - ERK1/2 IL-1β stimulation independent of DEN replication. Mast cell/Basophil - Post-ADE (72 hrs) significant increases for IL-1β, IL-6, not GM-CSF	(47) (40) (39) (42) (41) (38) (48)
Arteriviridae *(Arterivirus)*	* Porcine Reproductive & Respiratory Syndrome Virus (PRRSV) - (*Abortion, Respiratory disease in pigs*)	Alveolar macrophage - ADE-mediating viral epitopes mapped to N and GP5 proteins; ADE via FcγRI, FcγRIIb, FcγRIII; IFN-α, TNF-α expression decreased; IL-10 increased (mRNA, protein); IRF-1 IRF-3, NF-κB disrupted	(43) (45) (46) (44)
Filoviridae (Ebolavirus)	Ebola (*Hemorrhage, Shock*)	Granulocyte blasts (K562 cells) FcγRII, C1q-mediated ADE (via CR); FcγRIIa signaling via Src family protein tyrosine kinases (PTKs); Endosome uptake (phago-pinocytosis), Src phosphorylation	(49) (50) (51)
Coronaviridae (Beta-coronavirus)	SARS-CoV (1, 2) (Acute respiratory disease, Post-acute long-term fatigue)	Monocyte/Macrophage (THP-1; $CD68^{\pm}/CD14^{\pm}$ PBMC) - FcγRII required (intracellular domain); ADE infection achieved, but abortive in the longer term; IFN-α/β, MCP-1, IP-10, TNF, MIP-1 mRNA expression not altered post-ADE (1–72 h p.i.)	(52) (53) (54)

Virus details - NCBI Taxonomy Browser (www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi).
**Recently suggested nomenclature - Beta-arterivirus suid 1.*
For the history, and scientific reviews of ADE across virus families, see Taylor et al. (32), Tirado and Yoon (33), and Porterfield (34).

that immune responses are also required for cellular and tissue repair, which involve the pleiotropic nature of some cytokines that are normally associated with inflammation, like TNF (72, 73), in addition to other cytokine families with primary roles in healing, for example, the transforming growth factor (TGF) family. In fact, a member of the TGF super family, Activin B, has been identified in serum from ME patients as significantly different when compared to healthy volunteers (74). Activin proteins have many physiological roles, including repair, pro-, and anti-inflammatory functions (75).

In answering questions on why some individuals develop severe symptoms post-infection, and then in a proportion of cases intractable ME or long-COVID, while others display no short or long terms health impacts, surely is connected to individual differences in the regulation of the interactions discussed above. And within this milieu, the TOR family of proteins sit at the interface between the regulation of inflammation - immunity post-infection, and energy regulation both at the mitochondrial level, and associated pathways required for carbohydrate, lipid and amino acid catabolism.

DISCUSSION

While ADE is not the primary focus here, past investigations of ADE mechanism have identified a range of cellular pathways manipulated by viruses that may alter future cellular function, potentially leading to a long-term disturbance of homeostasis,

which can lead to chronic alterations in mitochondrial function and energy regulation, ultimately manifesting as multi-system disease. By linking patterns of post-infection cytokine dysregulation with observations on chronically increased TOR protein activity in cells from ME patients, the interface of TOR and inflammatory pathways is recommended as a topic for deeper investigation. Delving into these cellular processes will contribute insights into the mystery of why a virus infection can lead to a chronic health condition like ME in some individuals (estimated to be 11% in Australia) (7). The emergence of long-COVID brings a new urgency to these questions.

Of course, viruses do not need ADE entry to manipulate host immune-inflammatory responses to infection [(5) - includes explanations of virus-associated disruption of mitochondrial function, impact on immune cells, and discussion of infection and ME pathogenesis], but the impact associated with the expansion of cellular range to Fc-Receptor (FcR) or Complement-Receptor (CR) bearing cells, not normally permissive to infection, requires consideration. The strong disruption, and at times ablation of antiviral and inflammatory pathways, must have downstream impacts on later innate immune functions and the formation of adaptive immunity, particularly with the higher viral load allowing more FcR and CR cells to become infected, and their functions similarly impacted.

CONCLUSIONS

The history of ME features regular "outbreaks," which have been associated with virus infections. At the time of writing, the COVID (SARS-CoV-2) pandemic has revealed a sub-population of recovered patients who have developed long-term symptoms that resemble classic ME. Therefore, a perspective is presented herein that aims to link the viral manipulation of host antiviral and inflammatory-immune responses to mitochondrial function, with TOR proteins as the critical interface between deranged cytokine expression and energy regulation. Established for many virus families (**Table 1**), ADE post-infection is the particular perspective focus. ADE currently has renewed interest in relation to potential COVID vaccine safety, but in a more general context also raises questions on ME pathogenesis due to the dramatic consequences for immediate antiviral defenses, later innate immune responses, and thereafter guidance from ADE-impacted cells (e.g., antigen-presenting cells) for the formation of an appropriate adaptive immune response to support long term homeostasis.

The unraveling of the interactions between the viral manipulation of cells, bioenergetics and mitochondrial function will reveal the differences, at a cellular level, to explain why some individuals go on to develop chronic long-term health challenges like ME or long-COVID, while others do not.

AUTHOR CONTRIBUTIONS

The conception, research, analyses, and writing of this manuscript were all conducted by BAL.

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Adolescent and Young Adult ME/CFS After Confirmed or Probable COVID-19

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Introduction: Fatigue is a common acute symptom following SARS-CoV-2 infection (COVID-19). The presence of persistent fatigue and impaired daily physical and cognitive function has led to speculation that like SARS-CoV-1 infection, COVID-19 will be followed by myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS).

Methods and Results: We describe three adolescent and young adult patients who had confirmed or probable COVID-19 infections early on during the pandemic and were referred for evaluation to the Chronic Fatigue Clinic at the Johns Hopkins Children's Center. All patients reported orthostatic intolerance symptoms within the first 2 weeks of illness, and 10-min passive standing tests were consistent with postural tachycardia syndrome. After 6 months of illness, all three patients met criteria for ME/CFS. Clinical features of interest included strong histories of allergies in all three patients, two of whom had elevations in plasma histamine. Each demonstrated limitations in symptom-free range of motion of the limbs and spine and two presented with pathological Hoffman reflexes. These comorbid features have been reported in adolescents and young adults with ME/CFS.

Conclusion: ME/CFS can be triggered by COVID-19 in adolescents and young adults. Further work is needed to determine the pathogenesis of ME/CFS after COVID-19 and optimal methods of treating these patients. Our preliminary study calls attention to several comorbid features that deserve further attention as potential targets for intervention. These include neuromuscular limitations that could be treated with manual forms of therapy, orthostatic intolerance and POTS for which there are multiple medications and non-pharmacologic therapies, treatable allergic and mast cell phenomena, and neurologic abnormalities that may require specific treatment. Larger studies will need to ascertain the prevalence of these abnormalities.

Keywords: chronic fatigue syndrome, myalgic encephalomyelitis, dysautonomia, postural tachycardia syndrome, Hoffman sign, COVID-19, mast cell activation, neurodynamics

INTRODUCTION

The occurrence of chronic fatigue and other symptoms following infection with SARS-CoV-2 (COVID-19) has fueled speculation that the COVID-19 pandemic will trigger a wave of new cases of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (1, 2). As currently defined, the diagnosis of ME/CFS requires a duration of at least 6 months together with a substantial impairment in previously tolerated activities usually accompanied by profound fatigue, post exertional malaise (PEM), unrefreshing sleep, and either cognitive impairment or orthostatic intolerance (3). The diagnosis of ME/CFS requires a careful history and examination, and it should be brought into question if these symptoms are not present for the majority of the time and with at least a moderate severity.

Orthostatic intolerance refers to a group of clinical conditions in which symptoms of fatigue, lightheadedness, difficulty concentrating, and others are aggravated by quiet upright posture and are ameliorated by recumbency (4). Among those with ME/CFS, orthostatic intolerance is found in 90% of adults and over 95% of adolescents (3, 5–7). There has been some uncertainty about whether orthostatic intolerance is an early and primary contributor to ME/CFS symptoms, or if it develops as a secondary phenomenon due to reduced activity or some other aspect of disease pathophysiology. This question can be difficult to resolve because the onset of ME/CFS can be insidious. Even when there has been an obvious post-infectious onset, the diagnosis of ME/CFS is made long after the acute illness, making the timing of symptom recall subject to bias.

We recently evaluated three adolescents and young adults in whom COVID-19 illness had been confirmed with direct testing or was highly suspected based on the timing of the onset of symptoms in association with peak local and regional pandemic cases, close exposure to confirmed cases, along with characteristic clinical features such as anosmia. In all three, ME/CFS symptoms were prominent from the outset, as were symptoms and signs of orthostatic intolerance, consistent with an early contribution of circulatory disturbances to the pathogenesis of symptoms, before the onset of physiological changes due to inactivity. The preliminary findings of this case series have the potential to inform the investigation and treatment of what has been termed "long COVID" (8) or post-COVID-19 ME/CFS.

PARTICIPANTS AND METHODS

For this case series, eligible participants were individuals who had a confirmed or probable exposure to COVID-19 during the pandemic period and who had been referred to the Chronic Fatigue Clinic at the Johns Hopkins Children's Center after April of 2020. All individuals underwent a careful history and physical examination by a clinician with experience in the evaluation of ME/CFS. As part of routine procedure in the Chronic Fatigue Clinic, all individuals completed the unidimensional Wellness score which asks, "On a scale of 0–100 (with 0 being dying and 100 being the best a person can feel), how would you rate yourself on average over the last month?" This scale correlates well with longer questionnaires that measure health related quality of life (9).

Based on prior publications from our group and others about risk-factors for ME/CFS, the physical examination included an evaluation of joint hypermobility, screening maneuvers to identify limitations in symptom-free range of motion of the limbs and spine, a careful neurologic examination for evidence of myelopathy, orthostatic testing, and ascertainment of symptoms consistent with allergic inflammation and mast cell activation syndrome (10–13).

All patients had a general physical examination that included the nine-point Beighton score, a commonly used and reliable measure of joint hypermobility. Joint hypermobility was considered present if the Beighton score was four or higher. As part of the neurologic examination, all patients had an ascertainment of deep tendon reflexes and a Hoffman sign. The Hoffman sign was performed with the patient seated and with the head and neck in a neutral position. With the patient's distal interphalangeal joint of the middle finger supported by the examiner's index finger, the examiner's thumb made an abrupt downward flicking of the patient's distal phalanx. The Hoffman sign was considered positive if there was flexion of the patient's ipsilateral thumb or index finger (14). Patients were assessed for neurodynamic dysfunction and range of motion using the following maneuvers commonly used in physical therapy practice: seated slump testing, ankle dorsiflexion, passive straight leg raise, the upper limb neurodynamic test 1 (also known as the upper limb tension test with a median nerve bias), prone knee bend, and prone press-up. Methods for performing the examination maneuvers have been described in detail elsewhere (10, 11).

All patients were tested for orthostatic intolerance using a 10-min passive standing test (3, 15). Blood pressure and heart rate were recorded at 1-min intervals while the patient was supine for 5 min, and then again at 1-min intervals while the patient was standing upright and motionless for 10 min with the upper back resting against the wall and with the heels two to six inches away. At completion of standing, patients had repeat heart rate and blood pressure measurements for two further minutes in the supine position. Patients were asked when supine and at 1- to 2-min intervals while upright to report changes in symptoms on a 0–10 scale with 0 meaning absence of the symptom and 10 being the worst severity imaginable. The diagnosis of postural orthostatic tachycardia syndrome (POTS) for individuals 12–19 years required at least a 40 beat per minute (bpm) increase in heart rate between the lowest supine value and the peak while standing; for those 20 and older, a 30 bpm increase was required (16).

The Institutional Review Board of the Johns Hopkins Medical Institutes had waived informed consent for a retrospective study using data collected as part of routine care.

CASE REPORT (PATIENT 1)

A 19-year-old male resident of Florida with a past history of Gilbert syndrome and allergies developed a cough, sore throat,

headache, and fatigue on June 17, 2020. These symptoms began 3 days after a household exposure to a visiting relative who shortly thereafter had a positive SARS-CoV-2 RNA nucleic acid quantification test. Despite sleeping 3–4 h more than usual per night, this adolescent felt exhausted and flu-like, and his SARS-CoV-2 RNA nucleic acid quantification test was positive on June 18. He experienced a loss of sense of smell which persisted for several months. Both parents became ill at the same time, with confirmation of COVID-19 via a nucleic acid quantification test in his father and subsequent positive COVID-19 antibody tests in both.

Prior to his COVID-19 diagnosis, this patient was a college student and track and cross-country athlete, running on average 60–70 miles per week. Two weeks after the onset of his symptoms, his attempts to resume running resulted in an increased cough, labored breathing, and lightheadedness. By July 1, there was some improvement in the fatigue, and he was able to run three miles, however the cough remained. Throughout the rest of July, he continued to experience a decreased tolerance for running and an increase in post exertional malaise, characterized by lightheadedness, an increase in fatigue, and coughing. He also developed chest pressure, intermittent chest pain, and a significant increase in his heart rate after basic tasks such as walking to another room or showering. During very light activity (a game of cornhole, similar to bean bag toss) 2 months after the onset, his heart rate was 160–170 bpm for 20–30 min, followed by 3 days of PEM. A cardiac evaluation in mid-July, including an electrocardiogram (ECG), chest x-ray, and echocardiogram, revealed no abnormalities. A cardiopulmonary exercise test at 4 months after onset of illness showed a normal exercise ECG, but below average peak VO2 of 84% of predicted. A cardiac MRI showed no abnormalities. The troponin level was 0.009 mg/mL 6 weeks after the onset of illness.

We evaluated him 2 months after the onset of the COVID-19 infection, at which time his symptoms included constant fatigue, unrefreshing sleep, PEM after mild increases in activity, bi-frontal and bi-temporal headaches, chest pain, occasional cough, leg pain, insomnia, frequent awakening, and mild anxiety and depression. He also had a pre-COVID-19 history of mild asthma, allergic inflammation, and several food intolerances.

His physical examination showed a healthy-looking young man in no distress. He had a Beighton score of 3/9 for >10 degrees of hyperextensibility of the right elbow and both knees. His neurologic examination showed 2+ symmetrical deep-tendon reflexes with a bilaterally positive Hoffman sign. He had limited symptom-free range of motion on a seated slump test, lacking 30 degrees of full leg extension of the right and 60 degrees of the left. Passive straight leg raise end-range was limited at 35 degrees bilaterally. Upper limb neurodynamic test 1 lacked 50 degrees of elbow extension on the left and 40 degrees on the right. To investigate the lightheadedness and fatigue, a 10-min passive standing test showed a 70 bpm difference between his lowest supine and peak standing heart rate, consistent with a diagnosis of postural orthostatic tachycardia syndrome (**Figure 1**, **Table 2**).

A month after onset, his complete blood count showed a WBC of 5.1, hemoglobin 16.1, and platelet count of 255. His comprehensive metabolic panel was normal with the exception of a total bilirubin of 1.5 (reference range, 0.2–1.1 mg/dL) consistent with his prior diagnosis of Gilbert syndrome. His CK was normal at 79 (reference range, 44–196 U/L). The sedimentation rate was 6. In light of his allergic history, testing was also performed to evaluate the presence of mast cell activation disorder. Serum tryptase was normal at 5.4 (reference range, <11.0 mcg/L) but chromogranin A was mildly elevated at 144 (reference range, 25–140 mg/mL), and his plasma histamine was elevated to 4.2 (reference range, ≤ 1.8 mg/mL); plasma histamine remained elevated on repeat testing 3 and 7 months after the onset of the illness.

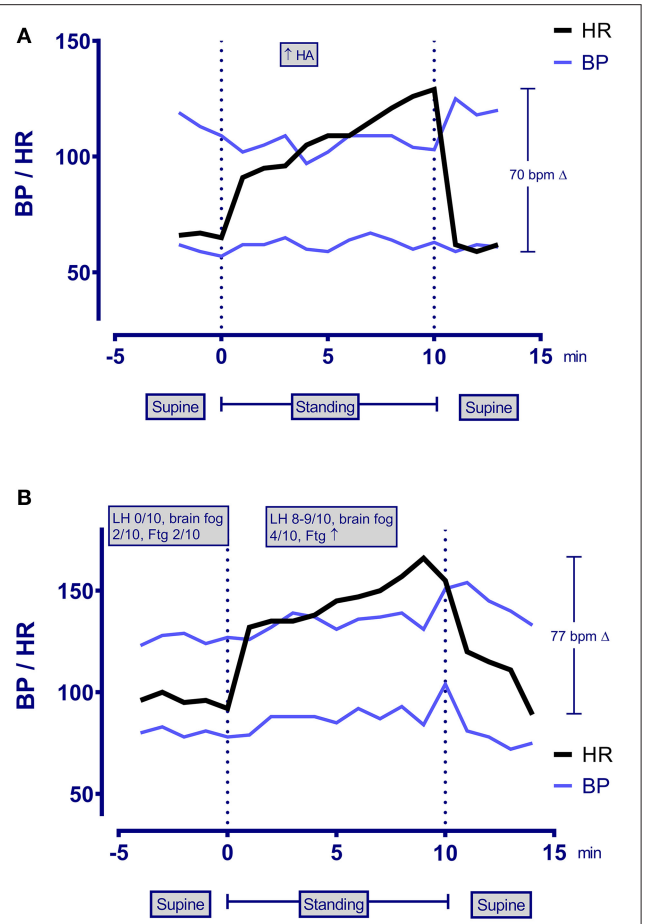

FIGURE 1 | Heart rate, blood pressure, and symptom changes during a 10-min passive standing test in patient 1 **(A)** and patient 2 **(B)**. LH, lightheadedness; Ftg, fatigue; bpm, beats per minute.

After 6 months of illness, he satisfied the Institute of Medicine criteria for ME/CFS. Seven months post-COVID-19, his ability to function as he had pre-illness remains markedly decreased. His main symptoms consist of persistent fatigue, limited tolerance of exercise, unrefreshing sleep, PEM, lightheadedness, and headaches. He now tolerates two to three 15-min walks daily without provoking excessive tachycardia or PEM. Current treatments include Lexapro 5 mg daily for post-illness anxiety, loratadine 10 mg daily and famotidine 40 mg twice daily for the

allergic symptoms and elevations in histamine, methylphenidate 10 mg each morning as a vasoconstrictor, along with compression garments and an increased sodium intake for POTS.

RESULTS

Participants

In addition to the patient reported above, two other individuals were referred to the clinic between August and October 2020. The demographic and clinical data on the three participants are displayed in **Table 1**. As discussed above, patient 1 had a confirmed COVID-19 infection. Patients 2 and 3 had close contact with an individual with confirmed COVID-19 infection and developed anosmia and dysgeusia lasting up to 5 months. Nucleic acid quantification testing for COVID-19 was performed relatively late, 1 month after onset of illness in patient 2. Nucleic acid quantification testing for patient 3 was not performed due to lack of availability, but two further household members developed similar symptoms within days of exposure to her. Of interest, antibody testing later was negative for patients 2 and 3.

Criteria for ME/CFS

As shown in **Table 2**, all three participants met criteria for ME/CFS 6 months after the onset of symptoms. ME/CFS symptoms had been present from day one of the illness. None of the participants had a prior medical history of ME/CFS. Symptoms of orthostatic intolerance were also present at an early point of the initial respiratory illness, from 1 day to 2 weeks. All had profound POTS on testing several months after the onset, as shown in **Table 2**, with the increment between lowest supine and peak standing heart rate within the first 10 min well-exceeding the diagnostic criteria, together with reproduction of orthostatic symptoms.

Other Clinical and Allergic Phenomena

Allergic symptoms and history were present in all participants as displayed in **Table 3**. Notably, one patient had a history of oral allergy syndrome, another had urticaria after exposure to citrus, and the third had cutaneous features consistent with mast cell activation (dermatographism and facial flushing). **Table 3** also displays the pertinent physical examination findings. Two of the three patients had an abnormal Hoffman sign. Patient 2 met criteria for hypermobile Ehlers Danlos syndrome. Neurodynamic testing measures commonly used in physical therapy to assess for changes in, among other things, neural gliding function in the trunk and limbs, revealed restricted range of motion findings in all three patients.

DISCUSSION

This case series describes one patient with confirmed SARS-CoV-2 infection and two with highly probable SARS-CoV-2 infection in whom the diagnostic criteria for ME/CFS were satisfied by month 6 of the illness. Fatigue has previously been reported as a common acute symptom of COVID-19, with a prevalence reaching 80% in the first month and remaining as high as 53% 2

TABLE 1 | Onset, diagnostic testing, and clinical features of confirmed and probable COVID-19 infection in the study participants.

Patient	Age (yrs)	Sex	Onset of COVID-19 symptoms	COVID-19 PCR		COVID-19 antibody		Exposure	Distinctive COVID-19 symptoms
				Result	Date	Result	Date		
1	19	M	6/17/2020	+	6/18/20	+IgG	1/19/21	Exposed to relative 3 days before onset. Other household contacts also developed COVID-19 symptoms and had positive PCR or antibody tests	Cough, sore throat, headache and fatigue at onset without fever followed by loss of sense of smell.
				+	7/01/20	+IgM	1/19/21		
				–	7/15/20				
2	30	F	3/19/2020	–	4/09/20	–	4/09/20	Exposed 2 days earlier to co-worker in an office two doors down the hall whose COVID-19 PCR test was positive	Mild cough, fatigue, and low-grade temperature elevation at onset followed by loss of taste and smell at day 5 (persisting for 5–6 weeks)
3	22	F	4/07/2020	Not performed		–	9/20/20	Exposed to a positive case and within days developed symptoms. In the next week, mother and boyfriend developed fever, myalgias, chills, headache, and shortness of breath	Low grade fever, sore throat, fatigue, and shortness of breath for 2 weeks.

PCR, polymerase chain reaction/nucleic acid quantification test.

TABLE 2 | Responses to the 10-min passive standing test and features of ME/CFS in the study participants.

Patient	Age	Orthostatic intolerance							ME/CFS*				
		Lowest supine HR	Peak HR standing	Δ HR	Supine BP	BP at peak HR	Symptoms during passive standing test	Onset of orthostatic symptoms during COVID-19	1. Substantial impairment in previously tolerated activities + fatigue	2. PEM	3. Unrefreshing sleep	4a. Cognitive impairment	4b. OI	Onset of fatigue	Wellness score at initial visit (0–100)
1	19	59	129	70	109/57	103/63	Headache	Week 1	x	x	x	x	x	Day 1	40
2	30	89	166	77	127/78	139/93	Fatigue Blurry vision Brain fog Lightheadedness Increased Respiratory rate	Day 2	x	x	x	x	x	Day 1	20
3	22	75	129	54	94/61	NA	Lightheadedness Fatigue	By week 2	x	x	x		x	Day 1	42

HR, heart rate; BP, blood pressure; PEM, post-exertional malaise; OI, orthostatic intolerance; ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome; Δ, change in heart rate between lowest supine and peak heart rate standing; NA, not available.

**The diagnosis of ME/CFS was made after a duration of 6 months of symptoms.*

months after the onset (17). Our work parallels the findings of a recent survey of 3,762 international COVID-19 patients by Davis and colleagues (8). They reported fatigue, PEM, and cognitive dysfunction as the three most common symptoms persisting after 6 months. Of the total survey population, 2,308 respondents reported tachycardia. Of all the respondents, only 8.43% were hospitalized. The probability of having fatigue was high from day one and the probability of having PEM peaked and plateaued around week six.

Several observations from our case series warrant further emphasis. First, all three individuals had relatively mild respiratory symptoms and none required hospitalization. This is consistent with observations from other groups that persistent fatigue following COVID-19 infection is independent of the severity of the initial infection (18). The potential for marked impairment in function after relatively mild respiratory illnesses contrasts with the emergence of ME/CFS after other infectious illnesses. In the 10–13% of individuals who meet criteria for ME/CFS 6 months following infectious mononucleosis, the risk of ME/CFS is related to the severity of the initial infection (19–21). The occurrence of ME/CFS after relatively mild viral illnesses raises the question of how many ME/CFS cases before the COVID-19 pandemic might have been due to mild, sub-clinical, or asymptomatic infections.

Second, as early as within the first 2 days and certainly within the first 2 weeks, all three developed symptoms of orthostatic intolerance and ultimately met criteria for POTS, which has been reported as a post-COVID-19 phenomenon by several groups (8, 22–25). Similar reports of dysautonomia followed the SARS-CoV-1 pandemic (26). While the 10-min passive standing tests to confirm the diagnosis of POTS were conducted 5–7 months after the onset of COVID-19, the orthostatic symptoms were present at an early point, suggesting that autonomic symptoms could have been a direct result of the viral infection rather than a consequence of inactivity or deconditioning. In those diagnosed with POTS following COVID-19 infection, there has been a variable onset of orthostatic symptoms. Miglis (22) reported palpitations developing on day two of the COVID-19 illness in a 26-year-old female nurse. She had noticed tachycardia by day seven of the illness. Kanjwal et al. reported a 36-year-old female who began to develop fatigue, dizziness, and palpitations with postural changes 3–4 weeks after the initial COVID-19 infection (25). Given the delay between the acute illness and the orthostatic symptoms, these authors speculated that autoimmunity was a likely mechanism for the onset of POTS, as has been reported by several groups (27–30). Novak (23) described a similar patient who developed orthostatic symptoms 1 month after the onset of acute COVID-19 infection, in whom intravenous immunoglobulin was partially successful. Further attention to the timing of the onset of orthostatic symptoms has the potential to help determine whether autonomic symptoms result from direct infection by the virus, sympathetic activation as part of the immune response, mast cell activation, or autoimmunity (31). In heterogeneous disorders like POTS, each of these mechanisms could be important for subsets of patients and might warrant different approaches to treatment. Based on these reports of

TABLE 3 | Past medical history and physical examination abnormalities.

Patient	Prior medical history	Allergic phenomena	Examination abnormalities		
			Beighton score (0–9)	Hoffman sign	Comments
1	Gilbert Syndrome	Allergies to pollens, grasses Immunotherapy for 3 yrs (ages 10–13) Oral allergy syndrome to carrots, cashews, and cherries Mild asthma Family history positive for allergic rhinitis and oral allergy syndrome Post COVID-19 plasma histamine 3.5 (normal ≤ 1.8 mg/ml)	3	+ bilateral	**Physical maneuvers:** Seated slump testing elicited stretch at 150° leg extension on the right, 120° on the left Passive straight leg raise elicited stretch at 30° bilaterally with end range 35° Upper limb neurodynamic test 1 elicited stretch and guarding at 130° of elbow extension on the left, 140° on the right Mild thoracic hypomobility on prone press-up
2	Presyncope (2× 10 years earlier) Migraine Post-pneumonia exhaustion (9 yrs earlier) Anxiety Discrete episode of depression (10 yrs earlier) with no intervening symptoms Dysmenorrhea	Allergic rhinitis (cat, dust, pollens) Urticaria after exposure to citrus in childhood Eczema Mild asthma Post COVID-19 plasma histamine 4.2 and 3.4 (normal ≤ 1.8mg/ml)	5	+ bilateral	**Neurological:** Brisk 3+ deep tendon reflexes at triceps and patella Fine resting tremor in hands **Physical maneuvers:** Upper limb neurodynamic test 1 elicited stretch at 160° of elbow extension on the left, 170° on the right Mild pectoralis minor tenderness L > R
3	Celiac disease Anorexia Dysmenorrhea	Facial flushing with activity Dermatographism Family history positive for histamine sensitivity	3	–	**Cutaneous:** Diffuse skin erythema Acrocyanosis in lower limbs **Physical maneuvers:** Mild pectoralis minor tenderness

orthostatic intolerance in patients with chronic symptoms post-COVID-19, and the high prevalence of orthostatic intolerance in those with established ME/CFS, we would recommend at least 10 min of orthostatic testing for all patients reporting chronic fatigue in the context of long COVID.

Third, range of motion impairments have been found more commonly in individuals with ME/CFS than in healthy controls and are important in the pathogenesis of symptoms (11, 32). The application of a longitudinal neural strain such as that imposed by a straight leg raise maneuver is capable of aggravating fatigue and other symptoms for at least 24 h (33). The mechanism for these range of motion impairments is unknown, but possible explanations include the result of previous musculoskeletal injuries, excessive guarding around hypermobile joints, post-infectious inflammation, and reduced activity in response to an illness (34). While two of our three patients presented with range of motion impairments, their pre-COVID-19 range of motion measurements are unknown. Therefore, it is impossible to tell whether these impairments preceded the onset of ME/CFS or were a result of the viral illness.

Fourth, all patients in our series had prominent histories of allergic inflammation. Afrin and colleagues and others (35–37) have hypothesized that mast cell activation can play an important pathophysiologic role in the hyperinflammatory response to COVID-19. Two of the three patients have had sustained elevations in plasma histamine, and both have had improvement in fatigue and cognitive dysfunction in response to treatment with drugs that block the H1 histamine receptor, consistent with mast cell activation. Further work is needed to determine the prevalence of similar allergic histories and evidence of mast cell activation syndrome (38) in others with prolonged symptoms for ME/CFS after COVID-19. If allergic inflammation and mast cell activation are common, it would be important to determine whether medications with antihistamine properties or medications that are capable of stabilizing mast cell membranes will prove effective in ameliorating the symptoms of post-COVID-19 ME/CFS.

Fifth, neuroanatomic abnormalities have been recognized in a subset of those with ME/CFS symptoms, including Chiari malformation, congenital or acquired cervical stenosis, and instability at the skull base or in the spine. Heffez (39) has described 270 patients with fibromyalgia among whom 64% had hyper-reflexia and 26% had a positive Hoffman sign (39). Our research group has reported a series of three patients in whom congenital or acquired cervical stenosis was a treatable cause of refractory orthostatic intolerance and other ME/CFS symptoms (12). The presence of abnormal Hoffman signs in two of our post-COVID-19 patients was unexpected after an acute illness. Long term study will be needed to determine whether these abnormalities were transient and related to the viral

infection or persistent and related to underlying neuroanatomic abnormalities that predisposed patients to prolonged symptoms and autonomic dysfunction.

LIMITATIONS

This report of three cases establishes the potential for adolescents and young adults who have mild respiratory illnesses from confirmed or probable COVID-19 to develop prolonged symptoms consistent with ME/CFS. While one patient had proven COVID-19 infection, we could not confirm the presence of COVID-19 in the other two, a problem that was complicated by the lack of availability of testing early in the pandemic. These two patients had highly probable COVID-19 based on their close temporal exposure to confirmed cases and based on characteristic symptoms such as anosmia and dysgeusia. A curious finding was that none of the two probable cases developed antibodies to COVID-19 in the convalescent phase of their illness. Whether impaired production of antibodies is a risk-factor for prolonged symptoms after COVID-19 or is related to a sampling anomaly will need to be assessed in larger samples. Because these patients were referred to a specialist clinic at a tertiary care center, we cannot know whether the clinical features we observed will prove to be common across the general population of those with prolonged symptoms after COVID-19.

CONCLUSION

Our evaluation of this sample of three patients suggests that ME/CFS can be triggered by confirmed or probable COVID-19 in adolescents and young adults. Komaroff and Bateman (1) predict that over 10 million new cases of ME/CFS will be triggered by COVID-19 globally. Further work is needed to determine the pathogenesis of ME/CFS after COVID-19 and how to treat these patients optimally to promote their return to their pre-COVID-19 quality of life. Our study identifies several comorbid features that could be treated including ROM limitations that could respond to manual forms of therapy, orthostatic intolerance and POTS for which there are multiple non-pharmacologic and pharmacologic therapies, treatable allergic phenomena, and neurologic abnormalities that may require specific treatment. If non-pandemic ME/CFS provides any guidance, the treatments for each patient are likely to vary based on the contributions of comorbid conditions. Whether post-COVID-19 patients are more homogeneous remains to be determined, but the expected number of new cases provides an opportunity to study diagnostic procedures, including standing and head-up tilt testing, as well as candidate therapies in an organized manner. These efforts also have the potential to inform the treatment of individuals with non-pandemic ME/CFS.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because: the main data from this case series is included in this article. Requests to access the datasets should be directed to prowe@jhmi.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Johns Hopkins Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LP wrote the initial draft and was involved in critical review of the final manuscript. SS, RLV, NP, RS, and PR were instrumental to the clinical care of patients, and have contributed to the writing and review of the manuscript. RLV contributed to the writing and critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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Theory: Treatments for Prolonged ICU Patients May Provide New Therapeutic Avenues for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

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We here provide an overview of treatment trials for *prolonged* intensive care unit (ICU) patients and theorize about their relevance for potential treatment of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Specifically, these treatment trials generally target: (a) the correction of suppressed endocrine axes, notably through a "reactivation" of the pituitary gland's *pulsatile* secretion of tropic hormones, or (b) the interruption of the "vicious circle" between inflammation, oxidative and nitrosative stress (O&NS), and low thyroid hormone *function.* There are significant parallels in the treatment trials for *prolonged* critical illness and ME/CFS; this is consistent with the hypothesis of an overlap in the mechanisms that prevent recovery in both conditions. Early successes in the simultaneous reactivation of *pulsatile* pituitary secretions in ICU patients—and the resulting positive metabolic effects—could indicate an avenue for treating ME/CFS. The therapeutic effects of thyroid hormones—including in mitigating O&NS and inflammation and in stimulating the adreno-cortical axis—also merit further studies. Collaborative research projects should further investigate the lessons from treatment trials for *prolonged* critical illness for solving ME/CFS.

Keywords: ME/CFS, treatment, suppressed endocrine axis, prolonged critical illness, oxidative and nitrosative stress, chronic critical care illness, non-thyroidal illness syndrome, post viral fatigue syndrome

INTRODUCTION

Critical illness refers to the physiological response to virtually any *severe* injury or infection, such as sepsis, liver disease, HIV infection, SARS-CoV-2 infection, head injury, pancreatitis, burns, cardiac surgery, etc. generally resulting in intensive care unit (ICU) hospitalization (1). *Prolonged* or chronic critical illness—a term applied to patients that survive *severe* injury or infection but fail to start recovering after a few days—is characterized by the suppression of multiple endocrine axes, irrespective of the nature of the original infection or trauma (2–7). This endocrine suppression is, however, not readily observable in single or average measurements of circulating tropic and non-tropic hormone concentrations (which are a function of both hormone release

and elimination from the blood stream); instead measurements of the *frequency* and *amplitude* of pituitary secretions (i.e., of the *pulsatility*) performed on ICU patients as often as every 10 min over 24 h are required to reveal the endocrine suppression (8). Pro-inflammatory cytokines play a role in inducing and maintaining the uniform suppression of the endocrine axes during *prolonged* critical illness—predominantly at the level of the hypothalamus and pituitary (9–15). Moreover, reciprocal relationships between cytokines, oxidative and nitrosative stress (O&NS), and thyroid hormone *function* appear to perpetuate illness (c.f. "vicious circle") (13, 16). These patterns are increasingly recognized as maladaptive and inhibiting patients' recovery, thus requiring treatment independent of the initial infection or trauma (5, 17–19). Moreover, it has been suggested that the persistence of the endocrine disturbances could also explain "post-intensive care syndrome (PICS)" (20); i.e., "the cognitive, psychiatric and /or physical disability after treatment in ICUs," including ICU-acquired weakness (21).

ME/CFS is a debilitating, multi-system disease of unclear etiology (22, 23). The most common peri-onset events reported by patients are infection-related episodes, stressful incidents, and exposure to environmental toxins (24). "Impaired function, post-exertional malaise (an exacerbation of some or all of an individual's ME/CFS symptoms after physical or cognitive exertion, or orthostatic stress that leads to a reduction in functional ability), and unrefreshing sleep" are considered to be core symptoms (25, 26). The severity of the symptoms varies: "very severely affected patients experience profound weakness, almost constant pain, severe limitations to physical and mental activity, sensory hypersensitivity (light, touch, sound, smell, and certain foods), and hypersensitivity to medications" (27). The disease can be completely incapacitating: "at least one-quarter of ME/CFS patients are house- or bedbound at some point in their lives" (25). Patients with milder symptoms experience a significant reduction in previous levels of functioning (23). The disease progresses over time: similar to critical illness, an early hypermetabolic state may culminate in a hypometabolic state with low energy production (28). The progressive nature of the disease makes establishing a set of diagnostic criteria or molecular markers particularly difficult. There are currently no effective treatments for ME/CFS (29–33).

In a previous publication (34) we argued that—without excluding possible predisposing genetic or environmental factors—the maladaptive mechanisms that prevent recovery in some ICU patients may also underlie ME/CFS. Specifically, these mechanisms are: (a) suppression of the pituitary gland's *pulsatile* secretion of tropic hormones, and (b) a "vicious circle" between inflammation, O&NS, and low thyroid hormone *function*. Here we provide an overview of past treatment trials for *prolonged* ICU patients which specifically address these mechanisms. We relate similar experimental trials to improve outcomes in ME/CFS in order to highlight the similarities in the treatment approaches for both conditions. As part of this overview we also draw on findings from fibromyalgia because ME/CFS and fibromyalgia are often jointly considered in the literature (35, 36); fibromyalgia is similarly a syndrome that is medically unexplained, often comorbid with ME/CFS, and "shares the core symptoms of fatigue, sleep problems, and cognitive difficulties" (37). Finally, we also suggest potential lessons to be learned from treatment trials for *prolonged* critical illness for the quest to solve ME/CFS.

The lessons-learned from the field of critical care medicine for ME/CFS may also be particularly relevant for the aftermath of the COVID-19 pandemic. Coronaviruses are associated with persistent inflammation (38) and endocrine dysfunctions (39)—elements that are central to both the pathologies of *prolonged* critical illness and ME/CFS. Many COVID-19 patients continue to experience a variety of debilitating symptoms despite successfully defeating the virus—termed "post COVID-19 syndrome" or "long COVID-19"– that resembles ME/CFS (40–45). Moreover, a recent analysis has shown that ME/CFS patients and COVID-19 recovery patients "share molecular signatures"—evidence of overlaps in immune and metabolic dysregulation (46).

COMPENSATION FOR AND CORRECTION OF SUPPRESSED ENDOCRINE AXES

Researchers have tried to correct suppressed endocrine axes in *prolonged* critically ill patients with the hope of reducing muscle wasting and mortality, and to aid recovery. The treatment trials can be grouped into two main approaches: (A) treatments with non-tropic *peripheral* hormones, and (B) the "reactivation" of the *central* endocrine glands. Whereas, the first approach compensates for suppressed endocrine axes by providing down-stream hormones into circulation, the second approach attempts to correct the endocrine axes themselves through interventions at the central level. We briefly summarize various treatment trials for each of these two broad approaches below. For each approach we also relate similar experimental treatment trials for ME/CFS and fibromyalgia in order to highlight the similarities in the quests to cure the two conditions, and to derive lessons for solving ME/CFS.

Abbreviations: ACTH, Adrenocorticotropic hormone; AVP, Arginine vasopressin; CIM, Critical illness myopathy; CIRCI, Critical illness-related corticosteroid insufficiency; CRH, Corticotrophin-releasing hormone; GH, Growth hormone; GHIH, Growth hormone inhibiting hormone; GHRH, Growth hormone releasing hormone; GHRP-2, synthetic Growth hormone releasing peptide; GnRH, Gonadotropin-releasing hormone; GSH, Glutathione; HPA, hypothalamus-pituitary-adrenal axis: "Adreno-cortical axis"; HPG, Hypothalamic–pituitary–gonadal axis: "gonadotropic axis"; HPS, Hypothalamic-pituitary-somatotropic axis: "Somatotropic axis"; HPT, Hypothalamic-pituitary-thyroid: "Thyrotropic axis"; HSPs, Heat Shock Proteins; ICU, Intensive Care Unit; IDO, Indoleamine 2,3-dioxygenase; IGF-1, Insulin like growth hormone-1; IGFBP, Insulin like growth hormone binding proteins; JAK/STAT, Janus kinase—signal transducer and activator of transcription; LDN, Low Dose Naltrexone; LH, Luteinizing hormone; LHRH, Luteinizing hormone-releasing hormone; ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; mtDNA, Mitochondrial DNA; NAC, N-acetylcysteine; NADH, Nicotinamide adenine dinucleotide; NTIS, Non-thyroidal illness syndrome; O&NS, oxidative and nitrosative stress; PICS, Post-intensive care syndrome; ROS, Reactive oxygen species; rhGH, Recombinant human GH; TRH, Thyrotropin-releasing hormone; TSH, Thyroid stimulating hormone; VIDD, Ventilator induced diaphragm dysfunction.

Approach A: Treatments With Peripheral Hormones

The use of *peripheral* hormones—notably glucocorticoids and other adrenal hormones, growth hormone (GH), insulin-like growth hormone-1 (IGF-1), thyroid hormones, and a combination of these hormones—have been trialed to improve outcomes in *prolonged* critical illness as well as in ME/CFS and fibromyalgia with varied successes as described below.

Treatments With Glucocorticoids and Other Adrenal Hormones

In Prolonged Critical Illness

Administration of large daily doses of hydrocortisone (200–300 mg) in patients during critical illness is quite common, particularly in sepsis or when cortisol levels are deemed low relative to the severity of the illness. The aim is to treat "critical illness-related corticosteroid insufficiency" (CIRCI) which is thought to "lead to an exaggerated proinflammatory response with increased tissue injury and organ dysfunction" (9, 47). Some researchers, however, have recently argued that such high doses of hydrocortisone may be counterproductive because they drive the inhibitory feedback loop inherent to endocrine axes, resulting in further central suppression of the axes (19). Moreover, large hydrocortisone doses heighten *catabolic* effects (i.e., the breakdown of molecules and tissues), especially if administered for too long [see review in (5, 48)].

In ME/CFS

Low production of adrenal hormones (i.e., partial hypoadrenalism) has been well-documented in ME/CFS (49–66). Several studies showed that a low dose of hydrocortisone could benefit ME/CFS patients, notably in reducing fatigue and disability scores [see reviews in (67, 68)]. Daily doses of 5–15 mg of hydrocortisone appear not to further suppress the adrenocortical axis (50, 69)—also called hypothalamic-pituitary-adrenal (HPA) axis—and may even improve the otherwise "blunted" responses of the pituitary to the signal from the hypothalamus (56). However, researchers have revealed that somewhat higher doses of hydrocortisone (25–35 mg per day) lead to a moderate decrease in endogenous adrenocorticotropic hormone (ACTH) and cortisol production in ME/CFS patients, via the negative feedback loop (50, 70). Consequently, there has been a debate since the late 1990s between researchers who argue that—despite improvement in symptoms—"adrenal suppression precludes the practical use of hydrocortisone" for ME/CFS patients (70), and those who stress that at low physiological doses hydrocortisone treatment for ME/CFS is safe and effective (67, 71–78).

The effects of supplementation with *other* adrenal hormones on ME/CFS symptoms has also been studied. Fludrocortisone (a corticosteroid) led to positive results in some trials (79–81), but not in others (82–84). In an uncontrolled pilot-study the supplementation with DHEA (an adrenal hormone with anabolic properties) led to a significant reduction in ME/CFS symptoms, including pain, fatigue, helplessness, anxiety, memory loss, and sexual problems (85). Finally, a recent study suggested pregnenolone sulfate (an endogenous neurosteroid derived from the adrenal hormone pregnenolone) may have therapeutic potential to restore the Transient Receptor Potential Melastatin 3 (TRPM3) ion channel function in natural killer cells in ME/CFS (86).

In summary, glucocorticoids and other adrenal hormones are used in practice to compensate for lower endogenous hormone production in both *prolonged* critical illness and ME/CFS. Treatments aim to manage inflammation and improve symptoms. However, their use is questioned by researchers in both fields because they tend to reinforce central HPA axis suppression.

Treatments With GH and IGF-1

In Prolonged Critical Illness

The hormone IGF-1 has been tested and applied in critical illness for decades, with positive results in reducing catabolism (i.e., muscle and protein loss), recovery of gut mucosal function, tissue repair, control over inflammatory cytokines, decreased protein oxidation, increased glucose oxidation, etc. [see review (87)]. However, research has shown that to avoid side effects doses must be physiological (i.e., not higher than regularly produced by the body). Some positive results have also occurred with administration of GH (or the synthetic version, rhGH) during critical illness [see reviews (87, 88)]. However, a large-scale double-blind randomized control study of rhGH infusions undertaken in 1999 resulted in increased mortality of critically ill patients (89). This led to the near cessation of the use of GH or rhGH in critical care. Other researchers argue that dosages in this study were too high, thereby overwhelming the negative feedback loops inherent to endocrine axes maintaining homeostasis (88). Finally, some promising trials have also been performed combining GH and IGF-1 in critical illness (90, 91). GH and IGF-1 have complementary roles in the balance between anabolic (i.e., the building of molecules and tissue) and catabolic activities (91).

In ME/CFS

There is also evidence for low GH secretion in ME/CFS (92, 93). A small placebo-controlled study found that treatment with GH injections in ME/CFS patients over 12 months was beneficial: a few of the patients were even able to resume work after long periods of sick leave (94). Evidence of relative GH deficiency in fibromyalgia (95–101) also spurred a series of placebo-controlled studies which demonstrated that GH injections over several months—in the form of physiological doses or doses adapted to increase IGF-1 to a specific level—reduced pain and improved quality of life scores in fibromyalgia patients (94, 102–105).

In summary, GH and IGF-1 have been trialed for both *prolonged* critical illness and ME/CFS with reports of beneficial outcomes. However, their use is not common in practice, notably because of the risks involved. Supplementation with these hormones also does not serve to relieve central endocrine axis suppression but would rather reinforce it.

Treatments With Thyroid Hormones

In Prolonged Critical Illness

Clinicians already began in the late 1970s to suggest thyroid hormone supplementation for their critical patients in an

attempt to increase survival rates (106–108). This came out of a realization that these patients suffered from a depressed level of thyroid hormone activity independent of the health of the thyroid gland—termed "non-thyroidal illness syndrome (NTIS)," "euthyroid sick syndrome" or "low T3 syndrome" (109, 110). Supplementation of thyroid hormone during critical illness continues to be the subject of intense debate (111–115). Results with thyroid hormone supplementation have been mixed [see reviews (116–118)]. The type of supplement (synthetic T4 or T3), the timing of treatment and the dosage could explain the variable, but often poor outcomes. Specifically, given that thyroid hormone conversion from its "*inactive*" (T4) to "*active*" (T3) form by deiodinase enzymes is impaired during illness through the actions of pro-inflammatory cytokines (13, 16, 114, 119, 120), it has been suggested that T3 supplementation may be more effective than T4 supplementation (121–123). In fact, given that T4 *up-regulates* the enzyme (deiodinase D3) which converts thyroid hormones into "*inactivated*" forms and *down-regulates* the enzyme (D2) responsible for thyroid hormone conversion to the "*active*" forms (124, 125), any T4 supplementation could exasperate NTIS. Yet, because of the short half-life of T3 and its normal circadian rhythm (126), the timing and periodicity of any T3 administration is likely to be an important determinant of its effect (127, 128). Finally, recognizing the fact that thyroid hormone uptake (i.e., transport into cells and binding by cellular receptors) is downregulated in tissue-specific ways during critical illness (13, 113, 119, 120, 129), thyroid hormone supplementation doses may have to be supra-physiological to achieve results (130, 131). In conclusion, "at present, no evidence-based consensus or guideline advocates thyroid hormone treatment of NTIS in patients who are critically ill," (117) but new calculated parameters derived from mathematical modeling of thyroid hormone *function* may in the future assist in identifying better therapeutic thyroid hormone interventions in patients (132).

In ME/CFS

Recent studies suggest the existence of low thyroid hormone *function* in ME/CFS—i.e., low impact of thyroid hormone on target glands or tissues despite "normal" TSH and T4 lab results (133, 134). Thus, thyroid hormone *function* in ME/CFS resembles the "euthyroid sick syndrome" (NTIS) described in the field of critical medicine (34).

In small placebo-controlled studies in the 1990s, Lowe et al., showed that supraphysiologic dosages of T3 (75–150 mcg/day) were safe and significantly effective in the treatment of fibromyalgia: "significant improvement in clinical symptoms were recorded in T3 phases compared to baseline and placebo phases" (135–138). Given that patients had been euthyroid (i.e., their serum TSH and T4 values did not indicate hypothyroidism), Lowe suggested that "euthyroid fibromyalgia is a clinical phenotype of partial peripheral resistance to thyroid hormone" (135, 139) (i.e., the uptake of thyroid hormones by transporters and receptors in cells is disturbed). In a subsequent placebo-controlled study, Teitelbaum et al. showed that euthyroid ME/CFS and fibromyalgia patients treated with T4 (Synthroid) or naturally desiccated thyroid hormone (Armor Thyroid)—in addition to adrenal hormones, vitamins, minerals, and antibiotics—also experienced significant improvements (140).

Moreover, for decades practitioners have been treating euthyroid patients suffering from ME/CFS and fibromyalgia symptoms with thyroid hormones (67, 73–78, 141–146); and several patients have written books to share their recovery stories (127, 147, 148). The treatments vary in the type of thyroid hormones used (e.g., natural desiccated thyroid, T3, or T4), the dosage (e.g., supra-physiological or physiological), the timing (e.g., circadian method, slow-release, or single dose) as well as the prescribed complementary vitamin and mineral supplements (149). Several practitioners emphasize the importance of providing adrenal hormones in tandem with thyroid hormones to enable the body to cope with an increase in metabolic rate (73–76). However, in the absence of a standard protocol, patients are discussing these treatment variations in a plethora of online discussion forums (149).

Several possible mechanisms have been proposed to explain positive outcomes of thyroid hormone supplementation in euthyroid patients (see section: Addressing low thyroid hormone *function*). In addition to their effects on mitochondrial activity, O&NS balance, immune function and neural activity, thyroid hormones stimulate ACTH secretion by the pituitary (127, 141, 150, 151). Supplementation with thyroid hormones might thus be directly relieving the central suppression of endocrine axes in *prolonged* critical illness and ME/CFS (see section: Reactivation of the adreno-cortical axis).

In this context it is necessary to mention that thyroid gland diseases (i.e., hypothyroidism, thyroiditis, and thyroid nodules) are frequently comorbid with ME/CFS (152–154). The standard medical practice in the case of underperforming thyroid glands is to prescribe levothyroxine (T4) with the aim of normalizing TSH levels. However, some researchers (in addition to the practitioners cited above) are increasingly warning that low thyroid hormone *function* in target cells (and associated symptoms) can persist despite the normalization of TSH levels with T4 treatment because of dysfunctions in the tissue-specific conversion and cellular uptake of thyroid hormones (132, 155–163)—particularly in the context of illness (as described above) or genetic polymorphism in thyroid hormone deiodinases and transporters (164–166). Consequently, these authors (and the many thyroid patient advocate groups around the world) promote treatments with T3 or T4/T3 combinations to ensure the adequate availability of the "active" thyroid hormone (T3) for target cells.

In summary, the use of thyroid hormones has been trialed for both *prolonged* critical illness and ME/CFS euthyroid patient groups. Although thyroid hormone supplementation suppresses endogenous thyroid hormone production via negative feed-back loops, their benefits may yet justify their use in the context of low thyroid hormone *function*. Positive results from early trials in ME/CFS and fibromyalgia—as well as anecdotal evidence described by ME/CFS practitioners and patients in numerous books—indicate that treatment for ME/CFS with thyroid hormone supplementation merits further investigation. The form of thyroid hormone supplementation (T3 vs. T4) is increasingly recognized as a determining factor in treatment success.

Treatments With Peripheral Hormone Combinations

In Prolonged Critical Illness

There is evidence that supplementation with a combination of peripheral hormones may lead to better survival and recovery in critical illness—compared to single hormone therapies. For example, the addition of GH and/or IGF-1 has been shown to mitigate the catabolic effects (e.g., protein wasting and osteoporosis) linked to high dose glucocorticoid treatments (167, 168). Moreover, critical care researchers have described the effects of hormones across endocrine axes (2, 169)—such as the stimulatory effect of GH on the T4 to T3 conversion (88) and the hypothalamic suppression of thyroid hormone production by high cortisol levels (11)—increasing the complexity in mitigating the effects of suppressed endocrine axes with peripheral hormones.

In ME/CFS

Similarly, ME/CFS practitioners treating patients with peripheral hormones typically prescribe a combination of hormones, including thyroid hormones, adrenal hormones (e.g., hydrocortisone, prednisone, pregnenolone, DHEA, and fludrocortisone) and even gonadal hormones (e.g., testosterone, progesterone and estradiol) (67, 73–78). Generally, the justification provided for this approach is the complementarity in the function of various hormones. The interactions between endocrine axes—such as the inhibitory effect of glucocorticoid on GH release (101)—described in ME/CFS and related studies also contribute to the rationale for peripheral hormone combination therapies (170–173).

In summary, trials using combinations of peripheral hormones often report beneficial outcomes in *prolonged* critical illness and ME/CFS exceeding those of single hormone therapies. However, the complex interactions between hormones during such trials remain largely unexplored.

Approach B: "Reactivation" of the Central Endocrine Glands

A number of critical illness researchers argue that instead of administering *peripheral* hormones, treatments to relieve suppressed endocrine axes in *prolonged* critical illness should target the central level of the endocrine axes (i.e., the pituitary and hypothalamus) (8, 19). The rationale is the following: (i) endocrine suppression during *prolonged* illness largely originates at the level of the hypothalamus (i.e., the hypothalamus is not sending the required signals to the pituitary); (ii) the pituitary and peripheral endocrine glands are in fact undamaged and could operate normally if given the signals by the hypothalamus; and (iii) by targeting the central level, treatments can avoid altering the rest of the endocrine axes—specifically, the negative feedback loops and adaptive peripheral metabolism of hormones remain intact, thus preventing the risk of toxic over-dosages. Proponents of treatments targeting the hypothalamus and/or pituitary thus argue that these may be more effective and safer than administration of the peripheral hormones. In the next paragraphs we describe trials to reactivate central endocrine glands from the field of critical medicine, and relate these to similar trials to improve outcomes in ME/CFS and fibromyalgia.

Reactivation of the Adreno-Cortical Axis (HPA Axis)

In Prolonged Critical Illness

In order to correct the suppressed HPA axes in *prolonged* critical illness, researchers have suggested stimulating the pituitary with corticotropin-releasing hormone (CRH) (174). CRH is the tropic hormone by which the hypothalamus signals to the pituitary to produce ACTH, which in turn signals to the adrenal glands to produce the various peripheral adrenal hormones (**Figure 1**). Researchers have shown that high levels of *free* cortisol during the *acute* phase of critical illness driven by peripheral mechanisms (specifically, a decrease in the abundance and affinity of the cortisol carrier molecules in circulation, and a slowing of cortisol breakdown in the liver and kidney) lead to a suppression of the release of CRH in the case of *prolonged* critically ill patients even after cortisol levels have returned to normal. Pro-inflammatory cytokines and O&NS likely play a leading role (8, 10, 18, 19, 174). Researchers liken this to the suppression of the HPA axis in patients on long-term glucocorticoid treatment. *Prolonged* critically ill patients (19, 175) and patients on long-term glucocorticoid treatment (176, 177) also both experience adrenal atrophy; it is the lack of pulsatile ACTH stimulation of the adrenal glands that results in their atrophy (178). Consequently, these critical illness researchers argue that, akin to patients that are being withdrawn from long-term glucocorticoid treatment, *prolonged* critically ill patients also require the reactivation of pituitary secretions. In the case of long-term glucocorticoid treatment, slowly weaning patients off glucocorticoids permits the pituitary to secrete ACTH which in turn promotes the regeneration of adrenals; this can take anywhere from 6 to 12 months (176, 177). In the case of *prolonged* critical illness, some researchers propose the administration of CRH may be necessary to reactivate ACTH synthesis by the pituitary (19, 174). Initial trials on *prolonged* critically ill patients show that the pituitary responds as expected to stimulation with CRH (174).

In ME/CFS

Echoing the findings in *prolonged* critical illness, researchers have found evidence suggesting that hypoadrenalism in ME/CFS is caused by a central deficiency of CRH (50, 52, 55, 179–181). Moreover, significant adrenal atrophy has been documented in ME/CFS patients (182) and is also surmised to be present in fibromyalgia patients (98). Researchers have proposed a "bi-stability model" that serves to explain the persistence of a suppressed HPA axis in ME/CFS (183–189)—summarized in our earlier publication (34). These researchers suggest various interventions to move patients from a "low-cortisol" to a "normal-cortisol" HPA axis steady-state. In the words of one team: "a well-directed push given at the right moment may encourage the axis to reset under its own volition" (184). Some suggest artificially dropping cortisol levels even further than they already are in ME/CFS patients could be such a push (184). Models show that, given the HPA axis' negative feedback loop, this is expected to spur an increase in ACTH secretion and, as a result, the HPA axis will naturally progress to the "normal-cortisol" HPA axis steady-state, allowing treatment to be discontinued. Similarly, others have modeled the effect of blocking the glucocorticoid receptors to reset the HPA axis

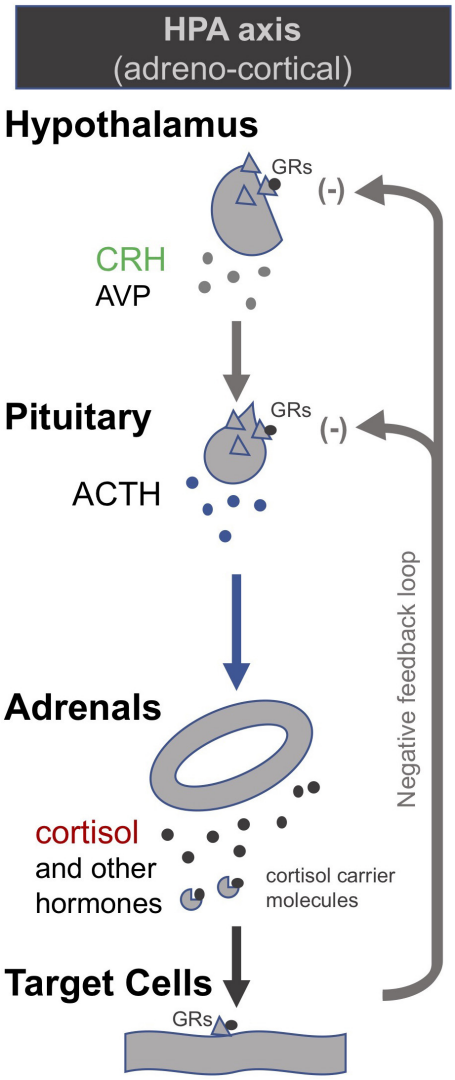

FIGURE 1 | The adreno-cortical axis (HPA axis) [modified from (34)].

in ME/CFS patients (185). They argue that this intervention renders the "low-cortisol" steady-state unstable and thereby favors a return to the "normal-cortisol" steady-state. Finally, other researchers who have included immune system aspects in their model calculated that an initial inhibition of Th1 inflammatory cytokines (Th1Cyt), followed by a subsequent inhibition of glucocorticoid receptor function, would allow a robust return to a "normal-cortisol" steady-state in patients suffering from Gulf War Illness (186). However, given that a chronic suppressed HPA axis leads to adrenal atrophy—the result of prolonged under-stimulation of the adrenal glands by ACTH (178)—a switch to a "normal-cortisol" HPA-axis steady-state is necessarily a gradual process paced by the capacity for adrenal regeneration (190).

In this context it is necessary to mention that some researchers have administered CRH to ME/CFS patients—not in order to assess therapeutic potential, but in order to evaluate HPA-axis dysfunction (51, 52, 56, 62, 191, 192). Several studies found that the response to CRH injection was blunted in ME/CFS patients compared to controls (i.e., ensuing cortisol or ACTH production were lower than in controls) (51, 56, 62), but this was not the case when CRH was combined with desmopressin (a synthetic analog of arginine vasopressin, AVP) which acts synergistically with CRH on the pituitary to stimulate ACTH secretion (52). Similar tests performed with fibromyalgia patients found an exaggerated ACTH response, but blunted cortisol response to CRH injection (98, 101, 170, 193). Finally, ME/CFS patients were found to have "a blunted serum DHEA response curve to i.v. ACTH injection" (54) and lower cortisol production (194). These studies generally discuss possible mechanisms for the blunted HPA axis response, including elevated levels of CRH-binding protein, enhanced sensitivity to the negative feedback of glucocorticoids (i.e., a higher abundance of glucocorticoids receptors at central level), secondary adrenal atrophy, etc. However, the therapeutic potential of CRH (or other pituitary secretagogues) to relieve the suppressed HPA axis in ME/CFS has generally not been considered.

In summary, researchers in both *prolonged* critical illness and ME/CFS have sought the reactivation of the HPA axis. Researchers from the field of critical medicine suggest the use of pituitary secretagogues; ME/CFS researchers suggest that an endogenous "push" could serve to revert the HPA axis to a "normal-cortisol" steady-state. Adrenal atrophy evidenced in both conditions—a result of chronic under-stimulation of adrenals by ACTH—implies that reversing hypoadrenalism in both conditions is a gradual process.

Reactivation of the Somatotropic (HPS) and Thyrotropic (HPT) Axes

In Prolonged Critical Illness

In the late 1990s van den Berghe and her team administered secretagogues that stimulate the pituitary to critically ill patients who had been in ICUs for several weeks, thereby supplementing signals produced by the hypothalamus (195, 196). Specifically, they administered thyrotropin-releasing hormones (TRH) and GH-releasing hormone (GHRH). These two secretagogues, respectively, target the hypothalamic–pituitary–thyroid (HPT) and the hypothalamic–pituitary–somatotropic (HPS) axes. TRH stimulates the pituitary to produce thyroid stimulating hormone (TSH), which in turn signals to the thyroid gland to produce thyroid hormones (197) (**Figure 2**). GHRH stimulates the pituitary to produce GH which in turn stimulates the production of IGF-1 mostly by the liver (in addition to direct effects of GH on some tissues). Nearly all of the IGF-1 hormones in the plasma are bound to IGF-binding proteins (IGFBP) (198) (**Figure 3**). As an alternative to GHRH the researchers also trialed the use of GHRP-2, an artificial ghrelin-like peptide that also stimulates the pituitary to produce GH.

These trials showed that each of these secretagogues can reactivate the secretion by the pituitary for the relevant endocrine axis, while keeping the negative feedback loops on the pituitary intact, thus preventing overstimulation of the endocrine glands. Specifically, the administration of GHRH or GHRP-2 reactivated

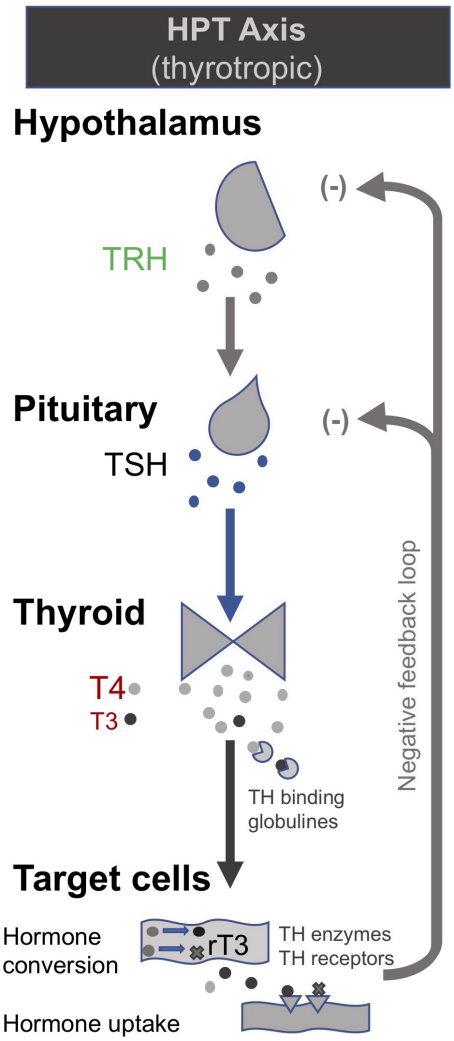

FIGURE 2 | The somatotropic axis (HPS axis) [modified from (34)].

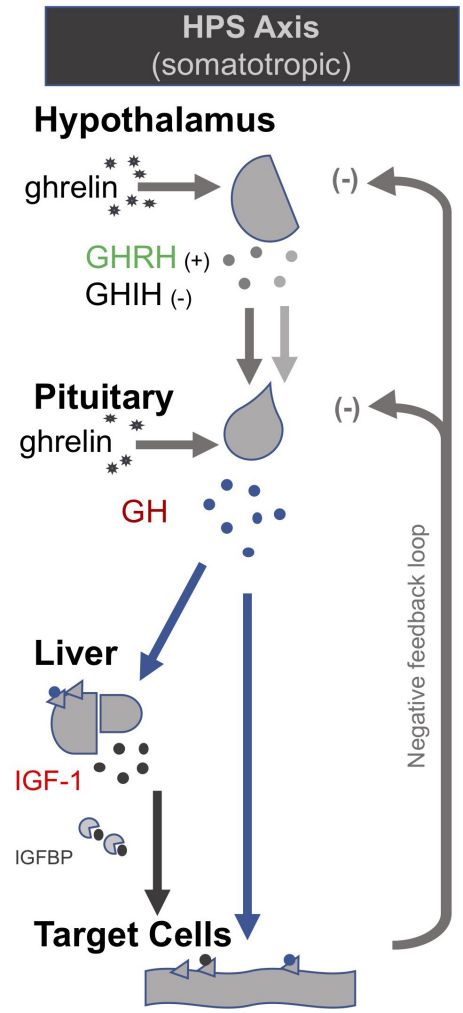

FIGURE 3 | The thyrotropic (HPT axis) [modified from (34)].

the pulsatile secretion of GH by the pituitary, and the plasma concentrations of IGF-1 and IGFBP-3 increased [Interestingly, GHRP-2 had a much stronger effect than GHRH, suggesting that the inactivity of ghrelin likely plays a key role in *prolonged* critical illness (8)]. Similarly, when the team administered TRH, the pulsatile secretion of TSH by the pituitary was reactivated, and the plasma concentrations of the peripheral hormones T4 and T3 increased. However, in the latter case reverse T3 (rT3)—an *inactivated* form of thyroid hormone—also increased; this is considered problematic because rT3 contributes to low thyroid hormone *function* (see section: Addressing low thyroid hormone *function*).

Crucially, the team showed that when *prolonged* critically ill patients were treated with a combination of the secretagogues to normalize GH and TSH secretion by the pituitary (i.e., GHRH or GHRP-2 in combination with TRH), plasma rT3 concentrations did *not* increase. This is likely because GH can deactivate the D3 enzyme which converts T4 into rT3 (88), and suggests that the normalization of the HPS axis is necessary to inhibit the production of rT3. Moreover, the administration of both secretagogues immediately inhibited *catabolism* (i.e., tissue break-down) and promoted *anabolism* (i.e., tissue building), thus halting the muscle and fat wasting of patients with *prolonged* critical illness. This has important both short- and long-term clinical consequences since the impaired neuromuscular function is considered to be the factor which most strongly correlates with the severely impaired quality of life in critical illness survivors several years after hospital discharge (199–202).

The treatments were only administered for 5 days for experimental purposes, and benefits ended a few days after the infusions were discontinued. Nonetheless, they demonstrated the possibility of reactivating suppressed endocrine axes in *prolonged* critical illness with secretagogues targeting the pituitary, as well as positive metabolic outcomes.

In ME/CFS

There are no comparable trials to reactivate the HPS and HPT axes in ME/CFS. One study did administer GHRH to ME/CFS

patients but only for the purpose of testing the function of the HPS axis; they found GH responses to stimulation with GHRH were no different in patients and controls, and also found no GH deficiency in ME/CFS (203). (These findings are consistent with our hypothesis that *pulsatile* pituitary GH secretions are suppressed in ME/CFS). There have, however, been attempts to reactivate pituitary GH secretions in fibromyalgia patients. Recognizing that the secretion of GH by the pituitary is controlled by both *stimulating* and *inhibiting* signals from the hypothalamus [i.e., both GH-releasing hormone (GHRH) and GH-inhibiting hormone (GHIH)], researchers treated fibromyalgia patients with pyridostigmine, a drug that inactivates the inhibiting effect of GHIH. Pyridostigmine reversed the impaired GH response to exercise in fibromyalgia patients, indicating a correction of an otherwise depressed HPS axis (97). However, it did not improve fibromyalgia symptoms (204)—this appears consistent with van den Berghe et al.'s findings described above, whereby the metabolic effects of GH only occur in combination with adequate thyroid hormone *function*. Others have administered GHRH and arginine to fibromyalgia patients—not in order to assess therapeutic potential, but in order to evaluate HPS-axis dysfunction (100).

In summary, researchers have demonstrated that the reactivation of centrally suppressed HPS and HPT axes in *prolonged* critical illness with pituitary secretagogues leads to beneficial metabolic effects. The reactivation of the HPS and HPT axes in ME/CFS for therapeutic purposes remains largely unexplored.

Reactivation of a Combination of Endocrine Axes

In Prolonged Critical Illness

Building on their earlier trials administering GHRH (or the synthetic peptide GHRP-2) and TRH in order to, respectively, stimulate the HPS and HPT axes as described above, van den Berghe et al. later also administered gonadotropin-releasing hormone (GnRH) to *prolonged* critically ill patients (205, 206). GnRH stimulates the pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn stimulate the gonads to produce estrogen, progesterone and testosterone (c.f. Hypothalamic–pituitary–gonadal axis: "HPG axis"; **Figure 4**). They found that the positive metabolic effect was strongest with the combination of secretagogues stimulating all 3 axes (i.e., with GHRP-2, TRH and GnRH). The authors write: "coadministration of GHRP-2, TRH and GnRH reactivated the GH, TSH and LH axes in *prolonged* critically ill men and evoked beneficial metabolic effects which were absent with GHRP-2 infusion alone and only partially present with GHRP-2 + TRH. These data underline the importance of correcting the multiple hormonal deficits in patients with *prolonged* critical illness to counteract the hypercatabolic state" (206) (**Table 1**).

In ME/CFS

We are not aware of any efforts to simultaneously reactivate the pituitary secretions for a combination of endocrine axes in ME/CFS or fibromyalgia in order to improve metabolic outcomes. Researchers have injected fibromyalgia patients simultaneously with CRH, TRH, GHRH as well as GnRH—however, this was not done in order to assess the therapeutic potential of secretagogues but to study the patients' endocrine dysfunctions (170). They found that the injection of the four releasing-hormones led to an "exaggerated" ACTH secretion compared to controls; this was not the case when CRH was administered alone [see above (101)]. They also highlighted the inhibitory effects of CRH on TSH and GH secretion. These results appear consistent with van den Berghe's findings that a combination of secretagogues most effectively improve metabolic outcomes during *prolonged* critical illness.

In summary, researchers have demonstrated that the concurrent reactivation of HPS, HPT and HPG axes with pituitary secretagogues in *prolonged* critical illness leads to greater beneficial metabolic effects than reactivation of just one or two of the axes. The concurrent reactivation of endocrine axes in ME/CFS for therapeutic purposes remains largely unexplored.

Intermediate Conclusions

There are important similarities in the efforts to correct endocrine suppression in *prolonged* critical illness and ME/CFS (**Table 2**). For both conditions, researchers have trialed supplementation with peripheral hormones, including hydrocortisone, GH and IGF-1, and thyroid hormone (including T4 and T3). Evidence exists of some benefits from these treatments for patients suffering from either illness, particularly when given in combination. However, difficulties with finding optimal dosing and risks of causing harm to patients have contributed to controversies and limited their application for both *prolonged* critical illness and ME/CFS. Moreover, administration of these peripheral hormones exasperates central suppression of the respective endocrine axes.

Researchers of *prolonged* critical illness have also trialed to reactivate endocrine axes at the central level. Unlike treatments with peripheral hormones, such an approach has the benefit that it addresses the central suppression of endocrine axes directly, and that peripheral endocrine glands are stimulated—this is particularly important for the adrenal glands which without stimulation by ACTH go into atrophy. Given that negative feedback loops and peripheral hormone metabolism remain intact, proponents argue that the approach is safer and more effective for restoring normal endocrine function. Trials on *prolonged* critical illness patients to reactivate pituitary secretions with secretagogues including CRH, GHRH (or the synthetic GHRP-2), and GnRH have had important initial successes not only in restoring *pulsatile* pituitary secretions of ACTH, GH, and LH, respectively, but also in achieving positive metabolic effects when administered in combination. Moreover, these trials also offer revelations about the interactions between the endocrine axes—including the insight that deleterious production of rT3 can be mitigated through the simultaneous reactivation of the HPT and HPS axes. These lessons-learned from the field of critical illness can complement and inform ME/CFS research. ME/CFS researchers, in turn, have proposed interventions to reactivate the HPA axis based on a "bi-stability model" with two HPA axis steady-states. However, the simultaneous reactivation of the endocrine axes in ME/CFS remains unexplored. The

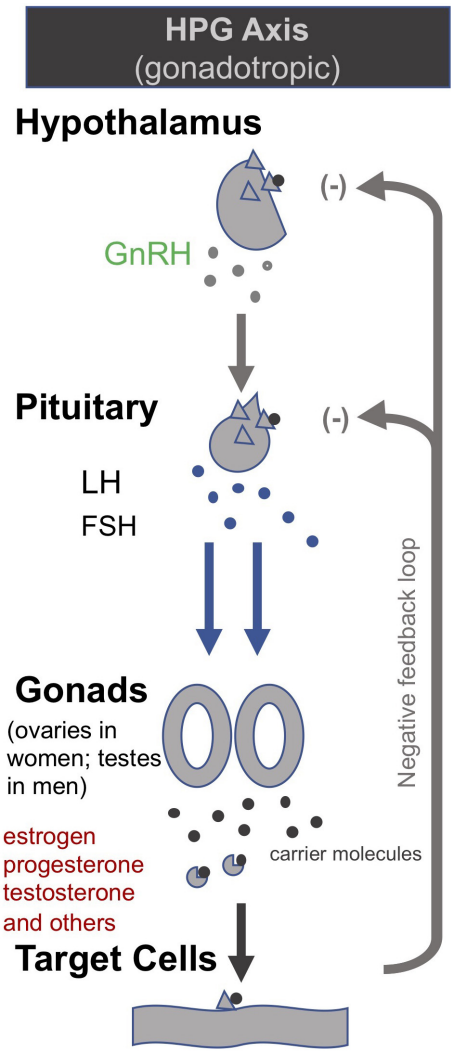

FIGURE 4 | The gonadotropic (HPG axis) [modified from (34)].

potential of treatments with pituitary secretagogue to correct central endocrine axes suppression—and to enable the reversal of secondary adrenal atrophy—should be assessed for ME/CFS.

INTERRUPTION OF THE "VICIOUS CIRCLE"

Critical illness researchers have proposed a model for how *prolonged* critical illness is perpetuated by reciprocal relationships between inflammation (notably pro-inflammatory cytokines), O&NS and reduced thyroid hormone *function* (13, 16). Simplified, this model suggests that (i) cytokines depress thyroid hormone *function*; (ii) low thyroid hormone *function* contributes to O&NS; and (iii) O&NS in turn stimulates the production of pro-inflammatory cytokines, thereby completing a "vicious circle." Moreover, reciprocal relationships between these elements and the suppressed endocrine axes [e.g., pro-inflammatory cytokines suppress ACTH release (9, 10); weakened adrenals permit excessive inflammatory responses] contribute to perpetuate a hypometabolic and inflammatory state (208), and thus help to explain why some critically ill patients fail to recover. Crucially, the same elements of such a "vicious circle" have also been documented in ME/CFS (134, 209–216)—as described in our earlier publication (34).

Treatment trials to improve survival and recovery from *prolonged* critical illness have often targeted one of the elements of this "vicious circle." In the following paragraphs we provide an overview of these various treatment trials using the "vicious circle" as a framework. We also relate analogous experimental treatments for ME/CFS and fibromyalgia in order to highlight the similarities in the quests to cure both *prolonged* critical illness and ME/CFS, and to derive lessons for solving ME/CFS.

Addressing Low Thyroid Hormone *Function*

In Prolonged Critical Illness

As described above (section: Treatments with thyroid hormones), clinicians began, as early as the 1970s, to suggest thyroid hormone supplementation for critically ill patients. The rationale was to correct what some considered a *maladaptive* hypometabolic state which was preventing recovery following *severe* infection or injury (106–108). Again, this approach remains controversial (111–115). Interestingly, there has been little research into pharmacological agents to correct the peripheral mechanisms, which to a large extent underpin the low thyroid hormone *function* during critical illness: i.e., the alterations in cellular thyroid hormone transporters, receptors, and (most crucially) deiodinases that convert thyroid hormones into their "active" and "inactivated" forms. Targeting these deiodinases could theoretically be an avenue for alleviating low thyroid hormone *function* during *prolonged* critical illness (217).

In ME/CFS

As described above, there are accounts of positive effects of thyroid hormone supplementation to address low thyroid hormone *function* in euthyroid ME/CFS and fibromyalgia (67, 73–78, 127, 135–145, 147, 148). Proponents generally believe that thyroid hormone supplementation serves to compensate for dysfunctions in the conversion of thyroid hormones (from "inactive" to "active" forms) and/or uptake at cellular level (139, 218–223), notably associated with inflammation in ME/CFS or fibromyalgia (224, 225).

However, the mechanisms by which positive metabolic effects were achieved with thyroid supplementation in ME/CFS or fibromyalgia are not entirely clear. Rat models show that T3 and T2 thyroid hormone injections can repair mitochondrial DNA (mtDNA) damage resulting from oxidative stress (226). In cells of patients with mtDNA mutations, administration of T3 led to a *reduction* in reactive oxygen species (ROS) production (i.e., oxidative stress) and a *reduction* in cytoplasmic Ca2+ (allowing for cellular signaling/regulation of enzymes and proteins). Moreover, cytochrome c oxidase activity (involved in ATP production) and ATP levels were increased. T3 also restored the mitochondrial membrane potential, complex V activity,

TABLE 1 | Summary of the treatment trials to reactivate the pituitary in *prolonged* critical illness as described by van den Berghe et al. (195, 196, 205, 206).

Target	Secretagogues used to stimulate the pituitary	Results in *prolonged* critical illness
HPT Axis	**TRH** (which stimulates the pituitary to produce TSH, in turn stimulating the thyroid gland)	**Reactivation of the HPT Axis** Normalized TSH secretion by pituitary Normalized T4 and T3 levels *Increased RT3*
HPS Axis	**GHRP-2** (artificial ghrelin mimetic which stimulates the pituitary to produce GH)	**Reactivation of the HPS Axis** Normalized GH secretion by pituitary Normalized IGF-1 and IGFBP-3 levels
	GHRH (which stimulates the pituitary to produce GH)	**Reactivation of the HPS Axis** Lower pituitary reactivation response than with GHRP-2
Combination HPS + HPT Axes	**GHRP-2 + TRH**	**Reactivation of the HPS and HPT Axes** Normalized GH secretion by pituitary Normalized IGF-1 and IGFBP-3 levels Normalized TSH secretion by pituitary Normalized T4 and T3 levels **RT3 levels do not increase!** **-> Inhibit *catabolism* and promote *anabolism***
Combination HPS + HPT + HPG Axes	**GHRP-2 + TRH + GnRH** (GnRH stimulates the pituitary to produce LH and FSH; trialed with men)	**Reactivation of the HPS, HPT and HPG Axes** As above and also normalized LH secretion by the pituitary **-> Strongest beneficial *metabolic* effect**

TABLE 2 | Summary of treatments proposed and trialed to correct for suppressed endocrine axes in critical illness, and ME/CFS and fibromyalgia.

Target	Approach A: Treatments with peripheral hormones	Approach B: "Reactivation" of the central endocrine glands
HPA Axis (adrenals)	***Prolonged* critical illness:** High dose hydrocortisone (5, 48)	***Prolonged* critical illness:** Administration of CRH to stimulate pituitary ACTH secretion (proposed) (174)
	ME/CFS or fibromyalgia: Low dose hydrocortisone [see reviews (67, 68)], fludrocortisone (79–84), DHEA (85), and pregnenolone (proposed) (86).	**ME/CFS or fibromyalgia:** - Suppress cortisol levels to reactivate ACTH secretion (modeled) (184) - Blocking of central glucocorticoids receptors (GRs) (modeled) (185) - Inhibition of Th1 cytokines followed by inhibition of GRs (modeled for Gulf War Illness) (186)
HPS Axis (growth hormone)	***Prolonged* critical illness:** Supplementation with GH and IGF-1 [see reviews (87, 88, 90, 91)]	***Prolonged* critical illness:** Administration of GHRH and GHRP-2 to reactivate pituitary secretion of GH (195, 196)
	ME/CFS or fibromyalgia: Supplementation with GH and IGF-1 (94, 102–105)	**ME/CFS or fibromyalgia:** Drug to inactivate GH inhibiting hormone (GHIH) (97, 204)
HPT Axis (thyroid)	***Prolonged* critical illness:** Supplementation w/thyroid hormones [see reviews (116, 117)]	***Prolonged* critical illness:** Administration of TRH to reactivate pituitary secretion of TSH (195)
	ME/CFS or fibromyalgia: Supplementation w/thyroid hormones (natural desiccated thyroid, T4, T3) (135–140) Anecdotal: (67, 73–78, 127, 141–145, 147, 148)	**ME/CFS or fibromyalgia:** *none?*
HPG Axis (gonads)	**Prolonged critical illness:** anabolic steroid (e.g., testosterone) (207)	***Prolonged* critical illness:** Administration of GnRH to stimulate pituitary release of LH (in men) (206)
	ME/CFS or fibromyalgia: as part of combined treatments (see below)	**ME/CFS or fibromyalgia:** *none?*
Combination of axes	***Prolonged* critical illness:** GH and IGF-1 in addition to hydrocortisone (167, 168)	***Prolonged* critical illness:** TRH + GHRP-2 + GnRH (see **Table 1**. above) (206)
	ME/CFS or fibromyalgia: Thyroid hormone + adrenal hormones (+ gonadal hormones) (67, 73–78)	**ME/CFS or fibromyalgia:** *none?*

and levels of manganese superoxide dismutase (an essential mitochondrial antioxidant enzyme). The authors conclude that "the results suggest that T3 acts to reduce cellular oxidative stress, which may help attenuate ROS-mediated damage, along with improving mitochondrial function and energy status in cells with mtDNA defects" (227). In theory, T3 supplementation could have similar impacts on relieving O&NS and improving mitochondrial function in *prolonged* critical illness and ME/CFS.

Moreover, T3 (but not T4) administration also stimulated Na-K-ATPase activity in rat models through non-genomic

pathways (the activity of this enzyme is critical for maintaining cellular ion gradients) (228). Others have found that T3 and T4 supplementation selectively affect GABA-evoked neurotransmission in rat models thus possibly producing profound alterations in brain activity (229, 230). In addition, it has been shown that thyroid hormones also have a neuroprotective effect (231) and regulate neurotransmission (232, 233). Moreover, clinical manipulation of thyroid hormone levels also modulates immune functions (234–237). Yet others have shown that T3 treatment of human cells caused decreased viral replication (238). Finally, as mentioned above, thyroid hormones also have a stimulatory effect on the HPA axis.

In summary, there is a history of clinicians in both the fields of critical illness and ME/CFS advocating for the use of thyroid hormones supplementation in euthyroid patients. Thyroid hormones affect mitochondrial activity, O&NS balance, immune function, neuroactivity, and stimulate ACTH secretion. The mechanisms by which supplementation with thyroid hormone can promote recovery from *prolonged* critical illness and ME/CFS require further investigations. Again, the form of thyroid hormone (T4, T3, or T2) appears to be a determining factor in physiological effects of treatments. The use of pharmacological agents to correct dysfunctions in the peripheral pathways of thyroid hormones remains unexplored in both illnesses.

Addressing Oxidative and Nitrosative Stress

In Prolonged Critical Illness

There have been a few trials to restore oxidative balance during critical illness, often with the aim of improving thyroid hormone *function* (i.e., relieving NTIS). In one case, researchers found that treating patients of acute myocardial infarction with n-acetyl-cysteine (NAC)—a precursor to the antioxidant glutathione (GSH)—could virtually eliminate the decrease in serum T3 levels and prevent the increase in serum rT3 which are characteristic of NTIS (239). They propose that supplementation with NAC relieves the competition for GSH between the thyroid hormone deiodinase and antioxidant enzymes, which would otherwise negatively affect thyroid hormone conversion and enable O&NS. Likewise, controlled experiments showed that administration of sodium selenite on human cells reduces cytokine-induced oxidative stress (240), and supplementation with selenium is associated with modest normalization of thyroid hormones during critical illness (241). Thus, *in-vivo* supplementation might similarly relieve competition for selenium required in the production of both thyroid hormone deiodinase and antioxidant enzymes.

Furthermore, cytokine-activated oxidative stress induced post-translational modifications and loss of the molecular motor protein myosin are important pathophysiological mechanisms underlying the severe muscle dysfunction and muscle wasting associated with the critical illness myopathy (CIM) and the ventilator induced diaphragm dysfunction (VIDD) observed in both experimental and clinical ICU studies in response to long-term mechanical ventilation and immobilization (242–246). Administration of the chaperone co-inducer BGP-15 upregulates Heat Shock Proteins (HSPs) and mitigates the muscle dysfunction associated with CIM and VIDD (247–249). This is consistent with HSP protection of muscle cells against the damaging effects of reactive oxygen species during exercise (250). Besides upregulating HSPs, BGP-15 also acts as a membrane stabilizer, protects mitochondria, and has anti-inflammatory effects (251). The anti-inflammatory effects are of specific interest since activation of the JAK/STAT signaling pathway has been reported in respiratory and limb muscles in both experimental and clinical ICU studies (252, 253). The JAK/STAT pathway is a signaling pathway for a wide range of cytokines and growth factors and its activation is a common feature of muscle wasting induced by the cytokine IL-6 (254). Previous anti-inflammatory interventions with BGP-15, the JAK1/2/STAT3 inhibitor Ruxolitinib, and the prednisolone analog Vamrolone have all shown positive effects on limb and respiratory structure/function (252, 253, 255).

In ME/CFS

Addressing antioxidant status is a common approach of some ME/CFS practitioners (73, 74, 211), notably with the aim of preventing mitochondrial damage (256). Pall—who described a "vicious cycle" between inflammation and oxidative stress in ME/CFS more than a decade ago (211, 257)—developed a treatment protocol based on a variety of antioxidants and anti-inflammatory agents. Moreover, placebo-controlled studies have shown that CoQ10—an important antioxidant in mitochondria—is beneficial to ME/CFS patients when provided in combination with the coenzyme NADH (258, 259). Following early positive results, a study is currently ongoing to determine the efficacy of NAC in neuroprotection against oxidative stress in ME/CFS symptoms (260). Furthermore, a placebo-controlled trial of the herbal medicine myelophil—with antioxidant and immunomodulatory properties—has had promising results in alleviating ME/CFS symptoms; the benefits may also derive from its modulatory effects on the HPA axis (68, 261).

Finally, akin to critical illness, it has been suggested (but not yet trialed) to incorporate the upregulation of HSP into future treatments for ME/CFS (262). Studies have shown that ME/CFS is also characterized by impaired HSP production (263) which—combined with O&NS and low-grade inflammation—could explain muscle dysfunction and exercise intolerance (264, 265).

In summary, there have been a few trials to mitigate O&NS in both *prolonged* critical illness and ME/CFS patients. This includes the use of various antioxidants and mitochondrial supports to rebalance oxidative stress, and the use of HSP to lessen the negative effects of O&NS. There is evidence of some beneficial results, but effects may be insufficient to interrupt detrimental and possibly self-perpetuating mechanisms.

Addressing the Production of Pro-inflammatory Cytokines and Inflammation

In Prolonged Critical Illness

As described above, clinical practitioners regularly seek to manage inflammation during critical illness—particularly in the

event of sepsis (see section: Treatments with glucocorticoids). Considering the relationship between pro-inflammatory cytokines and thyroid hormone *function* (9–15, 266), some researchers have tried unsuccessfully to cure NTIS (i.e., restore normal thyroid hormone *function*) by blocking IL-1 cytokine receptors (267). The unsuccessful result is perhaps not surprising, given that "cytokines are related to each other in a very complex network, and regulate positively or negatively the expression of other cytokines; it is, therefore, difficult to imagine how to interrupt this interplay and cascade of events" (268). Critical care researchers also debate using antivirals (269, 270) to treat viral reactivation observed in ICU patients (271–273).

Related to inflammation, a group of researchers has suggested inhibiting the kynurenine pathways during critical illness (274). In conditions of inflammation the indoleamine 2,3-dioxygenase (IDO) (which metabolizes tryptophan into kynurenine) is upregulated, and the kynurenine pathway preferentially produces neurotoxic metabolites (such as quinolinic acid) (274, 275). Increased kynurenine plasma levels thus precede the development and persistence of sepsis in critically ill patients (276, 277), and is associated with lower survival in ICU patients (274). Moreover, elevated kynurenic acid (also a metabolite of kynurenine) is associated with myelin damage leading to neuronal and cognitive dysfunction in critical illness (278). Based on the therapeutic literature from other diseases (275, 279–282), the critical illness researchers suggest inhibiting the IDO enzyme in order to curtail the production of neurotoxic kynurenine pathways metabolites.

In ME/CFS

Echoing the approaches in critical care, practitioners and researchers have also long sought to manage inflammation in ME/CFS (29, 73, 211). Trials include the use of Nexavir (Kutapressin) to reduce inflammation (283) as well as the administration of Low Dose Naltrexone (LDN) (284, 285) and IL-1 receptor antagonist (anakinra) (286) to reduce pro-inflammatory cytokines. There is evidence of some positive benefits from anti-inflammatory treatments for ME/CFS patients, notably with LDN.

Researchers have also trialed treatments which could have an indirect effect on inflammation. These include the use of cyclophosphamide (287) and monoclonal antibodies (rituximab) (288) which suppress the immune system, as well as immune adsorption (IgG depletion) and plasmapheresis (filtration of blood plasma) to reduce antibodies (289). Others have conversely tried to modulate the immune system, including through the use of toll-like receptor 3 (TLR3) agonists (rintatolimod/Ampligen) (290, 291), immune-stimulants such as Imunovir (292), and intravenous gamma globulin (293). There have also been trials targeting infections directly, including with antivirals (acyclovir and valganciclovir) (294–297) for chronic viral infections in ME/CFS patients (298–300). There is evidence of some positive benefits, at least for a subset of patients, from some of these studies, but results remain largely inconclusive or subject to controversies; readers are referred to reviews for details (29–32).

Finally, the modulation of kynurenine pathways has also been suggested as a therapeutic avenue for ME/CFS (301, 302). However, in contrast to the approach suggested by critical illness researchers, the initial emphasis of ME/CFS research is on enabling rather than inhibiting the activity of the IDO enzyme. Given that the downstream kynurenine pathways produce both beneficial and neurotoxic kynurenine metabolites (275)—and neurotoxic metabolites are preferentially produced in conditions of inflammation (274)—pharmacological agents that target specific enzymes of the kynurenine pathways may be required in order to maintain a beneficial balance of the various metabolites (303). A recent study demonstrated the safety of L-kynurenine supplementation in healthy volunteers (304); the impacts on ill patients will need to be further investigated.

In summary, many efforts in both *prolonged* critical illness and ME/CFS have focused on mitigating inflammatory processes and/or modulating the immune system in patients. There is evidence of some beneficial results from some of these studies, but effects appear insufficient to interrupt detrimental and possibly self-perpetuating mechanisms. Antivirals have also been trialed for both *prolonged* critical illness and ME/CFS (viral reactivation has been documented in both illnesses). In both fields there has also recently been discussion of modulating the kynurenine pathways to rebalance beneficial and neurotoxic metabolites.

Intermediate Conclusions

Treatment trials for *prolonged* critical illness and ME/CFS (or fibromyalgia) have both independently targeted low thyroid hormone *function*, O&NS, and pro-inflammatory cytokines and inflammation (**Figure 5**). Evidence exists for some benefits from these treatments in both conditions, but treatment trials have generally been limited in their scope, number and impact. Consequently, results have not translated into standard practices in either field. Further studies are required to fill gaps in the understanding of the physiological mechanisms behind some positive results, such as in the case of supplementation with thyroid hormones in both *prolonged* critical illness and ME/CFS.

ADDITIONAL CONSIDERATIONS

As mentioned in the introduction, we previously advanced the hypothesis that maladaptive mechanisms that prevent recovery in *prolonged* critical illness may also underlie ME/CFS—and propose that these mechanisms could underlie the perpetuation of illness in ME/CFS regardless of the nature of the peri-onset event (i.e., infection, stressful incident, exposure to environmental toxins or other) (34). Nonetheless, additional considerations must be taken into account when considering the relevance of treatment trials from critical care medicine for ME/CFS.

Firstly, the long disease duration in ME/CFS (relative to *prolonged* ICU patients) implies that dysfunctions that occur as a result of years of chronic disease must be considered as

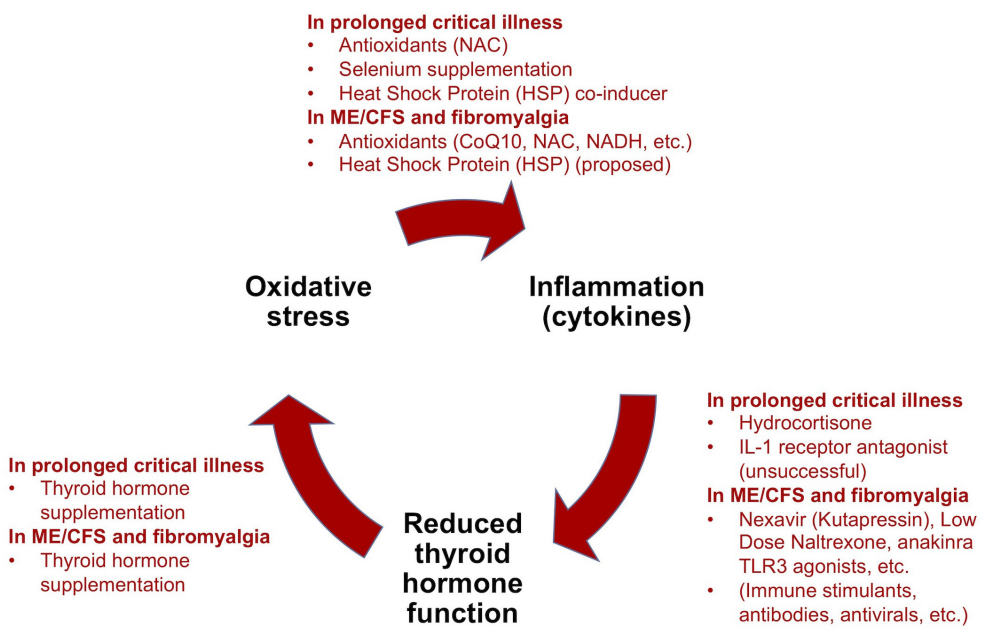

FIGURE 5 | Summary of treatment trials for *prolonged* critical illness using the "vicious circle" model as a framework, and analogous experimental treatments for ME/CFS and fibromyalgia [modified from (34)].

part of treatment approaches. Secondly, research suggests that dysfunctions change over time in ME/CFS patients (28) and that there may be ME/CFS disease sub-groups (305–307); this implies that disease subtyping is necessary in order to match treatments to the patients. Thirdly, given that ME/CFS patients—similar to *prolonged* critical illness patients—have multi-system dysfunctions (e.g., endocrine, immune, nervous system, etc.), the question of sequence and/or combination of treatments must be considered. Fourthly, the side-effects of treatments described in this paper may differ between *prolonged* critical illness and ME/CFS patients (and also across ME/CFS patients) not least because of differences in severity of illness and dysfunctions. Recognizing the differences in fragility and vulnerability to side-effects of patients, an assessment of the trade-offs of treatments is necessary. Finally, it is reasonable to hypothesize that patients would have to endure long treatment courses occurring sizable costs; yet the enormous total economic costs of ME/CFS [estimated at $36 to $51 billion annually in the USA (308)]—not to mention the high financial and emotional toll of the disease on the millions of patients and families worldwide—makes establishing and implementing effective treatments for ME/CFS a long overdue imperative.

In summary, ME/CFS and *prolonged* critical illness are not identical illnesses. Any discussion of the relevance of treatment trials from *prolonged* critical illness for ME/CFS should take into account the specificities of ME/CFS, notably the dysfunctions arising from years of illness, the progression of the disease over time, the possible existence of sub-groups of ME/CFS patients, and potential particular vulnerabilities to side-effects.

CONCLUSION

There are significant parallels in the treatment trials to aid recovery in *prolonged* critical illness and ME/CFS. Treatments proposed or trialed for both of these conditions have targeted (a) the correction of suppressed endocrine axes, and/or (b) inflammation, O&NS, and/or low thyroid hormone *function*. Treatment trials to date have been limited in scope and number; both *prolonged* critical illness and ME/CFS remain unsolved conditions. Incidentally, the parallels in the treatment trials would support the hypothesis that maladaptive mechanisms that prevent recovery in *prolonged* critical illness could also underlie ME/CFS.

From the brief overview and comparison of these trials provided here, we can derive some preliminary lessons to be learned. Notably, the early successes to reactivate the *pulsatile* secretions of the pituitary in *prolonged* critically ill patients with pituitary secretagogues—and the resulting positive metabolic effects—would indicate that this also could be an important avenue for ME/CFS treatments. The simultaneous reactivation of suppressed endocrine axes so far remains unexplored in ME/CFS. Conversely, the findings from ME/CFS related to the dysfunctions at the cellular and mitochondrial level can likely provide important complementary insights to the understanding of critical illness. In addition, the positive impacts from thyroid hormone supplementation described in some of the trials for both conditions merit further investigation.

Chiefly, given the similarities described above, an exhaustive analysis of the treatments already tried for either *prolonged* critical illness or for ME/CFS could help identify potential

approaches that could be immediately trialed for one or the other of these conditions. Moreover, active collaboration between critical illness and ME/CFS researchers to leverage their respective experiences could lead to improved outcomes for both conditions. More broadly—and given the similarities between *prolonged* critical illness, post-ICU syndrome, ME/CFS, fibromyalgia, and long-COVID—we suggest that collaborative efforts should be sought among the researcher community across these conditions in order to identify treatments mitigating the functional disability that they induce.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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The Enterovirus Theory of Disease Etiology in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: A Critical Review

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Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a complex, multi-system disease whose etiological basis has not been established. Enteroviruses (EVs) as a cause of ME/CFS have sometimes been proposed, as they are known agents of acute respiratory and gastrointestinal infections that may persist in secondary infection sites, including the central nervous system, muscle, and heart. To date, the body of research that has investigated enterovirus infections in relation to ME/CFS supports an increased prevalence of chronic or persistent enteroviral infections in ME/CFS patient cohorts than in healthy individuals. Nevertheless, inconsistent results have fueled a decline in related studies over the past two decades. This review covers the aspects of ME/CFS pathophysiology that are consistent with a chronic enterovirus infection and critically reviews methodologies and approaches used in past EV-related ME/CFS studies. We describe the prior sample types that were interrogated, the methods used and the limitations to the approaches that were chosen. We conclude that there is considerable evidence that prior outbreaks of ME/CFS were caused by one or more enterovirus groups. Furthermore, we find that the methods used in prior studies were inadequate to rule out the presence of chronic enteroviral infections in individuals with ME/CFS. Given the possibility that such infections could be contributing to morbidity and preventing recovery, further studies of appropriate biological samples with the latest molecular methods are urgently needed.

Keywords: myalgic encephalomyelitis, chronic fatigue syndrome, enterovirus, chronic infection, RT-PCR, serology, immunohistochemistry, cell culture

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INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex multi-system disease of unknown cause for which there is little insight into the molecular basis of disease progression, persistence and in rare cases - remission. The ME/CFS literature includes findings of patient immune system irregularities, abnormal cellular energy metabolism, and various altered autonomic nervous system manifestations including post-orthostatic tachycardia syndrome, orthostatic intolerance, and dysregulated hypothalamus pituitary adrenal axis. A hallmark symptom, required for many case definitions, is exercise intolerance or post-exertional malaise (PEM) (1, 2). The name of the illness itself is controversial, with one view holding that Myalgic Encephalomyelitis, a name

dating from a series of early outbreaks of the disease (3), defines an illness that is different than Chronic Fatigue Syndrome, a name created in 1988 through a U.S. government committee (4). A discussion of the case definition and nomenclature is outside of the scope of this article, so we will use "ME/CFS" despite of the possibility that the initial CFS case definition results in inclusion of individuals who would not have met earlier criteria for Myalgic Encephalomyelitis.

ME/CFS case documentation shows evidence of both sporadic events involving singular individuals and regional outbreaks involving significant fractions of affected communities, especially hospitals, schools, and military bases. Machine learning estimation of ME/CFS prevalence using large-scale medical claims data gives a frequency of diagnosis in the United States that falls somewhere between 1.7 and 3.38 million Americans (5) and world-wide, the prevalence may be as high as 65 million (6). ME/CFS is not a rare disease and therefore understanding of disease pathophysiology and discovery of standardized biological markers or tests are important to identify appropriate treatments.

The pattern of transmissibility, and acute symptom constellation reminiscent of a flu-like illness, led early investigators to hypothesize a viral theory of ME/CFS disease etiology. Indeed, a number of researchers have interrogated a diverse range of microbial pathogens as triggers and/or perpetuators of the ME/CFS disease state. These include but are not limited to Epstein-Barr virus, cytomegalovirus, parvovirus B19, Brucella, Toxoplasma, *Coxiella burnetti*, *Chlamydia pneumoniae*, human herpesviruses, enteroviruses, human T cell leukemia virus II-like virus, spumavirus, hepatitis C virus, and human lentiviruses (7–9).

Between the 1930s and 1960s, a number of globally occurring ME/CFS outbreaks, with a spatiotemporal incidence coinciding with poliovirus epidemics, appeared under the titles of "abortive or atypical poliomyelitis" transitioning to "benign myalgic encephalomyelitis" or "epidemic neuromyasthenia" as physicians sought a term to describe the symptom profile of affected individuals (3, 10, 11). A ME/CFS outbreak occurred in 1934 California and provides a representative example of clinical features experienced by patients during similar epidemics of the time. Briefly, the 1934 outbreak occurred among roughly 200 hospital employees, primarily female, who fell ill with what acutely appeared to be poliomyelitis. Epidemiological deviations from what is commonly expected in poliomyelitis epidemics included relatively high attack rates, low mortality rates, low paralytic rates and a high incidence in adults as opposed to young children. Symptoms of sufferers included significant diurnal temperature fluctuations, localized muscular weakness as well as pain and muscle tenderness. Patients further exhibited numbness, paresthesia, exercise intolerance, and recurrent systemic and neurological symptoms. Longitudinal tracking of a subset of these patients showed residual muscle alterations, fatigue, and mental changes. Electromyograms showed generalized, mild, motor neuron changes and observations indicated that recurrences could occur even after many years of relatively normal health (10, 12). The totality of these findings indicated an infectious agent although tests available at the time could not convincingly implicate a specific culprit.

Subsequent outbreaks displayed the same basic features of the 1934 outbreak with some distinct clinical presentations depending on the region (3, 11, 13). Overall, most epidemic outbreaks have occurred in mid-spring through early fall indicating a virus with seasonal epidemic trends may be involved. Seasonality is not rare for viruses; many types, including but not limited to echovirus, coxsackievirus and poliovirus-related species, are well-known to have strong outbreak seasonality peaking in the month of August or early fall (10, 14). Outbreaks occurring after 1934 that deserve notable mention based on similar clinical presentations and links to an enteroviral culprit are highlighted below:

- 1949–1953 Adelaide, Australia: Dr. R. A. Pellew conducted several animal studies using patient throat washings, feces and cerebrospinal fluid collected from the 1949–1953 Adelaide outbreak as inoculants into rhesus monkeys, rabbits, mice, and hen eggs. Investigation into two monkeys repeatedly inoculated with patient sample revealed minute red spots along the course of the sciatic nerve, infiltration of lymphocytes and mononuclear cells into nerve roots and nerve fibers showing patchy damage to the myelin sheath with axon swelling. Although similar to poliovirus inoculation outcomes, these monkeys displayed more widespread changes in additional areas of the nervous system with no evidence of damage to nerve cells in the brain and spinal cord. Additionally, severe myocarditis was found in one of the two monkeys studied – myocarditis being most commonly caused by enteroviruses (10, 15).
- 1948 Akureyri, Iceland: Incidence of over 1,000 cases during a 3 month period resulted in the naming of "Icelandic disease," which would later evolve to "benign myalgic encephalomyelitis" (16). Those who fell ill with the disease showed classical viral-type illness onset which later developed into a systemic form of the illness with symptoms including low fever and significant muscle tenderness/weakness. Due to the occurrence of concurrent local poliomyelitis epidemics, infectious disease testing was conducted but failed to indicate poliovirus, coxsackievirus or other known encephalitis viruses (10).
- 1956 Thorshofn/Egilsstadir, Iceland: Differential poliovirus vaccination responses between children exposed verses unexposed to the "Icelandic disease" indicated the etiological agent in ME/CFS may be a virus immunologically related to poliovirus. Children in a northeastern village of Iceland, Thorshofn, generated a slight rise in antibody production following vaccine administration whereas children from Egilsstadir, roughly 200 km south, had a much stronger immune response to polio vaccine administration. The difference between the two locations was that children from Eglisstadir were from an area which recently experienced a myalgic encephalomyelitis outbreak whereas children from Thorshofn were not (17). This indirect evidence of unknown prior immunity was also noted in the aforementioned Adelaide outbreak. This was evidenced by a 43% reduction

in polio cases in the south of Australia, where Adelaide is located, compared to regions such as New South Wales and Queensland that reported increased polio cases (18). Enteroviral cross-immunity is well-documented in the enterovirus field and suggests that children in ME/CFS affected areas had been exposed to an agent immunologically similar to poliovirus (19).

Similar epidemic events of ME/CFS have occurred globally over time where patients display acute symptoms are similar to some poliomyelitis-afflicted patients. The later phases of disease progression make evident several differences between ME patients and those with poliomyelitis. The occurrence of considerable symptom constellation overlaps between ME/CFS, poliomyelitis and other non-polio enterovirus-related clinical outcomes as well as similarity in epidemic seasonality is further circumstantial evidence for a relationship between ME/CFS and enteroviruses. One possibility for the co-occurrence of polio and non-polio enteroviral outbreaks may be the environmental source of enteroviruses, which often are contaminated bodies of water. If sewage is contaminating water, consumers may be exposed to multiple types of enteroviruses.

To date, the body of research investigating enterovirus infections in relation to ME/CFS supports an increased prevalence of chronic or persistent infections in several ME/CFS patient cohorts. The majority of early EV-related investigations occurred within the UK from the 1970s to early 2000s, starting with serological tests but advancing to molecular methods including immunohistochemical detection of enterovirus viral capsid protein (VP1) and viral genome detection using RT-PCR (3, 13). Although a significant number of early papers provided evidence for an association of chronic enteroviral infections with ME/CFS, research into the enteroviral theory of disease etiology largely died out in the early 2000s with a few exceptions (7, 20). One reason that enteroviral research in ME/CFS has languished is the difficulty of detecting virus after time has passed following an acute infection. Furthermore, because enteroviral infections are frequent and common, a large fraction of the population will have serological evidence of exposures. Another issue is that reports of association of other pathogens and environmental stresses led to the concept that many different types of insults could result in ME/CFS. We are offering a critical evaluation of current literature that may lead to further inquiry into the role of EVs in ME/CFS.

In this review, we will first cover what is known about enteroviruses in relation to tissue tropism and ability to persist in a chronic infectious state. Emphasis will be put on the aspects of ME/CFS patient pathophysiology that are consistent with an active, chronic enterovirus infection. We will provide a critical review of studies that were attempting to identify chronic EV infections. The studies will be categorized based on the research methodology employed and special emphasis will be put on the sample types used and limitations to the chosen methods. We hope this review may help guide future viral-related studies by highlighting the tissue types and approaches most likely to provide insight into the hypothesis that enterovirus infections are initiating and/or perpetuating the disease state in ME/CFS.

BACKGROUND REGARDING ENTEROVIRUSES

Enterovirus Classification and Basic Molecular Biology

Although poliovirus is the most well-known enterovirus, it belongs to only one of 15 total enterovirus species including enterovirus species A-L and rhinovirus species A-C. Of the true enteroviruses, species A-D are known to have caused a wide spectrum of severe and deadly epidemics in humans (21, 22).

The enterovirus genome consists of a single stranded positive sense RNA molecule roughly 7.5 kb in length (see **Figure 1**). Upon translation *via* host cell machinery, one full length polypeptide is produced and then proteolytically cleaved into the polyprotein products PI, P2, and P3. P1 encodes four structural proteins, VP1-VP4, forming the non-enveloped virion capsid. P2 and P3 are proteolytically cleaved into 10 non-structural proteins including 2A to 2C, 3A to 3D as well as precursors 2BC, 3AB, and 3CD. Viral genomic RNA is capped on its 5′ end with the viral-encoded protein VPg (3B) instead of a methylated nucleotide cap structure.

Enteroviruses gain cellular entry through binding to host cell receptors and undergoing receptor-mediated endocytosis. Cellular receptors vary between EVs and include CD155/poliovirus receptor, integrins αvβ6 and αvβ3, ICAM-1, ICAM-5, CD55/decay accelerating factor, KREMEN1, coxsackievirus and adenovirus receptor (CAR), scavenger receptor B2, P-selectin glycoprotein ligand-1, sialylated glycan, heparan sulfate, neonatal Fc receptor, and annexin II (24–28).

Upon cellular entry, translation occurs following ribosome binding onto a type I internal ribosome entry site (IRES) located within the 5′UTR of the viral genome. Replication occurs *via* the viral encoded RNA-dependent RNA polymerase (3Dpol) which forms the negative sense RNA complement that is used to create additional positive sense RNA genomes (29). During active infection the ratio of positive to negative strands is roughly 100:1, whereas chronic infections display a ratio closer to 1:1 (7).

5′ and 3′ UTR secondary structures recruit both viral and host cell proteins to aid in viral translation and replication (30). The 5′UTR of EVs contains a cloverleaf secondary structure, termed Domain 1, as well as an internal ribosome entry site (IRES) containing six major stem-loop structures (see **Figure 1**). The 5′UTR is required for initiating both negative and positive strand RNA synthesis. The 3′UTR also contains important secondary structures, two predominant hairpin loops, that are the essential structure of the origin or replication for negative strand synthesis. Proteins bound to the 5′UTR interact with others bound to the genome's polyadenylation sequence at the 3′ end, thereby promoting viral genome circularization. Circularization allows the 3′UTR secondary structures to act as the initiation site for 3Dpol binding and at the origin of replication (31).

The viral encoded RNA polymerase is error-prone due to lack of a proof-reading mechanism, resulting in high mutation rates throughout enteroviral evolution. Furthermore, intra- and inter-typic genetic recombination may occur between enteroviruses, leading to increased genotypic plasticity. Enterovirus genomes

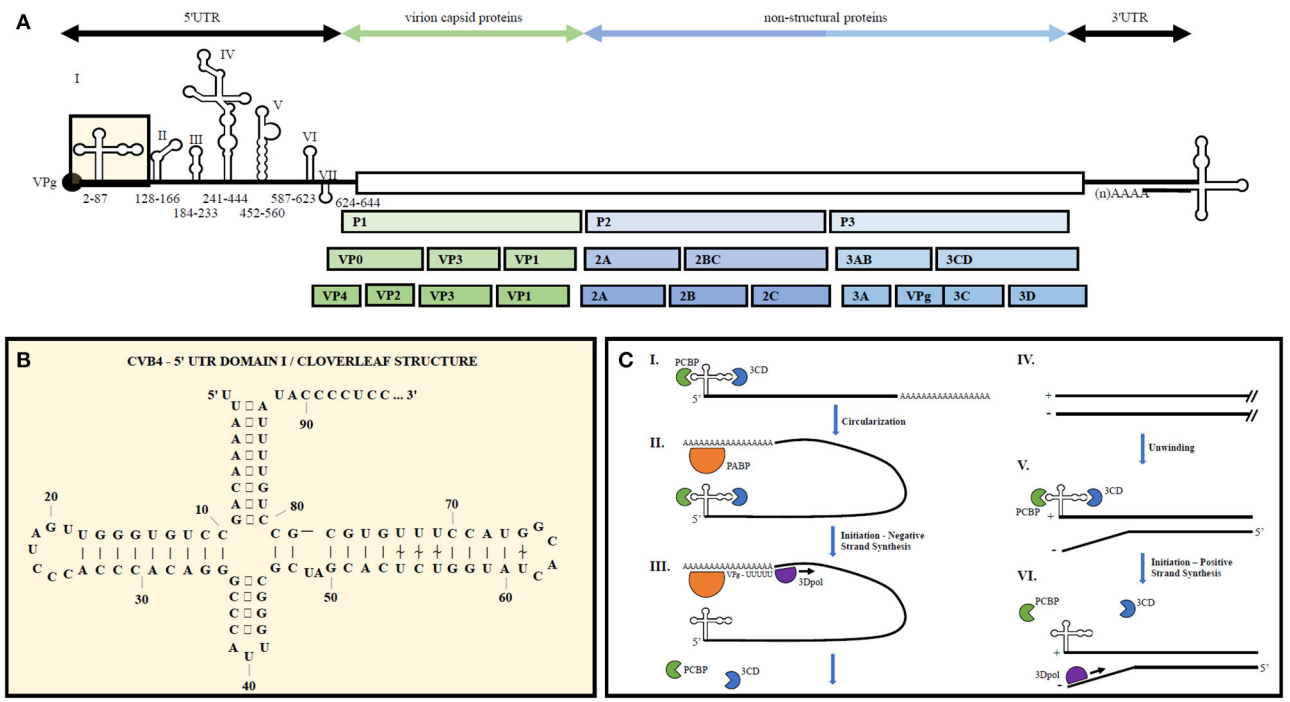

FIGURE 1 | Representative enterovirus genome structure with emphasis on 5′UTR Domain I and genome replication. **(A)** Graphical depiction of EV genome as well as proteolytic processing to produce all structural and non-structural proteins. Number ranges indicate nucleotide positions for domains 1–7 in the 5′UTR of CVB4. **(B)** 2D illustration of CVB4 Domain I secondary structure. Numbers indicate nucleotide positions. **(C)** From (23) by license: Creative Commons Attribution 4.0 International. An integrated model for enterovirus replication. Negative-strand synthesis is initiated by circularization of the positive-strand genome *via* a protein-protein bridge through the interaction of the ternary complex at the 5′-end (3CD and PCBP bound to the cloverleaf structure) and PABP bound to the 3′-poly(A)tail (I. + II.). CRE-mediated VPg-pUpU acts as primer of the reaction and the polymerase 3D synthesizes the new negative-strand (III.), resulting in a double-stranded intermediate (RF) (IV.). The positive-negative duplex RNA intermediate unwinds, so that the cloverleaf structure at the 5′-end of the positive-strand can form. 3CD and PCBP bind to the cloverleaf to form a ternary complex, which, in turn, will initiate positive-strand synthesis on the 3′-end of the negative-strand (V.). The primer, VPg-pUpU, is recruited and binds to the 3′-terminal AA of the negative strand, and the new positive-strand is synthesized by the polymerase, 3D (VI.).

frequently exhibit mosaic genomic sequences leading to a wide variety of genotypic and phenotypic diversity across enterovirus serotypes (32, 33).

Enterovirus Carrier-State vs. Steady-State Persistent Infections

Persistent enteroviral infections are generally agreed to occur in two forms, termed carrier-state and steady-state persistence. In carrier-state infections, high levels of infectious virus are produced with infection limited to only a small proportion of cells. Alternatively, steady-state infections show all cells are simultaneously infected but viral replication is slowed, leading to a non-lytic phenotype with low viral copy numbers per cell. Both types of persistent viral infections are known to occur across human enteroviral species and have been linked to multiple clinical conditions (34–42).

Research on CVB4 infections of pancreatic ductal-like cells (PANC-1) and murine cardiac myocytes (HL-1) shows productive viral replication (10^6-10^8 PFU/ml) is restricted to a limited subpopulation of cells in culture and are therefore examples of carrier-state infections *in vitro*. PANC-1 cells exhibiting resistance to lysis *via* subsequent CVB4 superinfection were determined to be those PANC-1 cells with downregulated coxsackie adenovirus receptor (CAR) expression that became dominant in culture within several passages (43–45). These findings together illustrate the host cell's influence in the co-evolutionary balance between host and virus as the host attempts to limit viral infection from spreading *via* reduction in viral entry receptor expression (44). CVB1 infection of PANC-1 cells also demonstrates that CVB1 drives downregulation of cellular proteins involved in mitochondrial energy metabolism. Mitochondrial dysfunction, oxidative phosphorylation, fatty acid alpha- and beta-oxidation, citric acid cycle and leucine and valine degradation pathways were significantly enriched among downregulated proteins detected by mass spectrometry. Interestingly, further investigation into the mitochondrial networks of PANC-1 infected cells revealed differential changes in mitochondrial network morphology based on the CVB1 (ATCC vs. 10796) strain used to generate carrier-state infections. CVB1 strain 10796 produced fragmented mitochondrial networks whereas uninfected cells or those infected with CVB1 strain ATCC both showed filamentous mitochondrial networks. Proteomic analysis further supported these findings by revealing a significant downregulation in mitochondrial proteins involved in fusion processes including mitfusion-1, mitofusion-2, and mitochondrial dynamin-like GTPase OPA1

in the strain 10796-induced persistent infection model (46). In addition to support for carrier-state coxsackievirus-induced infections in the pancreas and heart, *in-vitro* infection of human astrocyte cells also suggests persistent coxsackievirus infection could occur in the central nervous system (CNS) (47).

Steady-state infections are characterized by all cells in culture having low levels of non-lytic viral replication. Low levels of viral replication lead to decreased viral-induced inhibition of host cell protein synthesis and thus lead to the non-lytic phenotype. To date, multiple studies have shown a subset of enterovirus serotypes, including coxsackieviruses and echoviruses, are able to produce low replicative steady-state infections without cytopathic effect. This phenomenon may be caused by a number of factors including but not limited to 5′UTR terminal deletions that lead to replication deficiencies or reduced type I interferon response elicitation, faulty virion capsid formation due to incomplete capsid polypeptide processing, and alternative EV RNA mutations that lead to abnormalities such as stable and atypical double-stranded RNA complex formation that inhibits further viral positive strand synthesis (48–51). In the context of ME/CFS, 5′UTR terminal deletions and/or atypical dsRNA complex formation are notable, as they have been shown to occur in a proportion of ME/CFS patient cohorts in multiple studies (52–54). In a number of cases, chronic diseases with some overlap in symptom constellation with ME/CFS show substantial evidence of disease involvement by persistent infection EV variants. These chronic diseases include idiopathic dilated cardiomyopathy (IDCM) (35), chronic inflammatory myopathy (36), insulin-dependent diabetes mellitus (37–39), post-polio syndrome (40, 41), and chronic CNS inflammation and lesions (42). For example, one study of EV positive-IDCM heart tissue detected a positive to negative strand ratio ranging from 2 to 20 (35), while another demonstrated EV-negative to positive strand ratios of 1–5 in infected heart tissue (55). Furthermore, the median viral load in heart tissue was assessed to be 287 EV RNA copies/μg of tissue. Such a low amount presents a significant challenge when trying to detect persistent enteroviral infections in difficult- to-sample/invasive secondary tissue screening sites. Low levels of viral replication result in EV RNA levels so small that they may be past the lower limit of detection (51).

In reviewing the outcomes of persistent *in-vitro* EV infections, it is clear that EVs with the ability to create carrier-state infections are able to produce cellular outcomes that may be relevant to ME/CFS pathophysiology in an EV variant-dependent manner. As mentioned above, specific CVB1 variants (CVB1 10796) disturb mitochondrial network morphology and lead to a downregulation of proteins relevant to mitochondrial energy metabolism. In regard to EVs that produce steady-state infections, Echovirus 6 and Enterovirus 72 (hepatitis A) are both shown to cause persistent steady-state infections *in-vitro* (48, 56, 57). Echovirus 6 is also shown to cause persistent *in-vivo* infections and is associated with neurological disorders of encephalitis and meningitis (58). Unfortunately, literature surrounding mitochondrial outcomes relating to these two viruses is bleak at best. Echovirus 6 infection of cultivated monkey kidney cells shows mitochondria retain their shape but information on mitochondrial enzymology and mitochondrial membrane potential is absent (59). Although there is a serious lack of literature pertaining to enterovirus steady-state infections and mitochondrial dysfunction, persistent Echovirus 6 infections are associated with non-lytic viral RNA and alterations in capsid protein production including unprocessed capsid polypeptide V0 (49). Considering the large number of interactions between enteroviral encoded and host proteins, it is reasonable to assume a downregulation and variation in viral encoded protein production during steady-state infections could lead to a mitochondrial dysfunction phenotype different and less extensive than seen in enterovirus carrier-state infections, acute infections, or cells without infection.

Mitochondrial Abnormalities in ME/CFS Cells

There is recent literature that describes differences in immune cell metabolism between ME/CFS patients and controls (60–67). The relevance of these reports to possible dysfunction of mitochondria in tissues and organs is unclear. Immune cells alter their metabolism while responding to signals indicating a threat is present (68, 69). It is not known whether the altered mitochondrial metabolism is due to defective signaling or an appropriate immune response that is present in patients rather than healthy individuals, rather than an actual abnormality.

Early studies on 50 ME/CFS patient muscle biopsies found mitochondrial abnormalities described as branching and fusion of mitochondrial cristae upon ultrastructural examination in addition to swelling, vacuolation, myelin figures and secondary lysosomes indicating mitochondrial degeneration. The authors concluded their work was the first evidence that ME/CFS may be due to a mitochondrial disorder caused by a viral infection (70).

A few years later, right quadricep muscle biopsies from nine ME/CFS patients were assayed *via* electron microscopy, immunochemistry, mtDNA sequencing (as discussed earlier) and enzyme activity assays. The research group found mitochondrial structure abnormalities, inversion of the cytochrome oxidase/succinate dehydrogenase ratio and a reduction in some mitochondrial enzyme activities. The enzyme activity assay results indicate a reduction of the muscle oxidative property evaluated on multiple mitochondrial matrix enzymes including NADHtr, COX and succinate dehydrogenase. A reduction in mitochondrial enzyme activities was supported for cytochrome c oxidase and citrate synthetase as well (71).

Two recent studies found normal mitochondrial oxidative phosphorylation (oxphos) and normal respiratory chain complex activity compared to healthy controls. However, insight into mitochondrial oxidative phosphorylation was determined using plasma creatine kinase as a surrogate measure of oxphos in muscle (72, 73).

Another recent study used extracellular flux analysis *in vitro* to determine utilization of various substrates by skeletal muscle cells from patients vs. controls. This study found that muscle cells from ME/CFS patients had reduced oxphos in comparison to controls when supplied with glucose as a substrate, while

no abnormalities were detected when cells were supplied with galactose or fatty acids (74).

Overall, the literature surrounding mitochondrial dysfunction in ME. CFS patients is suggestive of bioenergetic abnormalities that are within the realm of possible cellular outcomes based on the nature of the persistent viral infection. Varied findings pertaining to mitochondrial function in ME/CFS muscle biopsies may be due to sampling bias as latent enteroviral infections within secondary infection sites may not be uniform and therefore discovery of a cellular pathophysiology would only be found if the correct tissue location were interrogated.

Enterovirus Cell and Tissue Tropism

Each enterovirus has a distinct cell and tissue level tropism that is governed by both host and viral factors, including cellular virus receptor availability, tissue-specific activity of IRES on viral RNAs, and innate immune antiviral activities such as interferon (IFN) response. Given these conditions, EVs as a whole display a wide spectrum of cell and tissue tropism leading to a wide array of disease outcomes. The diseases may appear as short-duration sicknesses such as the common cold and acute hemorrhagic conjunctivitis or may cause more serious diseases through infiltration into secondary infection sites such as organs, muscle or central nervous system (CNS), causing myocarditis, pericarditis, encephalitis, meningitis, pancreatitis, paralysis, and death (75).

CNS regulation of autonomic nervous system output occurs through multi-synaptic connections descending from the hypothalamus and midbrain to preganglionic neurons in the brainstem and spinal cord. The central autonomic system is further comprised of connections between a multitude of limbic system structures, such as the amygdala and hippocampus, to collectively regulate autonomic nervous system (ANS) outflow (76). The ANS is subdivided into the sympathetic, parasympathetic and enteric nervous systems, which act to control internal body processes such as blood pressure, heart and breathing rates, body temperature, digestion, metabolism, fluid retention, production of bodily fluids, urination, defecation, and sexual response (77).

ME/CFS patients have a number of pathophysiological traits that point to abnormalities in the ANS, including impaired blood pressure variability, orthostatic intolerance, high prevalence and severity of posturalorthostatic tachycardia syndrome (POTS), delayed gastric emptying, impaired thermoregulation in adolescent patients, loss of capacity to recover from acidosis on repeat exercise, abnormal cardiac output and altered brain characteristics in a wide variety of brain regions including the limbic system structures that govern the ANS (1, 78–81). These altered brain characteristics include reduced cerebral, brainstem, and cerebral cortex blood flow; impaired reciprocal connectivity between the vasomotor center, midbrain, and hypothalamus regions; increased neuroinflammation across widely distributed brain areas including but not limited to the hippocampus, thalamus, midbrain and pons; reduced cerebral glucose metabolism, and lower brain glutathione (1, 82–86). Many of the altered brain characteristics seen in ME/CFS patients are similarly reported in clinical cases associated with neurotropic enteroviruses. For instance, focal enterovirus encephalitis caused by coxsackievirus A3 is associated with focal hypoperfusion in the right frontal lobe that cleared upon patient recovery from the neurotropic enteroviral infection. This example case is largely similar to multiple SPECT studies indicating ME/CFS patients have significant hypo-perfusion in regions of the brain consistent with their patient-specific symptoms (87–92).

There is a diverse spectrum of tropisms for each enterovirus; some EVs are neurotropic in nature while others may be myotropic. Among human enterovirus families A-D, there exists a subset of EVs that are known to be neurotropic; these include EV71, multiple coxsackievirus group A members, all coxsackievirus group B members, poliovirus and EVD68, among many others. Not surprisingly, different neurotropic enteroviruses gain CNS access *via* alternative strategies and thus display distinct CNS tissue tropism. For example, poliovirus mainly infects and replicates in motor neurons in the anterior horn of the spinal cord, while EV71 primarily targets neuronal progenitor cells (NPSCs) and astrocytes (75). NPSC infection is particularly advantageous for viral dissemination, transmission, replication, and persistence. For instance, NPSC infection may expand CNS presence as the infected NPSCs differentiate into neuronal, astrocyte and oligodendrocyte lineages. Furthermore, NPSC migration following differentiation allows access into new CNS locations, and lastly, EV infection of NPSCs may trigger EV-specific genomic changes that allow the virus to persist in a latent state due to the quiescent cellular environment of non-activated NPSCs or NPSCs that have moved to a neuronal cell fate (93).

EVs gain access to the CNS through a diverse set of entry mechanisms including direct infection of brain microvascular endothelial cells, retrograde axonal transport following muscle infection, exosomal transport across the blood-brain barrier (BBB), and hitchhiking inside of migratory infected immune cells with BBB privilege (75). Infection outcomes can follow expected changes such as halting of host cell cap-dependent translational events and production of cytopathic effects causing tissue lesions. However, EVs may also establish a persistent/chronic infection producing atypical clinical outcomes, as may be the case in ME/CFS (75).

Several known EV-CNS infections display autonomic dysfunction symptoms reminiscent of those described in ME/CFS patients. Damage to the ANS is well-documented following poliovirus infection; postmortem histopathology routinely demonstrates damage to the reticular formation region of the brainstem whether or not the patient displayed spinal cord damage or paralysis (94). The reticular formation, a network of neurons located in the brainstem that project into the hypothalamus, thalamus, and cortex, plays a role as a cardiodepressor that lowers cardiovascular output. Post-polio syndrome (PPS) patients exhibit a high prevalence of hypertension and tachycardia while ME/CFS patients display high rates of POTS, which is accompanied by drop in blood pressure. The difference in autonomic dysfunction outcomes between ME/CFS and PPS patients may possibly be due to infection with genetically distinct EV serotypes with different neurotropism and thus different clinical manifestations. However, white matter brain lesions upon MRI, slowing

of electroencephalography outputs, clinical impairment of attention, and abnormal hypothalamic pituitary adrenal axis function are shared between patients with PPS and those with ME/CFS (95). Nevertheless, there is a controversy about whether the reports of excess white matter lesions in ME/CFS patients are instead related to age, a misdiagnosis of neurological disorder, or due to major depression. A quantitative summary of rigorous data pertaining to white matter lesions in ME/CFS reported no significant increase in the lesions (96), but studies that use more advanced neuroimaging methods are needed.

Three ME/CFS post-mortem brain autopsy studies found enteroviral genomic RNA and VP1capsid protein in the hypothalamus, brainstem, cerebral cortex, medial temporal lobe, lateral frontal cortex, occipital lobe, and cerebellum (97–99). These findings provide additional support that a persistent EV infection within patient limbic and extra-limbic tissues is possible and could be driving the ANS dysfunction observed in ME/CFS patients.

CNS infections by other EVs such as EV71 and the group B coxsackieviruses result in ANS dysfunctions reminiscent of ME/CFS pathophysiology. EV71 brainstem encephalitis occasionally induces symptoms of ANS involvement including fluctuating blood pressure, tachycardia or bradycardia, hypertension or hypotension and respiratory distress. EV71 CNS-specific clinical manifestations include myoclonic jerk, polio-like syndrome, lethargy, limb weakness, altered mental status, encephalomyelitis, encephalitis, aseptic meningitis, and rhombencephalitis (100, 101). Of these EV71-related ANS/CNS clinical manifestations, altered blood pressure regulation, altered heart rate regulation, myoclonic jerk, lethargy, limb weakness and altered mental status are reported in ME/CFS patients, indicating a large overlap in symptom constellations between ME/CFS patients and neurotropic EV infections (102, 103).

To summarize, some serotypes of EVs exhibit CNS tropism and have the ability to produce persistent viral infections that result in atypical and distinct chronic clinical outcomes. Another complicating factor is the production of EV quasispecies, a population of EVs with subpopulations that consist of specific genotypic variants, each with genotypically-dependent functional characteristics. The proportion of the different quasispecies in the overall population dictates infection initiation, progression and dynamics of clinical presentation (93).

DETECTION OF ENTEROVIRUSES

World Health Organization EV Surveillance Guidelines

The World Health Organization, in conjunction with the U.S. Centers for Disease Control, have published guidelines for enterovirus surveillance that details recommended procedures for specimen preservation as well as optimal methods for enterovirus detection and characterization. Although not all human enteroviruses can be propagated in cell culture, the guidelines state that multiple attempts should be made across a variety of cell lines including: primary African green, cynomolgus or rhesus monkey kidney cells (AGMK, CMK, RMK), rhesus monkey kidney (LLC-MK2), African green monkey kidney (Vero, BGMK, GMK), Madin Darby canine kidney (MDCK), human diploid cells lines (MRC-5, WI-38, SF), human embryonic kidney (HEK), human embryonic fibroblast (HEF), human epithelial carcinoma (HEp-2), and human rhabdomyosarcoma (RD) cells (104).

The guidelines further state the preferred and alternative sample types to use in cell culture inoculation depending on the clinical syndrome noted in patients. Based on the occurrence of encephalitis and respiratory clinical syndromes in a large proportion of ME/CFS cohorts, preferred sample types include brain tissue and broncho-alveolar lavage, with alternatively approved sample types, including cerebrospinal fluid (CSF), feces, throat swab, oropharyngeal swab, nasopharyngeal swab, and rectal swab (104).

Approaches and Limitation of EV Detection Strategies Employed in ME/CFS Studies

Across the enterovirus and virus literature at large, a number of methodologies are used to detect the presence of enteroviral infection in patients. In the early years of virus detection, biological approaches such as serological testing and cell culture methods were employed. Isolation *via* cell culture requires patient samples to be inoculated into enterovirus-susceptible cell lines and then examined periodically for the presence of viral-induced changes such as cytopathic effect (CPE), which is described as cells becoming rounded, refractile and shrinking before detaching from the cell surface. The identity of the isolated virus was then confirmed/typed *via* tests such as neutralization of infectivity with serotype-specific antisera or immunochemistry using fluorescent antibodies. The main disadvantages to cell culture are that inoculation depends on quality of the patient sample and requires variable and sometimes extended time periods to allow detection (105, 106). Some enteroviruses, especially persistent enterovirus variants, do not produce CPE in cell culture. Without CPE, screening for viral nucleic acid or protein would be necessary.

Serological testing is confounded by several factors. First, enteroviruses often produce clinical disease before the appearance of antibodies, making their detection retrospective. Furthermore, enteroviruses and rhinoviruses have extensive antigenic heterogeneity and lack cross-reacting antigens, so that many different antigens would be needed to detect anti-EV antibodies (105–107). Virus antigen detection can be achieved both by immunohistochemical detection and ELISA. Viral antigens such as VP1 exhibit sequence similarity between serotypes, which is an advantage in detection of enteroviruses, but also means that serotype identification is not feasible solely from reaction with a VP1 antigen. Commercial labs with serological tests for EVs are far from comprehensive. For instance, the Enterovirus IgG/IgA/IgM ELISA kits sold *via* Virotech Diagnostics detects 14 (CVA9, CVA16, CVB2, CVB4, CVB5 and Echo 5, 11, 15, 17, 22, 23, 25, 33) of the roughly 120 known EV serotypes (108). The Enterovirus Antibody Panel lab test provided by ARUP Laboratories similarly detects 14 EV serotypes (CVA9, CVB1-6, Echo 6, 7, 9, 11 and

30, poliovirus types 1 and 3) although the serotypes differ slightly (109). Negative detection of EVs *via* these commercially available serological tests does not conclusively eliminate the possibility of an EV infection. Other companies, such as SERION Diagnostics and Immuno-Biological Laboratories also sell enterovirus-specific ELISA kits but with the added benefit of using recombinant antigens. The recombinant antigens are made from conserved and subtype specific domains across a subset of human enteroviruses and are therefore likely to demonstrate antigens for all known human enteroviruses. These kits have an increased comprehensive nature, but a positive detection cannot reveal exactly which EV serotype is the culprit in question.

A serological method for detection of antibodies to enteroviruses that has not yet been employed in ME/CFS is the peptide array, which is comprised of tiled peptides corresponding to a virus family. Such an array designed to probe human herpesviruses has been used to compare ME/CFS patients to healthy controls and individuals with other diseases (110). An enterovirus peptide array was successfully used to detect antibodies against EV-68 in some samples of cerebrospinal fluid and serum from patients with acute flaccid myelitis (111).

The most popular detection method for identification of enteroviruses is RT-PCR, with amplification directed at conserved regions of the enterovirus genome, including those encoding the 5′UTR, 3Dpol and VP1. VP1 is the region of choice to conduct enterovirus typing. However, low sequence similarity amidst the approximately 120 enterovirus serotypes means that no one primer set is robustly comprehensive so that RT-PCR methods would have a lower chance of identifying novel EV serotypes than unbiased sequencing. RT-PCR experiments that use primers directed at the 5′UTR of enteroviruses can be problematic if the enterovirus contains mutations within the primer binding region, as is known to happen during persistent infection. Traditional RT-PCR approaches have reduced ability to identify novel enteroviruses that could be etiological agents in new diseases.

Northern blots using sequences complementary to EV genomic regions to detect viral RNA in a gel are similarly confounded by a lack of comprehensiveness, as the probe sequence might fail to hybridize to EV serotypes that have sufficient variation in targeted sequences. For greater sensitivity and breadth, many researchers have instead used an unbiased RNAseq approach to detect enterovirus nucleic acids in patient samples. In terms of disadvantages, RNAseq is expensive and requires significant read depth in sequencing to identify low copy transcripts among the sea of nucleic acids that are being sequenced. Capture approaches have been developed to enhance sensitivity and increase breadth of viral detection (112–114) (**Table 1**).

Critical Review of EV Detection in ME/CFS by Method Used

Tissue Culture Reports

To date, ME/CFS studies reporting the use of tissue culture for EV detection have used CSF and feces in 1 and 4 studies, respectively (115–118). The singular CSF study reported two EV infections in a cohort of 4 patients, while the 4 fecal studies reported an increased EV infection prevalence in 2 of 4 studies, with cohorts ranging from a 22–25% prevalence across patient cohorts (**Table 1**).

Although the prevalence of EV infections in these studies was generally shown to be significantly increased compared to healthy control cohorts, limitations in patient sample types and cell culture models may have led to findings that underrepresent the prevalence of EV infections in patient cohorts. Of the five cell culture studies, one study used only one cell type (115), 3 studies used two cell types (115–117) and one study used three cell types (118).

The most comprehensive study, which utilized three cell culture types, included green monkey kidney cells, RD cells and HeLa cells, which together supply a diversity of enterovirus receptors including CAR, CD155 and DAF. These cultures therefore detect a wide diversity of enteroviruses although the system is still not totally comprehensive. No enterovirus-positive fecal samples were found within a cohort of 12 ME/CFS patients (118) when the triple-cell culture method was used. EVs may be absent in these patients, but lack of detection might also be attributed to the presence of an enterovirus that uses an alternative receptor as well as the low likelihood of detecting EV infections in the stool samples of chronically ill patients with persistent infections in secondary sites such as muscle and brain tissue. Furthermore, the investigators were searching for CPE, and EVs present in chronic infections commonly undergo genetic changes which reduce CPE. An example of the inadequacy of CPE is a report that inoculated cell cultures were negative for CPE production in human fetal lung fibroblast and tertiary monkey kidney cell cultures but were nevertheless positive upon RT-PCR (119).

Two studies utilized Hep-2, VERO, and monkey kidney tissue cultures for identification of enterovirus from CSF and feces from 4 and 76 patients, respectively. Innes (115) identified enterovirus in 2 of 4 CSF samples and one of 4 feces samples (115). Yousef et al. (116) found that 17/76 patients tested positive for enterovirus infection while only 2/30 controls tested positive (116).

Studies reporting the absence of enterovirus infections in ME/CFS patient cohorts using tissue culture approaches had small sample sizes and incomprehensive cell culture systems. Small sample sizes along with the fact that EVs harboring 5′UTR deletions do not produce CPE means that no definitive conclusion can be made about the absence of EVs from the data in these studies. Furthermore, fecal samples usually identify only acute enterovirus infections and not chronic ones that might be in secondary infection sites. Nevertheless, some studies that screened suboptimal sample types with culture methods did find an increased prevalence of EV infections, which might have been due to inclusion of patients who were still in the acute phase of illness.

Serological Testing for EVs

A wide variety of serological tests for detection of EVs have been developed. Studies between the 1970s and late 1990s that screened for EV infections in ME/CFS patients largely focused on serological testing. The diversity of testing employed

TABLE 1 | Compilation of enterovirus-specific ME/CFS studies listed by tissue type and sub-grouped based on EV detection methodology.

	Positive studies	Total studies	Prevalence in positive cohorts
Blood	20	24	8–100%
Serological test	16	20	8–90%
PCR	4	5	18–100%
RNAseq	0	2	N/A
Muscle	8	11	13–53%
PCR	6	9	13–100%
Northern blot	4	4	21–50%
VP1 immunohistochemistry	0	1	N/A
Throat swab	1	1	17%
PCR	1	1	17%
Stomach tissue	2	2	82%
PCR	1	1	37%
VP1 immunohistochemistry	2	2	82%
dsRNA immunohistochemistry	1	1	64%
Heart Tissue	1	1	N/A
PCR	1	1	N/A
Cerebrospinal fluid	1	2	N/A
Tissue culture	1	1	50%
EV IgG ELISA	0	1	N/A
Brain tissue	3	3	N/A
PCR	2	2	N/A
VP1 immunohistochemistry	2	2	N/A
Feces	2	4	22–25%
PCR	0	1	N/A
Tissue culture	2	4	22–25%
Electron microscopy	0	1	N/A

Many studies utilize multiple detection methods resulting in the total number of studies not equaling the number of studies based on each method.

in a total of 20 serological-based ME/CFS studies included neutralization, complement fixation, micro-metabolic inhibition, ELISA, indirect immunofluorescence, and VP1 antigen detection tests. In total, 16 of the 20 studies found an increased prevalence of CVB signals in ME/CFS cohorts with positive findings ranging from 8 to 90% compared to the positive findings in healthy control cohorts that ranged from 0 to 65% (**Table 1**) (115–118, 120–135).

The vast majority of studies evaluated the presence of antibodies directed only against CVB enteroviruses, with a few exceptions in which echo30- and echo9-directed IgG antibodies were screened *via* ELISA (118). A notable study was performed in 1997, in which neutralization tests for 11 enteroviruses (CVB1-6 and echo 6, 7, 9, 11, 30) found that 100 out of 200 tested patients had elevated enteroviral titers (136).

Although serological testing in ME/CFS cohorts generally shows an increase in the prevalence of EV antibodies, the findings often lack clinical specificity as a high prevalence of EV antibodies are found in the general population from previous exposure. In a retrospective study, it cannot be known whether the enterovirus infection occurred before or after ME/CFS disease onset without having paired sera from both time periods.

Immunohistochemistry to Detect EV Capsid Proteins and dsRNA

The enteroviral capsid protein VP1 is commonly used for identification of enteroviral virions in ME/CFS patient tissues. In total, 5 studies have used this technique on a variety of patient sample types, including muscle, gastrointestinal, and brain tissue (**Table 1**, **Supplementary Table 1**) (20, 54, 98, 99, 137). Of these, 4 out of 5 studies identified the presence of VP1 capsid proteins in patient tissue. The muscle tissue study did not detect VP1 staining in samples of a cohort of 30 ME/CFS patients, despite RT-PCR signals that indicated the presence of EV-RNA in 13 of the same 30 patients. The authors suggested that the difference in PCR and VPI immunochemistry resulted from persistent but latent enteroviral infection in patient muscle tissues, in which no detectable amount of virion particles were being produced (137).

The remaining 4 studies showed positive VP1 staining in both gastrointestinal and brain tissues (20, 54, 98, 99). Gastrointestinal samples exhibited positive staining rate of 82% in two patient cohorts ($n = 165$, $n = 416$). Comparative cohorts for these two studies were healthy controls ($n = 34$) and patients with functional dyspepsia (FD) ($n = 66$), which displayed a positive VP1 staining rate of 20 and 83%, respectively (20, 99). Both the ME/CFS and FD patient cohorts showed dsRNA staining for 64

and 63% of patients, respectively (54). Because persistent/chronic EV infections with reduced CPE and viral replication typically have a 1:1 ratio between enteroviral positive and negative RNA strands, finding a high rate of dsRNA in patient tissues indicates the likely presence of persistent enteroviral infections. One study found VP1 in fibroblasts of small blood vessels in the cerebral cortex and in a small fraction of glial cells in brain (98), while another detected VP1 instead in the pontomedullary junction, medial temporal lobe, lateral frontal cortex, occipital lobe, cerebellum and midbrain (99).

Molecular Approaches to Detect EV Infections

We identified 24 reports of the use of either Northern Blot ($n = 4$) (52, 138–140), RT-PCR ($n = 18$) (20, 53, 97, 99, 117, 118, 132, 134, 137, 141–145) or RNAseq ($n = 2$) (146, 147) across multiple sample types including blood, feces, muscle, brain, heart, gastrointestinal tissue and throat swabs. In a few cases, a single publication used RT-PCR on multiple sample types; thus, there are 20 independent studies amongst the 24 reports. Seventeen of the twenty publications report detection of EVs in patient samples or indicate an increased prevalence of EV infections compared to control cohorts (**Table 1**, **Supplementary Table 1**).

The 4 Northern blot studies used muscle tissue biopsies and were all positive for viral RNA, indicating an EV prevalence between 21 and 50% in ME/CFS with control cohorts showing a prevalence between 0 and 1% (52, 138–140). The two RNAseq studies were negative for the presence of EV in blood, whether or not blood was taken before or after an exercise stress that exacerbated subject symptoms (146, 147). While RNAseq is a more comprehensive approach to enterovirus detection than Northern blots, these studies cannot be directly compared since one used muscle tissue and the other assayed blood samples.

With regard to EV studies that applied RT-PCR methods, 5 of the 17 reports indicated no significant difference in EV prevalence between ME/CFS and control cohorts. The 5 reports were performed on peripheral blood leukocytes (132), muscle tissue (118, 141, 142), and feces (119). A list of all 8 PCR approaches/methods, indicating the primer sets employed in RT-PCR experiments, was first compiled, and then each PCR set was examined for its effectiveness for detection of all 117 known EV serotypes (**Table 2**, **Supplementary Tables 2, 3**). *In-silico* PCR was run with conservative allowances (1 mismatch and no mismatches within 2 base pairs of the 3′end) as well as less conservative allowances (4 mismatches with mismatches being allowed on the 3′end) to give a range of possible experimental results, given that one *in-silico* PCR experiment does not likely represent the true *in-vitro* PCR outcomes. The less conservative *in-silico* experiments resulted in predicted binding to multiple locations, sometimes over 15 locations along an EV genome, and thus were not likely to represent results that would be gained from an actual experiment. Examining the conservative *in-silico* PCR experiments that used 1 mismatch and 0 allowed mismatches within the 3′end of the primer (**Supplementary Table 2** indicated methods 1, 3, 5, 7, and 8 are low in their comprehensive nature with 52, 50, 21/0, 39, and 65 EVs being amplified out of 117, respectively. Interestingly, four (118, 119, 132, 141) of the five (118, 119, 132, 141, 142) studies indicating a lack of EV presence by RT-PCR used primer sets from methods 1, 7, and 8, which amplify 44, 33, and 56% of known human enteroviruses, respectively. Therefore, an EV infection could have been present and simply escaped detection due to the primer sets employed. One study reported 20.8% ($n = 48$) of the ME/CFS cohort to have detectable EVs compared to 0% ($n = 29$) of controls even though method 5 was used, in which round 2 PCR primers amplify 0% of EVs under conservative PCR parameters and 3% of EVs under less conservative parameters. Either that reported primer sequence does not function as expected in the *in-silico* PCR or the particular EV that was detected in these patients is one of the few able to be observed with method 5 primers. Poor *in-silico* PCR amplification using method 5 was caused by the primer OL253 (5′-GATACTYTGAGCNCCCAT-3′) used in the second round PCR. First round primers, OL252 and OL68, as well as second round primer OL24 had binding rates to the EV serotypes with only OL253 lacking *in-silico* hybridization. Overall, RT-PCR experiments with low rates of positive *in-silico* PCR amplification are strongly correlated with publications indicating insignificant differences in EV prevalence between controls and patients (**Table 2**, **Supplementary Tables 2, 3**).

As mentioned earlier, EVs are known to exhibit mutations in the 5′UTR that result in replication deficiencies. Interestingly, all 8 PCR methodologies used primer pairs targeting the 5′UTR with the exception of method 5 whose reverse primers target the VP4 and VP2 genomic regions (see **Figure 2**). This is an important consideration as patients infected with EV variants exhibiting 5′UTR deletions may not be successfully targeted by the primer sets employed across these PCR methodologies. In conclusion, PCR studies aimed at identifying EVs in ME/CFS have been crippled by the use of incomprehensive primer sets that target potentially deleted portions of the viral genome.

DISCUSSION

Multiple aspects of the ME/CFS pathophysiology, especially related to autonomic dysfunction, are reminiscent of chronic neurotropic enterovirus-related diseases and clinical outcomes. This fact, in conjunction with the enterovirus-like seasonality of ME/CFS epidemics, often occurring in spatiotemporal incidence with known poliomyelitis epidemics of the time, gives strong justification for the conclusion that enteroviruses have been etiological agents in ME/CFS outbreaks.

Many ME/CFS patients in a variety of studies indicate a viral-like illness immediately preceded their ME/CFS symptoms. However, surveys also indicate that patients ascribe their onset to a variety of other reasons, including emotional stress, life events, recent travel, accidents, toxic substances, or mold (148, 149). However, some of these events and exposures could merely be coincidental and actually be due to an enteroviral infection that was unnoticed or very mild, given that many enteroviral infections are asymptomatic (150). The COVID19 pandemic has made it obvious that persistent symptoms can arise from mild or asymptomatic infections (151). Were the existence of

TABLE 2 | *In-silico* PCR amplification results using primers reported throughout enterovirus-specific ME/CFS publications.

References	PCR primers and probes	No. amplified EVs: 1 mismatch	No. amplified EVs: 4 mismatches
(132, 141)	1: EP1, EP4 and EP2 (probe)	52/117	92/117
(142)	2: EP1, EP4	85/117	112/117
(53, 134, 144, 145)	3: 1 (EP1, EP4), 2(P6, P9)	(85/117), (50/117)	(112/117), (112/117)
(97)	4: Primer 2, Primer 3 and Probe	102/117	112/117
(143)	5: 1(OL252, OL68), 2(OL24, OL253)	(21/117), (0/117)	(111/117), (3/117)
(20, 99, 137)	6: RNC2, NC1, E2 and Probe	89/117	108/117
(117)	7: 1(Primer 1, Primer 4), 2(Primer 2, Primer 3 and Probe)	(77/117), (39/117)	(103/117), (75/117)
(118)	8: 1(Primer 1, Primer 2), 2(Primer 3, Primer 4)	(65/117), (65/117)	(110/117), (110/117)

Nested RT-PCR studies (methods 3, 5, 7, and 8) are reported as PCR amplification results for both rounds of PCR (1st round), (2nd round). Values indicate number of EVs with a positive PCR amplification out of 117 total EVs tested for amplification. In-silico PCR was performed using Geneious Prime v2019.1.1. Primer design uses a modified version of Primer3 v2.3.7. Primers could bind anywhere on sequence (1 mismatch): in-silico PCR testing allowed 1 mismatch to be tolerated between the primer and its complementary site on the EV transcript. The 1 allowed mismatch was not allowed to occur within 2 base pairs of 3′ end. The 1 mismatch PCR parameter therefore indicates an in-silico PCR amplification with low forgiveness for incomplete complementarity and reduces the likelihood of off target amplification events (4 mismatches): in-silico PCR testing allowed 4 mismatches to be tolerated between the primer and its complementarity site on the EV transcript. All 4 mismatches could occur at any site within the primer and EV transcript complementarity region. The 4 mismatch PCR parameter therefore indicates an in-silico PCR amplification with high forgiveness for incomplete complementarity and increases the likelihood of both on and off target amplification events.

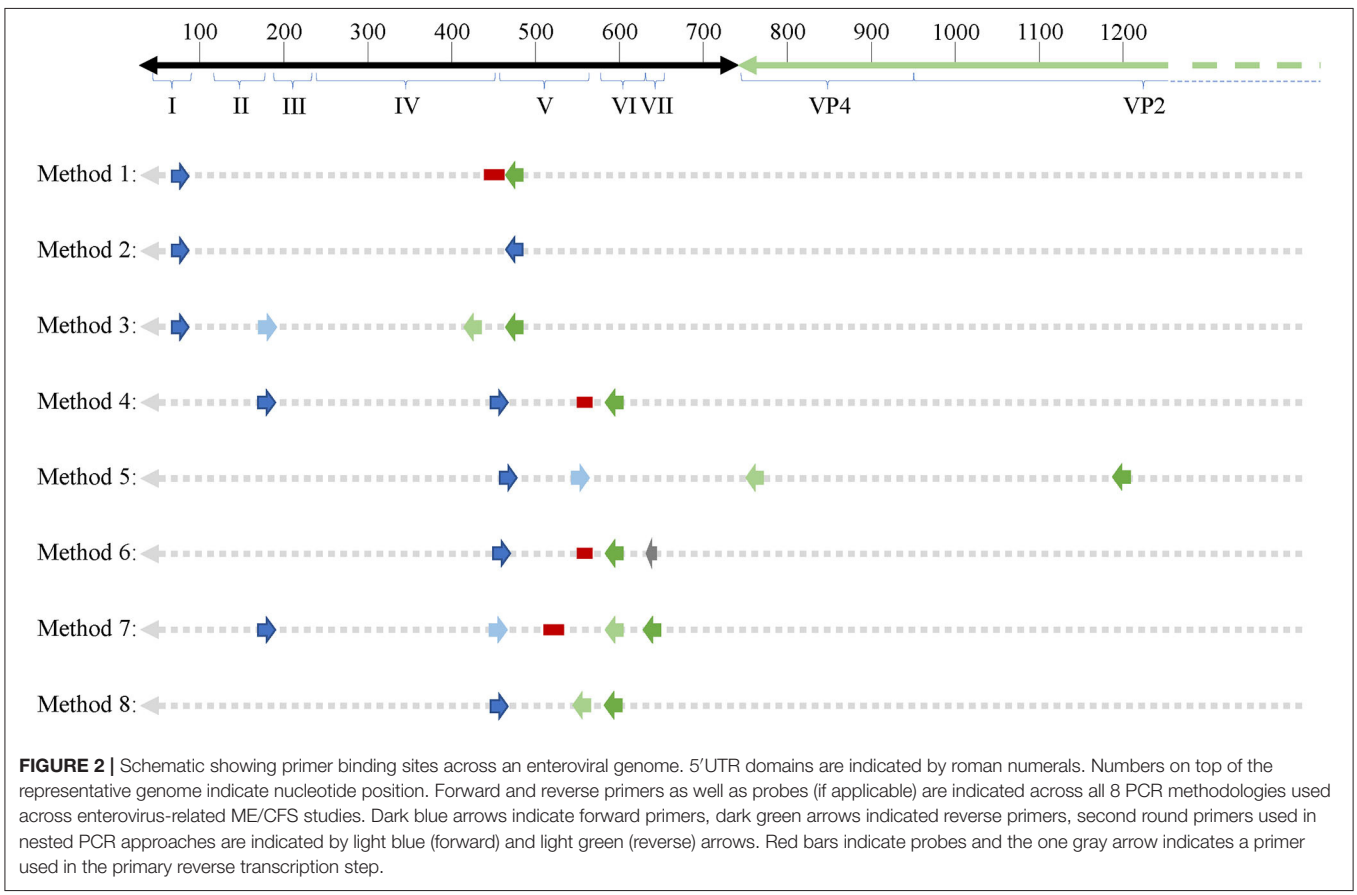

FIGURE 2 | Schematic showing primer binding sites across an enteroviral genome. 5′UTR domains are indicated by roman numerals. Numbers on top of the representative genome indicate nucleotide position. Forward and reverse primers as well as probes (if applicable) are indicated across all 8 PCR methodologies used across enterovirus-related ME/CFS studies. Dark blue arrows indicate forward primers, dark green arrows indicated reverse primers, second round primers used in nested PCR approaches are indicated by light blue (forward) and light green (reverse) arrows. Red bars indicate probes and the one gray arrow indicates a primer used in the primary reverse transcription step.

SARS CoV-2 not known, many of the individuals with long-lasting symptoms of COVID 19 might readily have ascribed their mysterious illness to some other factor than viral infection.

Post-acute viral syndromes may not all fit the diagnostic criteria recommended by the U.S. Institute of Medicine (IOM) for ME/CFS (152), as the victims of a number of viral infections have not been thoroughly investigated over long time periods. Further, even the definition of ME/CFS or SEID itself may be lumping together disparate phenomena (153). The last report on the 2003 SARS outbreak patients exhibiting long-term symptoms followed them up to only 2 years later (154). At this writing, individuals who contracted SARS-CoV-2 and did not recover completely have been ill no more than 14 months, and many are displaying not only symptoms required for the IOM definition

of ME/CFS, but additional ones, suggesting that further study may differentiate them at the molecular/biochemical level from individuals with pre-2020 ME/CFS. New information from ongoing studies of the consequences of COVID19 may indicate that the definition of ME/CFS will need to be refined to distinguish it from post-acute SARS-CoV-2 syndrome, even though a number of symptoms overlap. Notably, Gulf War Illness victims have symptoms that overlap with ME/CFS, but a number of studies are able to distinguish them from individuals with ME/CFS who did not participate in Gulf War era military activities (155–158).

A relatively small number of viruses have been identified as possible triggers for ME/CFS, making the concept held by some, that "any virus" can lead to ME/CFS, unsupported by evidence. One of the few studies of viral triggers of fatiguing syndromes is being carried out in Australia, namely the Dubbo study of post-infective fatigue syndromes, which follows individuals with diagnosed Ross River virus, Epstein-Barr Virus (EBV), as well as Q fever (a bacterial rather than viral infection) (9, 159–161). Given the geographic limitation to Ross River virus exposure, it is not likely that it is a major cause of ME/CFS worldwide.

There appears to be a special relationship between herpesvirus infection and ME/CFS, as recently reviewed (8, 162). Whether this is actually a relationship between enteroviral and herpesviral infection is not known. Several studies have documented that a certain percentage of people who contract mononucleosis from Epstein-Barr virus infection will still be ill 6 months or more, exhibiting symptoms diagnostic of ME/CFS (163, 164). Surveys often indicate that a proportion of patients believe their ME/CFS followed an acute case of mononucleosis or other type of herpesvirus infection (149, 164, 165). However, given that enteroviruses are known often to cause mild or asymptomatic infections, it is possible that individuals who report ME/CFS after mononucleosis or other herpesviral infections may have also had an inciting enterovirus infection before or after the herpesvirus infection. In fact, one may speculate that an undetected enteroviral infection could make an individual more susceptible to symptomatic cases of EBV infection, for example. Most individuals are infected with EBV as children, yet a number of patients have reported an adult-onset EBV infection as triggering their ME/CFS. Perhaps these adult cases are actually mis-diagnosed reactivated infections. Indeed, there are several reports of reactivated herpesvirus infections in ME/CFS patients (166, 167). Furthermore, a few studies have discovered impaired immunological response to EBV in ME/CFS patients (168, 169). Is this impaired response due to a prior or ongoing enteroviral infection? Whether or not herpesviruses may incite ME/CFS or merely take advantage of immune disruptions caused by enteroviral infections, they may contribute to the symptoms of the illness, and may prevent recovery, as illustrated by a subset that improves upon anti-herpesvirus drug treatment (170–172).

Our review emphasizes that EV-related ME/CFS literature indicates that some patients exhibit chronic enteroviral infection. Furthermore, our review highlights a number of experimental weaknesses (cohort size, tissue type interrogated, methodological approach, etc.) that exist across the EV-ME/CFS literature for studies both supporting or opposing increased EV infection prevalence in ME/CFS patients vs. healthy controls. Those studies that do not support an increased prevalence of EV infections in ME/CFS patient cohorts using RT-PCR are especially confounded with issues related to incomprehensive RT-PCR primer design. Considering that the majority of patient samples interrogated have been collected from patients in the chronic stage of illness, too few studies have been directed at more appropriate secondary infection tissue sites that would give insight into the possibility of persistent myotropic or neurotropic enteroviruses. Indeed, the majority of studies interrogating muscle tissue and all studies we have identified interrogating brain tissue or cerebrospinal fluid *via* PCR or tissue culture have found detectable signs of EV infection. It is evident that more research must be conducted in order to determine whether or not the majority of pre-2020 ME/CFS cases have arisen from EV infection. At the time of this writing, there have been a number of anecdotal reports of individuals experiencing remission of long-term COVID19 symptoms after receiving anti-SARS COV2 vaccines. Such a therapy will not be possible for any ME/CFS patients whose illness is due to chronic infection unless the persistent virus is identified.

Moving forward, studies aimed at identifying chronic EV infections in ME/CFS patients need to consider quality and types of samples to interrogate as well as methodological approaches to employ. The key samples suggested to interrogate further would include brain tissue, cerebrospinal fluid, and muscle biopsy samples. As of now, we could identify only 5 studies reporting on the assessment of either brain ($n = 3$) or cerebrospinal fluid ($n = 2$) and these studies are either on individual patients or cohorts of up to 7. Muscle biopsies have been chosen as the source of patient tissue sample in a total of 11 identified studies, but problems in RT-PCR primer design, small cohorts and few biological tissue replicates means the conclusions of the 8 studies reporting an increased EV prevalence in ME/CFS cohorts may be underrepresenting the true prevalence. Furthermore, the 3 studies indicating a lack of increased prevalence may have been unable to identify the EV serotype in question.

In terms of methodological approaches, RT-PCR with optimal primer sets and or RNAseq with target capture enrichment should be utilized as the methodology of choice for EV detection specifically. Both experimental approaches may be modified to allow detection of both positive and negative strand viral transcripts and are also advantageous in their ability to detect low copy number transcripts. Targeted RNAseq has the increased benefit of being completely comprehensive for the enteroviral family, allowing complete genomic sequencing as well as an increased likelihood of identifying novel EV serotypes possibly at play in an illness such as ME/CFS whose inciting pathogen remains unidentified.

AUTHOR CONTRIBUTIONS

AO'N: examined efficacy of published primers *in silico*. AO'N and MH: reviewed literature and wrote the paper. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.688486/full#supplementary-material

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Herpesviruses Serology Distinguishes Different Subgroups of Patients From the United Kingdom Myalgic Encephalomyelitis/Chronic Fatigue Syndrome Biobank

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The evidence of an association between Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) and chronic herpesviruses infections remains inconclusive. Two reasons for the lack of consistent evidence are the large heterogeneity of the patients' population with different disease triggers and the use of arbitrary cutoffs for defining seropositivity. In this work we re-analyzed previously published serological data related to 7 herpesvirus antigens. Patients with ME/CFS were subdivided into four subgroups related to the disease triggers: S_0-42 patients who did not know their disease trigger; S_1-43 patients who reported a non-infection trigger; S_2-93 patients who reported an infection trigger, but that infection was not confirmed by a lab test; and S_3-48 patients who reported an infection trigger and that infection was confirmed by a lab test. In accordance with a sensitivity analysis, the data were compared to those from 99 healthy controls allowing the seropositivity cutoffs to vary within a wide range of possible values. We found a negative association between S_1 and seropositivity to Epstein-Barr virus (VCA and EBNA1 antigens) and Varicella-Zoster virus using specific seropositivity cutoff. However, this association was not significant when controlling for multiple testing. We also found that S_3 had a lower seroprevalence to the human cytomegalovirus when compared to healthy controls for all cutoffs used for seropositivity and after adjusting for multiple testing using the Benjamini-Hochberg procedure. However, this association did

not reach statistical significance when using Benjamini-Yekutieli procedure. In summary, herpesviruses serology could distinguish subgroups of ME/CFS patients according to their disease trigger, but this finding could be eventually affected by the problem of multiple testing.

Keywords: disease trigger, cutoff value, stratification, Epstein-Barr virus, human cytomegalovirus, varicella-zoster virus, human herpesvirus-6, herpes simplex virus 1 and 2

INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a complex disease with unknown cause whose patients experience persistent fatigue that cannot be alleviated by rest and suffer from post-exertional malaise upon minimal physical and/or mental activity (1). Disease prevalence has been estimated around 0.4% after pooling data from different epidemiological studies (2). However, this estimate might be conservative (3, 4) due to poor societal recognition of the disease including amongst health professionals (5), the inexistence of an objective disease-specific biomarker for the corresponding diagnosis (6), a small number of well-designed epidemiological studies (7), and limited funding opportunities for more comprehensive and integrative research (8).

The etiology of ME/CFS and its pathogenesis remains a topic under intense debate with the proposal of many competing hypotheses (9–16). However, there is a general consensus that the disease could be initiated by a combination of genetic predisposing factors (17–20) and environmental triggers (e.g., exposure to toxins, chronic emotional and physical stress) (10, 21). In this regard, a large proportion of patients report an acute infection at the onset of their symptoms (22, 23). Herpesviruses such as the Epstein-Barr virus (EBV) and the human herpesvirus-6 (HHV6) were considered to be the main candidates for the causative agents of ME/CFS due to their high prevalence in adults and their reactivation observed in patients (11, 24, 25). To understand the role of these viruses on ME/CFS, many serological investigations were conducted with inconclusive or even contradicting findings (24). Possible reasons for this contrasting evidence could be related to disease misclassification and selection bias (26, 27), the necessity of dividing patients into different subtypes (28), the low number of patients recruited (6), or differences in the antigen and experimental assays used (29). An additional but often ignored reason is that serological studies are typically based on arbitrary cutoff values for identifying seropositive individuals or high antibody responders, as illustrated in two serological studies on herpesviruses (30, 31).

Recently, the analysis of serological data from the United Kingdom ME/CFS Biobank (UKMEB) did not find any association between ME/CFS and the presence of antibodies against chronic infections by different herpesviruses (32). In this work, we re-analyzed these data by dividing the ME/CFS patients into four subgroups related to non-infection vs. infection disease triggers. We also performed a sensitivity analysis of the association between ME/CFS and each herpesvirus as a function of the cutoff defining seropositivity.

MATERIALS AND METHODS

Study Participants

All study participants are part of the UKMEB as described before (33). In summary, the data refer to a cohort of 226 patients with ME/CFS and 99 healthy controls (HC); note that the sample size of the healthy controls is in line with the ones used for this group by current serological studies on the role of herpesviruses on ME/CFS, as summarized elsewhere (24). At biobank enrollment, patients had to fill in a symptom's assessment questionnaire in which they were asked a specific question about whether they had an infection at the disease onset. This question had four categories of response, which we used to divide the patients into the following subgroups: subgroup S_0– she/he did not know whether she/he had an infection at the disease onset (n = 42, 18.5%); subgroup S_1– she/he did not have an infection at the disease onset (n = 43, 18.9%); subgroup S_2– she/he had an infection at the disease onset, but this infection was not confirmed with a lab test (n = 93, 41.0%); subgroup S_3– she/he had an infection at the disease onset and this infection was confirmed with a lab test (n = 48, 21.1%). In the participant questionnaire, patients were also asked to narrate the factors that could have triggered or contributed to the disease. Given that this was an open question, we only performed a brief description of the respective responses.

All individuals had age between 18 and 60 years old. Patients with ME/CFS were referred for a possible inclusion in the UKMEB by general practitioners working in the National Health System (NHS) of the United Kingdom. The respective diagnosis was confirmed using the 1994 Centers for Disease Control and Prevention (CDC) (34) or the 2003 Canadian Consensus Criteria (35) by the UKMEB dedicated clinical research team, according to their designed clinical protocol (36). Participants were excluded if they were taking any anti-viral drug or any medication that could alter their immune function in the three preceding months. Healthy controls were either family members or friends of the recruited patients with ME/CFS, or they were volunteers recruited by advertisement within Higher Education Institutions. Detailed information about exclusion and inclusion criteria of the UKMEB and additional information about recruitment and sample processing can be found elsewhere (36, 37).

Herpesviruses Serology

Serological data and the respective laboratory procedures were previously described in the original study (32). However, given that the main focus of this early study was cellular immunology,

the description of herpesviruses serology was kept to a minimum. We have now provided some additional details.

The following commercial ELISA assays from Demeditec Diagnostics (Kiel, Germany) were used to quantify the plasma concentrations of IgG antibodies against the following viruses: the human cytomegalovirus (CMV; Prod. Ref. DECMV01), EBV-VCA antigen (Prod. Ref. DEEBVG0150), EBV-EBNA1 antigen (Prod. Ref. DE4246), herpes simplex virus-1 (HSV1; Prod. Ref. DEHSV1G0500), herpes simplex virus-2 (HSV2; Prod. Ref. DEHSV2G0540), Varicella-Zoster virus (VZV; Prof. Ref. DEVZVG0490). The commercial ELISA-VIDITEST from VIDIA (Vestec, Czech Republic) was used for the IgG quantification against HHV6 (Prod. Ref. ODZ-235).

Antibody quantification was expressed in arbitrary units per milliliter (U/ml). According to manufacturer's instructions, seropositivity was considered for all samples with concentration $\geq$ 12 U/ml for HSV1, HSV 2, VZV, CMV and EBV antigens. Likewise, individuals with antibody concentrations against HHV6 $\geq$ 12.5 U/ml were considered seropositive.

Statistical Analysis

To compare the age of the participants, the age of disease onset, and the disease duration of different study groups and/or subgroups of ME/CFS patients, we used the non-parametric Kruskal-Wallis test. To compare the gender distribution in the same subgroups, we applied the Pearson's χ^2 test for testing independence in two-way contingency tables. For simplicity of the analysis, we only reported frequencies and the respective percentages of different disease triggers in the subgroups of ME/CFS that mentioned the occurrence of such triggers.

We previously performed thorough analyses of different cutoff values for seropositivity to each viral antigen (38, 39). These earlier analyses were based on the comparison and the selection of different scale mixtures of skew-normal distributions and four different criteria to define seropositivity. In accordance with a sensitivity analysis, instead of selecting a fixed cutoff, we here allowed this cutoff to vary between 10 U/ml and 100 U/ml with a lag of 1 U/ml. For each cutoff of a given antibody, we first estimated the unadjusted seropositivity odds ratio (OR) and the area under the Receiver Operating Characteristic curve (AUC) between each ME/CFS subgroup and the healthy controls using a logistic regression model in which seropositivity status of the individuals and a group indicator were the outcome and the covariate, respectively.

We then adjusted these ORs and AUCs using a similar logistic regression model but including age, gender, and a group indicator variable as the respective covariates. In both unadjusted and adjusted analyses, the effect of healthy controls was set as the reference of the group indicator variable. We calculated the *p*-values of the Wald's score test to assess the significance of different log-ORs of each subgroup of ME/CFS in relation to the group of healthy controls. The significance level of each executed test was set at 5%. In **Supplementary File**, one can find a detailed description of the likelihood function of the regression models used and how the ORs (or the log-ORs) are related to the parameters of these models.

To investigate the impact of multiple testing on the results, we adjusted the raw *p*-values of the Wald's score tests in order to ensure a false discovery rate of 5%. With this purpose, we used the Benjamini-Hochberg (BH) and Benjamini-Yekutieli (BY) procedures under the assumption of independent and positively correlated tests, respectively (40, 41). In this analysis, adjusted $p < 0.05$ were indicative of statistically significant associations.

Finally, we estimated the statistical power of the detected associations using a parametric Bootstrap approach (42). For each antibody, we used the following algorithm: (i) determine the optimal seropositivity cutoff by maximizing the likelihood ratio statistic as a function of this parameter when comparing the above logistic models with and without the group indicator covariate; (ii) generate the seropositivity data resulting from the optimal cutoff; (iii) estimate a logistic model including the group indicator as the only covariate (unadjusted analysis) or a logistic model including age, gender and group indicator variables as the respective covariates (adjusted analysis) using the seropositivity data obtained in (ii); simulate 1,000 data sets using the seropositive probability estimates obtained from models fitted in (iii); (iv) calculate the power of the association between seropositivity and each study group by the proportion of simulated data sets in which the association was deemed significant at the 5% significance level using the Wald's score test as described above.

The statistical analysis was conducted in the R software version 4.0.3. In particular, the estimation of the logistic regression models was done using the "glm" command and the analysis based on the AUC was conducted using the package "pROC" (43). In the multiple testing analysis, the raw *p*-values were adjusted by the BH and BY procedures using the package "MASS" (44). The corresponding scripts are available from the first or the corresponding author upon request.

RESULTS

Basic Characterization of Study Participants

The four subgroups of ME/CFS had the same age distribution approximately (Kruskal-Wallis test, $p = 0.30$) with means of 44.6, 40.7, 43.3, and 40.9 years old for S_0, S_1, S_2, and S_3, respectively (**Table 1**). The respective mean ages of disease onset were 32.1, 30.2, 31.3, and 27.3 years old, while the mean disease durations were 12.7, 11.6, 12.1, and 13.5 years for the same subgroups. The differences in these variables were not statistically significant ($p = 0.55$ and 0.21, respectively). Similarly, the percentages of female patients ranged from 70.8% to 80.6%, but they were not statistically different (Pearson's χ^2 test, $p = 0.62$).

Overall, the percentage of severely affected patients significantly differed among the subgroups (Pearson's χ^2 test, $p = 0.003$). In particular, the percentage of these patients in S_0 and S_1 was approximately 9%. This value was in clear contrast with the 30% of severely affected patients belonging to S_2 and S_3, both groups related to infection triggers.

In terms of the number of narrated disease factors or triggers reported in the participant's questionnaire, the subgroup S_1 had

TABLE 1 | Basic characteristics of study participants where patients with ME/CFS were split into four subgroups according to the responses about their disease triggers in the symptoms' assessment questionnaire.

	Healthy controls ($n = 99$)	Subgroups of ME/CFS patients				Comparison (*P*-values)	
		S_0 ($n = 42$)	S_1 ($n = 43$)	S_2 ($n = 93$)	S_3 ($n = 48$)	ME/CFS subgroups	ME/CFS subgroups + Healthy controls
Female (%)	73 (73.7)	33 (78.6)	33 (76.7)	75 (80.6)	34 (70.8)	0.62	0.69
Mean age (IQR)	41.9 (32–51.5)	44.6 (35.0–53.8)	40.7 (28.0–52.0)	43.3 (35.0–53.0)	40.9 (32.0–50.3)	0.30	0.44
Mean age of disease onset (IQR)		32.1 (21.9–43.5)	30.2 (20.2–39.3)	31.3 (22.1–39.1)	27.3 (18.9–36.2)	0.21	-
Mean disease duration (IQR)	-	12.7 (5.30–17.90)	11.6 (4.2–15.9)	12.1 (5.5–16.5)	13.5 (6.0–19.2)	0.55	-
Disease severity at recruitment							
Mild/moderate (%)	-	38 (90.5)	39 (90.7)	64 (68.8)	34 (70.8)	0.003	-
Severely affected (%)	-	4 (9.5)	4 (9.3)	29 (31.2)	14 (29.2)	-	-
Number of self-reported disease triggers							
Single	-	-	19 (44)	52 (56)	32 (67)	<0.001[a]	-
Multiple	-	-	10 (23)	35 (38)	11 (23)	-	-
Missing	-	-	14 (33)	6 (6)	5 (10)	-	-

S_0, Do not know; S_1, Non-infection trigger; S_2, An infection trigger but not confirmed with a lab test; and S_3, An infection trigger confirmed with a lab test.
[a]*Pearson's χ^2 test including the missing as a category for the number of disease factors/triggers.*

TABLE 2 | Frequency and the respective percentage within brackets of specific disease factors or triggers narrated by patients from the subgroups S_1 ($n = 43$), S_2 ($n = 93$), and S_3, ($n = 48$) in the participant's questionnaire.

Narrated disease trigger	Subgroups of ME/CFS patients			Total (%)
	S_1 (%)	S_2 (%)	S_3 (%)	
Glandular Fever; tonsilitis; EBV infection	1 (2)	25 (27)	22 (46)	48 (21)
Respiratory infection; pneumonia	1 (2)	6 (6)	3 (6)	10 (4)
Flu-like infection or illness	2 (5)	20 (22)	4 (8)	26 (11)
Gastrointestinal infection	0 (0)	6 (6)	3 (6)	9 (4)
Tropical infections	0 (0)	1 (1)	2 (4)	3 (1)
Other infections including unspecified viral infections	2 (5)	33 (35)	13 (27)	51 (22)
General stress or anxiety	6 (14)	11 (12)	3 (6)	20 (9)
Stress due to personal events	9 (21)	6 (6)	3 (6)	18 (8)
Stress at work or school	4 (9)	5 (5)	2 (4)	11 (5)
Vaccinations	0 (0)	4 (4)	6 (12)	10 (4)
Chemical exposure	1 (2)	6 (6)	0 (0)	7 (3)
Accidents/Injuries/Surgeries	7 (16)	2 (2)	2 (4)	11 (5)
Pregnancy/Childbirth/Postnatal/ Hysterectomy/Endometriosis	6 (14)	0 (0)	0 (0)	6 (3)
Other	4 (9)	6 (6)	0 (0)	7 (3)

the lowest percentage of patients reporting a single factor or trigger for their disease (44%) when compared to infection-related subgroups S_2 and S_3 (56 and 67%, respectively; **Table 1**). The same subgroup was the one with the highest percentage of missing data to this question (33% for S_1 vs. 6 and 10% for S_2 and S_3, respectively). Overall, the distribution of the number of reported disease factors or triggers was significantly different among subgroups S_1, S_2, and S_3 (Pearson's χ^2 test, $p < 0.001$) mostly due to differences in the amount of missing data.

These subgroups of ME/CFS patients were well matched for gender and age with respect to the healthy control group (Pearson's χ^2 and Kruskal-Wallis tests, $p = 0.69$ and 0.44, respectively).

Disease Factors or Triggers Narrated by Different Subgroups of ME/CFS Patients

When the 184 patients belonging to the subgroups S_1, S_2, and S_3 were asked to narrate the factors or triggers of their disease in the participant questionnaire, 103 (56%) and 56 (30%) of them reported single and multiple factors (or triggers), respectively. However, 25 patients (14%) did not mention any specific trigger or factor contributing to their disease. In total, there were 14 distinct categories of disease triggers narrated by the patients. These categories were consistent to the ones reported by previous epidemiological studies about possible triggers of ME/CFS (22, 23).

The following non-infection factors or triggers were mentioned by patients mostly belonging to the subgroup S_1: stress subdivided into general anxiety (9%, $n = 20$), personal (8%, $n = 18$) or professional-related stress (5%, $n = 11$); accidents/injuries/surgeries (5%, $n = 11$); pregnancy, childbirth and other problems related to women's reproduction system (3%, $n = 6$), and other non-infection triggers (**Table 2**). The remaining factors were related to microbial infections and/or infectious diseases: upper respiratory tract infections – glandular fever (GF), tonsillitis, EBV infections, or throat infection (21%, $n = 48$); lower respiratory tract infections – chest infection or pneumonia (4%, $n = 10$); flu- or cold-like illnesses (11%, $n = 26$); gastrointestinal problems and related infections (4%; $n = 9$); and tropical infectious diseases – Dengue fever and schistosomiasis (1%, $n = 3$); and other viral or bacterial infection, and unspecified infections (22%, $n = 51$). Note that 6 patients from subgroup

S_1 mentioned an infection in the narrative question about the factors or triggers of their disease. However, the same patients also reported other possible non-infection triggers, such as trauma, bereavement, and stress. We speculate that these patients attributed a higher likelihood to these non-infection disease triggers when answering the related question in the symptoms' assessment questionnaire. Interestingly, patients belonging to the subgroup S_3 reported the highest percentage of disease factors or triggers consistent with an EBV infection (46%, $n = 22$). Patients from subgroup S_2 also self-reported a high frequency of EBV-related factors or triggers (27%, $n = 25$), but closely matched by a flu-like infection or illness (22%, $n = 20$).

Serological Data Analysis by Subgroup of ME/CFS Patients

We then compared the serological data of these ME/CFS subgroups of patients with the group of healthy controls (**Figure 1**). In this analysis, we intended to investigate the impact of cutoff on the resulting seropositivity OR and AUC between each subgroup of ME/CFS patients and the group of healthy controls.

With respect to unadjusted analysis, the AUCs were in most cases estimated between 0.50 and 0.60 (**Figure 2A**). This finding suggested that serological data had limited predictive power to discriminate the seropositivity of subgroups of ME/CFS patients from that of healthy controls. The highest estimated AUC was approximately 0.75 for VZV when comparing the seropositivity of the subgroup S_2 to healthy controls using cutoffs below 15 U/ml.

According to the OR estimates, we could not find any significant association of subgroups S_0 and S_2 with herpesviruses serology (**Figures 2B,C**). The only exception was a putative association between the subgroup S_0 and the antibodies against EBV-VCA using a cutoff of 37 U/ml. Interestingly, before correcting for multiple testing, we found significant negative associations (i.e., negative log-ORs) between the subgroup S_1 and antibodies against EBV-VCA, EBV-EBNA1, and VZV depending on the cutoff used (**Figures 2B,C**). These negative associations suggested decreased seroprevalences to these herpesviruses in this subgroup when compared to the group of healthy controls. When controlling for multiple testing, these associations were not considered statistically significant using either BH or BY procedures (**Figures 2D,E**).

We also found a significant negative association between subgroup S_3 and CMV seropositivity (**Figures 2B,C**), which suggested decreased antibody levels in this subgroup of patients in relation to group of healthy controls. The corresponding AUC was estimated around 0.65 for most of cutoffs (**Figure 2A**). This association was consistent across the range of cutoffs specified for the analysis and even after controlling for multiple testing using the BH procedure based on the assumption of independent tests (**Figure 2D**). However, the statistical significance of the association was lost after using the BY procedure based on the assumption of positively correlated tests (**Figure 2E**).

Similar findings were obtained when adjusting for possible confounding effects of age and gender (**Supplementary Figures 2A–E**). This agreement between unadjusted and adjusted analyses can be explained by a good matching between the different ME/CFS subgroups of patients and healthy controls in terms of age and gender (**Table 1**). However, the significance of adjusted ORs was slightly reduced due to these putative confounding factors (**Supplementary Figure 2C**).

Finally, we estimated the statistical power related to the identified associations using the optimal seropositivity cutoff for each herpesvirus antibody. For the unadjusted analysis, these optimal cutoffs varied from 11 U/ml to 90 U/ml (**Supplementary Figure 1** and **Supplementary Table 1**). Similar optimal cutoffs were obtained for the analysis adjusting for age and gender (**Supplementary Figure 3** and **Supplementary Table 1**) with the exception of EBV-EBNA1 for which the optimal cutoffs were 72 U/ml and 88 U/ml for the unadjusted and adjusted analyses, respectively. The maximum power (∼90%) was obtained for the association between CMV seropositivity and ME/CFS subgroup S_3 in either unadjusted or adjusted analyses (**Supplementary Figure 4**). A high power (∼75%) was also obtained for the associations between VZV seropositivity and ME/CFS subgroup S_1. The remaining associations between each study group and herpesvirus seropositivity had a power that did not exceed 60%. In conclusion, the manufacturer's seropositivity cutoffs were not the most adequate to maximize the chance of finding an association of ME/CFS subgroups with the herpesviruses serology and only three associations between the study groups and herpesviruses seropositivity had a high statistical power.

DISCUSSION

In contrast with the original study where we could not find differences related to herpesviruses serology between healthy controls and ME/CFS patients divided according to their disease severity (32), our re-analysis of the same data identified two subgroups of ME/CFS patients (S_1 and S_3) in which such differences are now statistically significant. This new finding was only possible due to the stratification of patients according to a question related to the occurrence of an infection at disease onset together with a sensitivity analysis of the seropositivity cutoff used. Patients' stratification or subtyping was performed in line with past recommendations for ME/CFS research (28). Following this recommendation, we previously performed an immunological investigation based on a stratification of ME/CFS patients according to the severity of their symptoms (32). By using this stratification, we showed perturbations in the T-cell compartment, namely, in effector $CD8^+$ T cells and in the mucosal-associated invariant T cells. In another study using similar stratification of the samples from the UKMEB, the levels of the cellular stress biomarker GDF15 were found to be increased in severely affected patients at different time points (45). We speculate that other immunological perturbations could be detected if our alternative stratification could have been used. This investigation will be carried out in the near future.

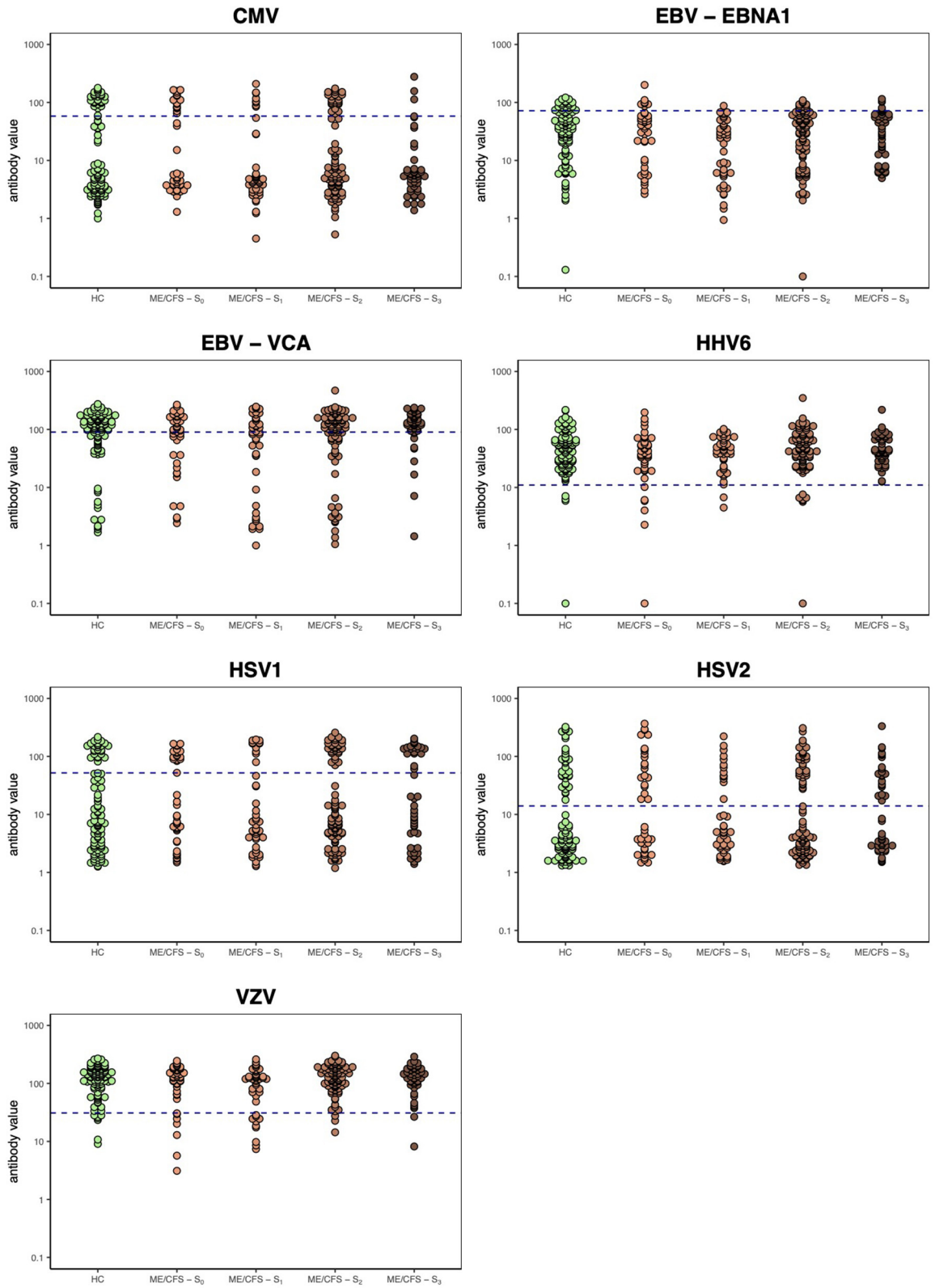

FIGURE 1 | Herpesvirus serology data per study group including the four ME/CFS subgroups. Horizontal dashed lines represent the optimal seropositivity cutoff for the unadjusted analysis according to the maximization of likelihood ratio statistic for testing the significance of the group indicator covariate in the logistic models (see **Supplementary Figure 1**).

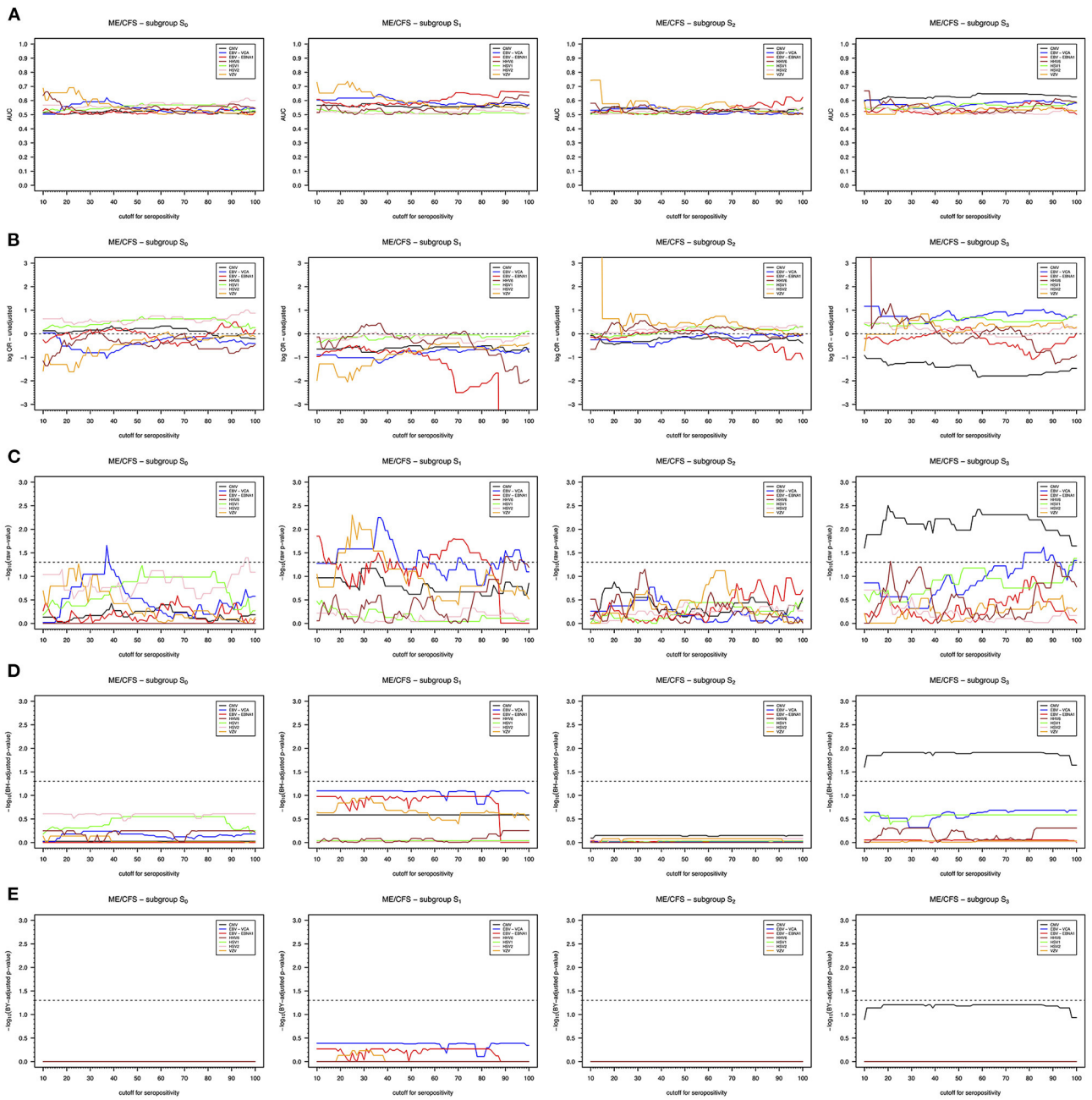

FIGURE 2 | Unadjusted association analysis of seropositivity to different herpesvirus antigens as function of the cutoff defining seropositivity. **(A)** AUC of the probabilities of being seropositive estimated in each subgroup of ME/CFS patients when compared to the same probabilities estimated in the group of healthy controls. **(B)** Log-ORs of being seropositive in each subgroup of ME/CFS patients in relation to the group of healthy controls. **(C)** Raw *p*-values of Wald's score test for the significance of log-ORs. **(D)** The corresponding BH-adjusted *p*-values. **(E)** The corresponding BY-adjusted *p*-values. For convenience, raw and adjusted *p*-values were transformed as $-\log_{10}(p\text{-value})$. The dashed lines in the respective plots represent the threshold referring to the 5% significance level [i.e., $-\log_{10}(0.05)$; **C**] or the 5% false discovery rate. **(D,E)** Cutoff values in which $-\log_{10}(p\text{-values})$ are above these thresholds provided evidence for significant associations.

In line with our findings, evidence has been emerging that the occurrence of an acute infection at the onset of disease symptoms is indeed a key stratifying factor to detect genetic and immunological differences between subgroups of ME/CFS patients when compared to healthy controls (17, 46). However, the simplistic approach of dividing patients according to non-infection and infection triggers might not be sufficient to obtain relatively homogeneous subgroups of ME/CFS patients, which affects the statistical power to detect any disease-specific effects. Besides the limited choice of antibodies against different

herpesvirus-related antigens, the large heterogeneity in infectious triggers seems a possible explanation for the lack of association between the subgroup S_2 and herpesviruses seropositivity. Notwithstanding not having their infection trigger confirmed in the lab, patients from this subgroup reported the highest proportion of flu-like illnesses, which could have been caused by the influenza virus, the rhinovirus, or the respiratory syncytial virus (47). It is then conceivable that these patients exhibit different pathological mechanisms of ME/CFS according to the causative virus, some of which without any direct impact on the antibody responses against herpesviruses. To overcome these problems, we recommend the collection of infection-trigger data as detailed and accurate as possible.

Our most consistent association was obtained between CMV seropositivity and patients from the subgroup S_3. These patients tended to be less seropositive to this herpesvirus when compared to healthy controls, irrespective of the seropositivity cutoff value used. Previously, different serological investigations did not provide conclusive evidence for the role of CMV on ME/CFS pathogenesis, as reviewed elsewhere (24). The lack of or the use of an inadequate stratification could also explain these past findings. In this regard, the unveiled association was obtained in a subgroup in which the accuracy of the reporting might be the highest, because the disease-triggering infections were supposedly confirmed in the lab. However, we cannot ignore the fact that this subgroup has a large fraction of patients whose disease trigger was related to an EBV infection, one of the most reported causative agents of ME/CFS. Therefore, it is possible that our finding resulted from a coincidence between a low-resolution patient's stratification and a random enrichment of a specific infection trigger in one of the subgroups.

A supposedly decreased seropositivity (or antibody levels) to CMV in an EBV-infection trigger could be explained by the hyperregulation hypothesis (11). According to this hypothesis, a possible pathological mechanism of ME/CFS is related to an expansion of regulatory $CD4^+$ T cells (Tregs) driven by an autoimmune response against a viral antigen that mimics a self-antigen. This expansion of Tregs upon herpesvirus infection or reactivation locks the (adaptive) immune system in an active state of hyperregulation where different infections are more difficult to be cleared from the body. Frequent infections are in fact reported by patients with ME/CFS (33). The question is then how the expansion of Tregs could affect antibody responses against CMV. The so-called follicular Tregs might hold the answer to this question. These specialized Tregs are derived from Treg precursors with the ability to migrate to germinal center reactions (GCRs) to inhibit the respective antibody production and antibody maturation (48). In particular, experiments with animal models demonstrated that the amount of IgG antibodies against different foreign antigens is increased in immunized mice depleted of follicular Tregs (49, 50). In this line of thought, it is reasonable to assume that an increased proportion of Tregs in ME/CFS could be translated into an increased proportion of follicular Tregs. This increase could in turn decrease the antibody production derived from GCRs. We can then hypothesize that an EBV infection triggered an autoimmune response that disrupted the normal balance between Tregs and effector $CD4^+$ T cells; a peptide of the viral EBNA6 was found to share a high sequence homology with the human lactoperoxidase and thyroid peroxidase (30). The disruption of this balance could lead to an increase of both natural and follicular Tregs. A possible consequence of this increase is a diminished antibody production against a posterior CMV infection or reactivation. Note that several peptides from CMV were also found as putative candidate for molecular mimicry with human proteins (51). Similar to the situation of immunosuppression, a reduction in the humoral immunity against CMV would render ME/CFS patients more susceptible to a possible reactivation of this virus (52). It is worth noting that the role of follicular Tregs was never investigated in ME/CFS patients.

Another interesting finding is the possible association between the subgroup S_1 and EBV and VZV seropositivity. This subgroup refers to patients who reported non-infectious triggers, mostly related to stressful or stress-related events. It is also a group where ME/CFS was triggered in many women who had problems during and after pregnancy, had difficult childbirth or had disorders related to women's reproduction system. In line with this finding, stressful conditions and events such as the ones experienced by astronauts increase the chance of herpesvirus reactivation, specifically, EBV, VZV and CMV (53). Reactivation of latent herpesvirus infections could be explained by an increase in production of stress-related hormones together with an inflammatory cytokine signature that debilitates the immune system. This subgroup is then expected to have a higher prevalence of active herpesvirus infections than the remaining subgroups of ME/CFS patients and healthy controls. Given that this subgroup could represent $<$50% of the patients (22, 23), it is likely to have insufficient statistical power to detect any differences in herpesvirus reactivation rates between ME/CFS and healthy controls even in the case of a proper stratification of the patients' populations. This limitation is yet another reason that could explain the inconsistent findings on herpesvirus reactivation across many studies on ME/CFS.

We did not find any association between the subgroup S_0 and herpesviruses seropositivity. This subgroup represented 18.5% of the patients' cohort, a value compatible with the percentages of patients that did not report any disease triggering event from past epidemiological studies [10%, (22); 24%, (54)]. The sample size of this subgroup was not very large and, therefore, we cannot rule out that our lack of associations could be simply due to insufficient statistical power to detect putative associations between this subgroup and herpesviruses seropositivity.

In our association analysis, we allowed the seropositivity cutoff to vary within a given range of possible values, similarly done in a recent study of molecular mimicry between Anoctamin 2 and EBNA1 in multiple sclerosis (55). This analytical approach seems reasonable given the difficulty to choose the best seropositivity cutoff among the different criteria and methods available, as illustrated in the earlier analyses of the same data (38, 39). This approach is also in line with several discussions about seropositivity estimation and the sensibility to use a fixed cutoff (56–59). However, varying the cutoffs defining seropositivity might increase the chance of false positives due to the multiple testing problem. We attempted to overcome this problem by

controlling the false discovery rate, but the small sample size of each subgroup of ME/CFS did not allow to reach statistical significance of the detected associations when using the BY procedure based on the assumption of multiple positively correlated tests. On the other hand, our power calculations suggested a high probability of detecting some of the associations under the assumption that they were actually true. Therefore, the correct application of cutoff-varying approach should include a thorough assessment of a putative multiple testing problem together with a power calculation in order to assess the statistical consistency of the findings.

As final remarks, we should also note that cutoffs for detecting associations between herpesviruses and ME/CFS might vary from one lab to another and with the serological kits used. In addition, a high cutoff for the data might not define seropositivity *per se*, but rather a high antibody response whose detection could be the primary objective of the analysis (30, 31). The use of a high cutoff is also in accordance with a modeling approach where seropositivity might be subdivided into different levels (60–62). Therefore, our sensitivity-like approach seems to have the capacity to detect further serological associations beyond the ones based on the classical seroprevalence. Such a capacity could increase the chance of reaching scientific reproducibility. We then recommend the routine use of this approach in future serological investigations of ME/CFS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this paper will be made available from Eliana M. Lacerda upon request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by London School of Hygiene & Tropical Medicine (LSHTM) Ethics Committee (Ref. 6123) and the National Research Ethics Service (NRES) London-Bloomsbury Research Ethics Committee (REC ref. 11/10/1760, IRAS ID: 77765). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NS conceived this research. HM and NS supervised this research. J-SL and JC generated the serology data. AG and EL compiled and curated the data related to ME/CFS triggers. EL and LN were responsible for data collection of the UKMEB. TD, AG, JA-A, and NS performed the data analysis. FW, CS, LN, and HM helped in data interpretation. TD, AG, and NS wrote the manuscript. All authors have revised, read, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.686736/full#supplementary-material

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Salivary DNA Loads for Human Herpesviruses 6 and 7 Are Correlated With Disease Phenotype in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a complex chronic condition affecting multiple body systems, with unknown cause, unclear pathogenesis mechanisms, and fluctuating symptoms which may lead to severe debilitation. It is frequently reported to have been triggered by an infection, but there are no clear differences in exposure to, or seroprevalence of, any particular viruses between people with ME/CFS and healthy individuals. However, herpes viruses have been repeatedly hypothesized to underlie the chronic relapsing/remitting form of MS/CFS due to their persistence in a latent form with periodic reactivation. It is possible that ME/CFS is associated with herpes virus reactivation, which has not been detectable previously due to insufficiently sensitive testing methods. Saliva samples were collected from 30 people living with ME/CFS at monthly intervals for 6 months and at times when they experienced symptom exacerbation, as well as from 14 healthy control individuals. The viral DNA load of the nine humanherpes viruses was determined by digital droplet PCR. Symptoms were assessed by questionnaire at each time point. Human herpesvirus (HHV) 6B, HHV-7, herpes simplex virus 1 and Epstein-Barr virus were detectable within the saliva samples, with higher HHV-6B and HHV-7 viral loads detected in people with ME/CFS than in healthy controls. Participants with ME/CFS could be broadly separated into two groups: one group displayed fluctuating patterns of herpesviruses detectable across the 6 months while the second group displayed more stable viral presentation. In the first group, there was positive correlation between HHV-6B and HHV-7 viral load and severity of symptom scores, including pain, neurocognition, and autonomic dysfunction. The results indicate that fluctuating viral DNA load correlates with ME/CFS symptoms: this is in accordance with the hypothesis that pathogenesis is related to herpesvirus reactivation state, and this should be formally tested. Herpesvirus reactivation might be a cause or consequence of dysregulated immune function seen

in ME/CFS. The sampling strategy and molecular tools developed here permit such large-scale epidemiological investigations.

Keywords: ME/CFS, human herpesvirus, digital droplet PCR, DNA viral load, Clinical specimens

INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a disease of unknown aetiology, causing persistent, or recurrent incapacitating fatigue; a hallmark symptom is post-exertional malaise (PEM). Other symptoms, which are present to a varying degree in different individuals, include pain and disturbances in immune function along with lymphadenopathy, unrefreshing sleep, cognitive difficulties, and dysfunction of the endocrine and autonomic nervous systems (1, 2). Despite a prevalence in Europe ranging from 0.1 to 2.2% (3), there is still no diagnostic test for ME/CFS, hampering research into its cause and pathogenesis, and development of treatments. While the risk of developing ME/CFS is likely to be multifactorial, in ∼50% of cases ME/CFS symptoms appear after a "viral-like" illness, and it is possible that an infectious disease can trigger ME/CFS onset. However, no single causative pathogen has been identified for ME/CFS, despite extensive research (4).

Although, there have been reports that bacterial infections such as Lyme disease (5) or Q fever (6) can trigger ME/CFS, it is mostly commonly viral infections, especially those from the herpesvirus family, which have been more widely associated with ME/CFS (4). There are nine members of the human herpesvirus (HHV) family which naturally infect humans: herpes simplex virus-1 and -2 (HSV-1, HSV-2), varicella-zoster virus (VZV), which is the causative agent of chicken pox and shingles, Epstein-Barr virus (EBV), which causes of infectious mononucleosis, human cytomegalovirus (HCMV), HHV-6 including subtypes HHV-6A and HHV-6B, HHV-7, and Kaposi's sarcoma virus (KSHV). These viruses are all of potential interest in ME/CFS due to their ability to latently infect either neurons or immune system cells, both of which may be affected in ME/CFS. There has been speculation that Epstein-Barr Virus (EBV) is involved in ME/CFS pathogenesis for many years (7, 8), although, reports are inconsistent (9, 10). Reports of the potential involvement of CMV in ME/CFS are also mixed, with higher anti-CMV antibodies reported in serum in ME/CFS compared to healthy controls (HCs) in one study (11) but no correlation in another (12). HHV-6 and HHV-7, which are from the *Roseolovirus* genus of the *Beta-herpesvirinae* subfamily, cause widespread long-term persistent infection with reported population prevalence of >90% (13) with infection usually occurring within the first 3 years of life. In early studies, there was serological indication of HHV-6 reactivation with higher specific anti-HHV-6 serum antibody concentration in people living with ME/CFS than in HCs (14, 15) but these data have been contradicted in other studies (16). Similarly, HHV-7 infection is commonly detectable in people living with ME/CFS, but the detection rate of HHV-7 DNA is similar in ME/CFS patients and in HCs (17).

Thus, reports of association between herpesvirus infection and/or reactivation and ME/CFS are inconsistent. This may be due partly to small sample sizes in some studies, and to heterogeneity within and between ME/CFS populations included in different studies. We did not find any difference in seroprevalence or antibody titres against the eight herpesviruses between people living with ME/CFS and HCs in our previous work (18). However, a hallmark feature of herpes viruses is their life cycle in which, following an acute infection, they remain in the host in a latent persistent form, and can reactivate following immune system disturbance. Measurements of antibody titre by ELISA and qPCR analysis of viral DNA concentration in blood may be too insensitive to fully distinguish reactivation events from latent infection, and a more sensitive analysis might enable the full characterisation of herpesvirus reactivation events in ME/CFS, leading to a definitive answer regarding the role of herpesvirus infections in this condition. Digital droplet PCR (ddPCR) permits absolute quantification of nucleic acids (19). This is achieved *via* an emulsion process in which the fluorescent probe-based PCR reaction is partitioned into ∼15,000 highly uniform, one nanolitre volume, reverse (water-in-oil) micelles that are stable at high temperatures: each droplet is essentially an independent nano-PCR. Enumeration of the number of positive and negative droplets post-PCR gives a sensitive and precise readout of the template DNA concentration.

In this study, we hypothesised that ME/CFS may either follow an acute infection with a herpes virus, with symptoms being triggered by a virus reactivation followed by a state of "aberrant homeostasis" (20), or that in people with ME/CFS due to another cause (infectious or not) reduced control of herpes virus latency leads to periodic reactivation and exacerbation of symptoms. Although, herpes virus infections are common, it is possible that people living with ME/CFS experience prolonged or more frequent reactivation events than healthy individuals and are unable to consistently contain a latent infection. We aimed to develop highly sensitive and specific ddPCR assays for the humanherpes family viruses, and use them to test for correlation between viral load in saliva, an accessible sample likely to be involved in transmission (16), and the clinical manifestations of disease over time, particularly during disease exacerbation.

MATERIALS AND METHODS

Study Participants

Potential ME/CFS study participants were recruited to the UK ME/CFS Biobank (UKMEB) (21) through the UK National Health Service or referred *via* patient support groups with ME/CFS. They were required to have a confirmed medical diagnosis of ME/CFS and were re-assessed by clinical research staff to ensure compliance with the Canadian Consensus (22) and/or CDC-1994 ("Fukuda") (1) criteria, which were the study case definitions. Non-fatigued HC participants were recruited as friends or family of people with ME/CFS or by advertisement

in Higher Education Institutions and GP practices. Participants were aged between 18 and 60 years and provided written informed consent. Exclusion criteria included having taken anti-viral or immunomodulatory drugs within the previous 3 months, chronic comorbid disease (including hypothyroidism; hyperthyroidism; cancer; rheumatoid arthritis; fibromyalgia; other serious rheumatic disease, such as lupus or polymyositis (but not osteoarthritis); heart disease, (such as heart failure); severe COPD or other severe ongoing respiratory disease; severe anaemia; kidney failure; diabetes; Addison's or Cushing's disease; any of bipolar disorder, schizophrenia, major depression, anorexia, or bulimia; multiple sclerosis; Parkinson's Disease; Myasthenia Gravis or other serious neurological disease (except for ME/CFS); sleep apnoea/narcolepsy), a history of infectious disease such as tuberculosis or hepatitis B or C, excessive consumption of alcohol or recreational drugs, pregnancy, and morbid obesity. Inclusion criteria included HHV DNA detected in either plasma or PBMC (DNA positive) or HHV DNA not detected in either sample (DNA negative (as described below, 5.2). Ethical approval was granted by the London School of Hygiene & Tropical Medicine (LSHTM) Ethics Committee (Ref. 6123) and the National Research Ethics Service (NRES) London-Bloomsbury Research Ethics Committee (REC ref. 11/10/1760, IRAS ID:77765). Peripheral Blood Mononuclear Cells (PBMC) and plasma samples were cryopreserved and stored from all UKMEB participants (21) and accessed for this study.

Longitudinal Analysis

Selected participants from the UKMEB cohort provided further written informed consent for this longitudinal study. Participants were sent GeneFiX™ Saliva 2 ml DNA Collection tubes containing DNA stabilisation buffer and asked to return filled saliva tubes by post monthly for 6 months as well as on the first, third and fifth day of any disease exacerbation episode including acute illness or significant worsening of ME/CFS symptoms.

Clinical symptom assessment forms (**Supplementary Figure 1**), containing 58 questions with graded answers, were completed and returned at the same time as each saliva sample. From these data, symptom subgroup scores were developed, for immune, neuroendocrine, autonomic, neurocognition, pain, sleep, and post-exertional related symptoms; the Canadian Consensus Criteria assesses these domains for ME/CFS diagnosis (22). The scores ranging from "0" to "100" represent a weighted average of the reported symptoms on each domain, which are reported as "absent, mild, moderate, or severe."

DNA Extraction and Digital Droplet PCR

DNA was extracted from stored plasma samples using the QIAamp MinElute Virus Spin Kit (Qiagen), and from PBMC and saliva using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. Each DNA extraction run included a seronegative control sample and a Clinical Virology Multiplex I [National Institute for Biological Standards and Control (NIBSC)] positive extraction control: this comprised Adenovirus serotype 2, BK virus, CMV, EBV, HSV-1, HSV-2, HHV-6A, HHV-6B, JC virus, Parvo B19 virus, and VZV.

For assay development and to generate standard curves, titrated commercial quantitated DNA was used: HSV-1, HSV-2, VZV, & HHV-8 were purchased from Vircell (Granada, Spain), HHV-6A, HHV-6B, and HHV-7 were from ABI (Advanced Biotechnologies Inc, MD, USA) while EBV and CMV were from the National Institute for Biological Standards and Control (NIBSC).

Each 20 μl ddPCR reaction mix contained 10 μl ddPCR™ Supermix for Probes (Bio-Rad Laboratories, Hercules, USA), 9 μl of template DNA, 1 μl of 20X primer and probe mix at a final concentration 900 nM (for primer) and at 250 nM (for probe). Positive (commercial viral DNA) and negative (water) controls were included in every ddPCR run. Primer and probe sequences are shown in **Supplementary Table 1**, and were obtained from publications (23–28). The reaction mix was partitioned in oil-in-water with 70 μl of droplet generator oil using a QX-100 droplet generator (Bio-Rad). The generated droplets (around 40 μl) were transferred to ddPCR™ 96-Well Plates (Bio-Rad) using a multichannel pipette and covered with a pierceable PCR plate seal using a PX1™ PCR Plate Sealer (Bio-rad). PCR amplification was performed with the following thermal cycling: 95°C for 10 min, 40 cycles consisting of 94°C for 30 sec (denaturation) and 60°C for 1 min (extension), followed by 98°C for 10 min and holding at 12°C. After that, droplets (positive and negative) were analysed using a QX100 droplet reader (Bio-rad) and the target DNA concentration was calculated and presented as copies/μl in the reaction mixture using QuantaSoft software (Bio-Rad). The ddPCR result was determined as positive when the DNA samples showed at least three positive droplets from 10,000 to 15,000 droplets, according to the manufacturer's recommendation.

Herpesvirus Serology

Commercial ELISA kits were used to measure plasma concentrations of IgG to HSV-1, HSV 2, VZV, HCMV, EBV viral capsid antigen (VCA), EBV nuclear antigen 1 (EBNA 1) [from Demeditec Diagnostics (Kiel, Germany)] and HHV-6 [from VIDIA (Vestec, Czech republic)]: these data have been reported previously (18), and are included here for comparison. The antibody concentrations were calculated from standard curves. For qualitative evaluation of IgG antibodies to HSV-1, HSV-2, VZV, CMV, EBV VCA, EBV, EBNA-1, the concentration was interpreted as: concentration $\leq$8 U/ml = negative, concentration $\geq$12 U/ml = positive, and concentration between 8 and 12 U/ml = equivocal. For qualitative evaluation of IgG antibodies to HHV-6, concentration $\leq$10.5 U/ml was considered negative, while concentration $\geq$12.5 U/ml was considered positive and concentration between 10.5 and 12.5 was considered equivocal.

Data Analysis

Statistical analysis was conducted using GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA). Continuous variables (viral loads, viral persistency and symptom scores) were described using median and interquartile ranges (IQR) values or min

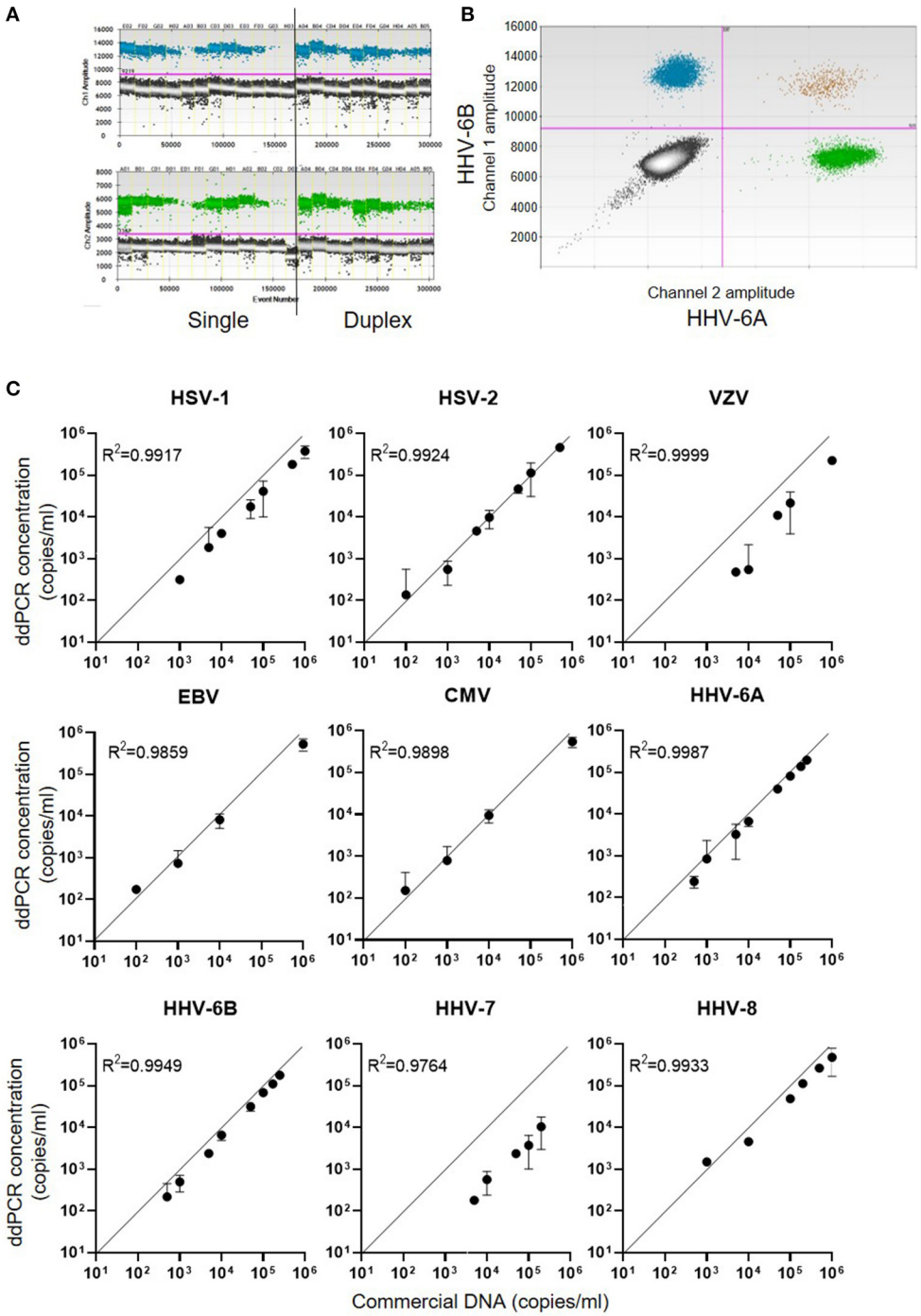

FIGURE 1 | DNA concentration measured by ddPCR assays for the nine human herpes viruses. **(A)** Example 1D dot plot data file for HHV-6A and HHV-6B ddPCR assays. The X-axes show quantities of commercial viral DNA in different assay plate wells (E02, F02, G02, etc.,). Assays were run either singly or in duplex as shown. The Y-axes indicate the fluorescence intensity within each PCR droplet, with the pink line indicating the threshold for positivity. **(B)** Example representation of 2D dot plot displaying duplex ddPCR of HHV-6B (FAM labelled, channel 1) and HHV-6A (HEX labelled, channel 2). **(C)** Standard curves obtained for each of the nine human herpesviruses, indicating the concentration of serial dilutions of commercial DNA on the X-axes, and the calculated concentration of viral DNA from the ddPCR assay on the Y-axes.

to max as indicated. Mann-Whitney test was used for viral load comparison of each HHV between clinical groups. The frequencies of HHV were compared using the chi-square test. For correlation analysis between HHV viral load and numerical clinical symptom scores or IgG titre from serological tests, the Spearman's correlation was utilised. Differences where $P < 0.05$

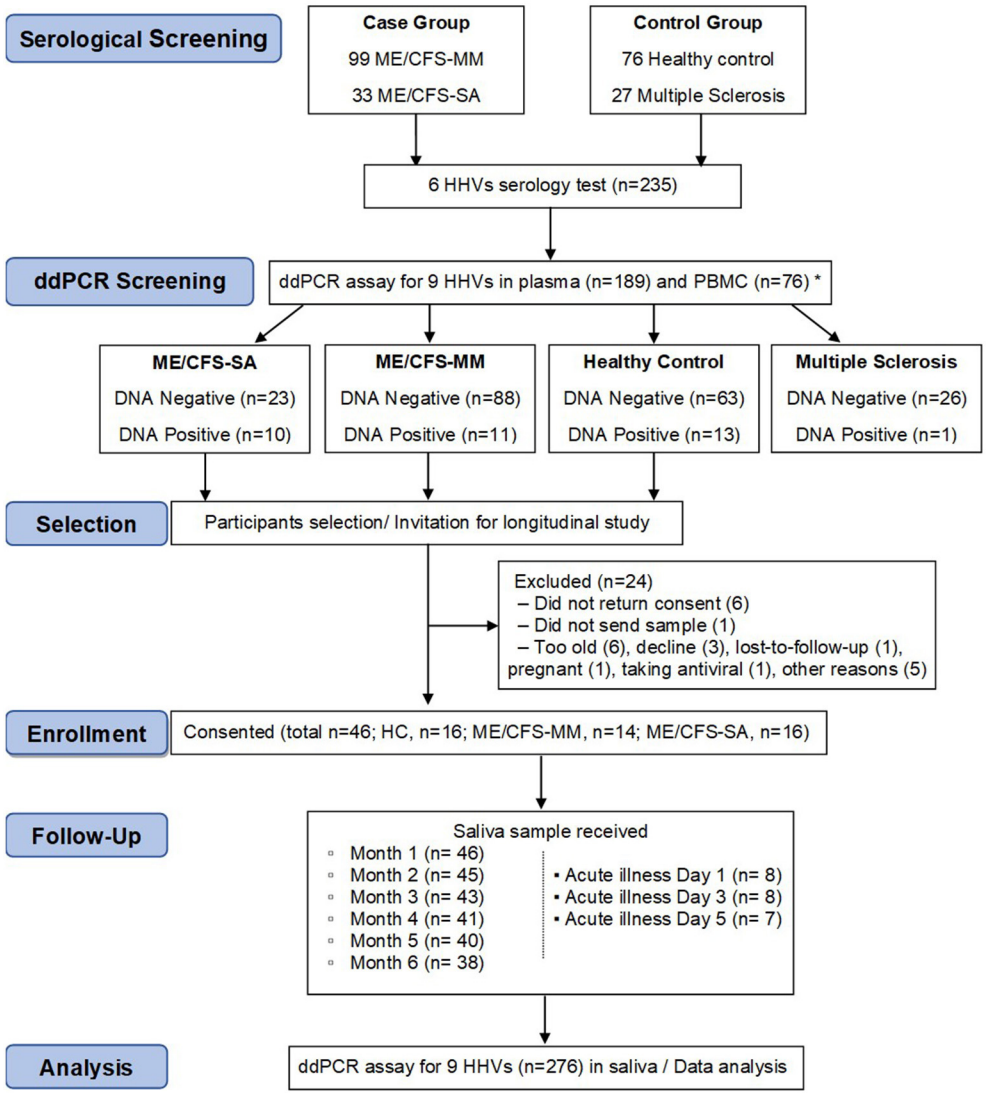

FIGURE 2 | Flow diagram of participant recruitment and follow up in the longitudinal saliva clinical study. Serological test ($n = 235$) and consecutive ddPCR assay in the plasma ($n = 189$) and PBMC ($n = 76$) were performed for HHV screening purposes. In total, 46 participants were enrolled into a 6-month longitudinal study to determine salivary HHV. Saliva samples (monthly and day 1, 3, and 5 of an acute illness or worsening of disease episode in people with ME/CFS, $n = 276$) were then analysed for HHV DNA concentration by ddPCR assay, with results compared with symptoms scores. *30 participants were tested for both plasma and PBMC.

are deemed significant, and in graphs *indicates $P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

RESULTS

Development of Sensitive and Specific ddPCR Assays for Human Herpes Viruses

For each of the nine members of the human herpes family of viruses, ddPCR assays were designed to run in duplex using specific primers and probes which were coupled to either FAM or HEX reporter labels (**Supplementary Table 1**). Examples of single and duplex ddPCR assays for HHV-6A and HHV-6B are shown in **Figure 1A**, with different proportions of positive and negative droplets reflecting the starting concentration of commercial DNA. In duplex assays spiked with both HHV-6A and HHV-6B DNA, the majority of ∼15,000 droplets measured were PCR-negative for both viruses, with substantial positivity observed for each individual virus but very few droplets deemed to have dual positivity (**Figure 1B**), as expected. Reproducible precise standard curves were obtained for each of the human herpesviruses (**Figure 1C**). Of note, the viral DNA copy number detected by ddPCR for HHV-7 and VZV was lower than the commercial DNA quantity description, over-estimates of DNA concentration by absorbance spectrophotometry are common (29).

Study Participant Selection: Detection of Human Herpes Virus DNA in Plasma and PBMC

The aim of the longitudinal clinical study was to test whether the concentration of HHV DNA in saliva reflected disease severity: in order to achieve this, we needed to identify potential participants who had previously been exposed to HHVs (**Figure 2**). Previously (18), concentrations of IgG antibodies against HSV-1, HSV-2, VZV, EBV, CMV and HHV-6 in plasma samples from 132 people with ME/CFS, 76 HC participants and 27 people with multiple sclerosis (MS) in the UKMEB were measured by ELISA. Serum samples (189) with high IgG titre to CMV (≥90 U/ml) or EBV (≥150 U/ml for VCA or ≥90 U/ml for EBNA), or that were seropositive to ≥ five out of six HHVs measured, were screened by ddPCR to detect potential active (lytic) replication of any of the nine HHVs. HHV DNA was rarely detected, with HSV-2, HHV-6A and HHV-6B detected in plasma from one or two subjects and the remaining HHVs always negative (**Table 1**). We therefore, screened PBMC samples, which were available from 76 potential participants; EBV was detected in 8 subjects, CMV in 1 subject and HHV-7 in 25 subjects (**Table 1**), probably reflecting latent infection. A single HHV DNA was detected in most cases of DNA positivity, whereas, co-detection of HHVs occurred in three individuals (1 HC: CMV &HHV-7; 2 ME/CFS_SA: EBV & HHV-7) (**Supplementary Table 2**). There was no significant correlation between EBV DNA copy number and either anti-EBNA 1 IgG ($P = 0.64$) or anti-VCA IgG ($P = 0.19$), possibly due to low DNA positivity rates. Correlation between HHV-7 DNA copy number and IgG concentration could not be tested, due to lack of serological assay availability. The participant characteristics are shown in **Supplementary Table 2**.

Longitudinal Study: Clinical Analysis of HHVs in Saliva From People Living With ME/CFS

Candidates were selected for invitation to participate in the longitudinal study of saliva HHV (**Figure 2**) if any HHV DNA was detected in either plasma or PBMC (DNA positive) or if HHV DNA was not detected in either sample (DNA negative); all invited participants were seropositive for all six HHVs tested. MS patients, who also have samples stored in the UK ME/CFS Biobank, were not included in the longitudinal study due to low HHV DNA positivity in plasma and PBMC. A total of 70 candidates were invited to participate in the study, of whom 46 were enrolled; their blood DNA results reflected those of the group as a whole (**Supplementary Table 2**). Participants were requested to post a saliva sample and a completed patient questionnaire (**Supplementary Figure 1**) to LSHTM monthly for 6 months, and on the first, third and fifth day of any disease exacerbation episode including acute illness or significant worsening of ME/CFS symptoms. From December 2017 to June 2018, 276 saliva samples were collected. The monthly sampling return rate was 92.7, 83.3, and 97.9% in HC, ME/CFS_MM (mild/moderate) and ME/CFS_SA (severely affected) groups, respectively. Additionally, one ME/CFS_MM and six ME/CFS_SA participants sent samples during episodes of acute illness/worsening of symptoms: one ME/CFS_SA sent samples for two episodes (**Figure 2**).

As anticipated, the symptom questionnaires revealed absent or mild symptoms across all the domains in the healthy participants, with the exception of Sleep Dysfunction, which was reported at mild to moderate levels in over half the group at some points during the study period (**Supplementary Figure 2**). In contrast, all participants reported a wide variety of symptoms across all the domains, ranging from mild to severe and fluctuating through the 6 months. Post-exertional malaise, neurocognitive, autonomic, and immune system dysfunctions were reported as more severe in the severely affected participants, whereas, pain, sleep and neuroendocrine dysfunction were more similarly reported across participants with mild/moderate or severe ME/CFS (**Supplementary Figure 2**).

HHV Prevalence and Persistence Over Time in Saliva From People With ME/CFS

The concentration of DNA for each of the nine HHVs was measured in saliva samples from ME/CFS patients (14 MM cases; 16 SA cases) and 16 HCs by ddPCR and the prevalence and shedding pattern of HHVs investigated over time. Throughout the study time course, HSV-1, EBV, HHV-6B, and HHV-7 were detected in at least some participants, whereas, the remaining HHVs were undetectable throughout. Most saliva samples contained one, two or three HHVs. At the start of the study (month 1), HHV-7 was the most prevalent HHV in saliva of all participants, whereas, HSV-1 was detected from only 1 participant in each group (**Figure 3A**) and the prevalence of EBV and HHV-6B differed between the groups (EBV: 56% (9/16) in HC, 29% (4/14) in ME/CFS_MM, 38% (6/16) in ME/CFS_SA; HHV-6B: 25% (4/16) in HC, 29% (4/14) in ME/CFS_MM, 56% (9/16) in ME/CFS_SA). HHV prevalence remained largely unchanged throughout the 6 months of follow up with HHV-6B being the only virus to differ in prevalence between the groups, being borderline significantly more highly prevalent in severely affected ME/CFS patients than in healthy controls (**Supplementary Table 3**).

To investigate viral persistence, the proportion of samples from each participant that were HHV-positive over the time course was determined (**Figure 3B**). HHV-7 was consistently detected in all samples from the majority of participants throughout the 6 months (median for all 3 groups = 100%), whereas, HSV-1, EBV, and HHV-6B were only intermittently detected. HHV-6B was detected significantly more frequently in the ME/CFS_SA participants (median: 33.3%, IQR: 16.7–75%), than in HC (median: 8.33%, IQR: 0–41.7%; $p = 0.049$), but not in ME/CFS_MM participants (median: 22.5%, IQR: 0–70.8%; $p = 0.28$). In contrast, there was no significant difference in the frequency of detection of EBV or HSV-1 in HC compared to people with ME/CFS (**Figure 3B**).

TABLE 1 | Human herpesvirus DNA in plasma and PBMC with anti-HHV IgG concentration.

ddPCR		HHV serology						Prevalence ddPCR-positive (%)	Clinical Group of ddPCR positive samples (*n* =)
		Positive		Negative		Border			
		Number (*n* =)	IgG median (IU/ml)	Number (*n* =)	IgG median (IU/ml)	number (*n* =)	IgG median (IU/ml)		
HSV-1									
PLASMA	Positive	0	–	0	–	0	–	0	–
	Negative	115	131.00	62	4.60	12	10.19		
PBMC	Positive	0	–	0	–	0	–	0	
	Negative	34	115.70	40	2.34	2	9.91		
HSV-2									
PLASMA	Positive	0	–	1	2.87	0	–	0.5	MS (1)
	Negative	100	93.45	82	2.84	6	10.50		
PBMC	Positive	0	–	0	–	0	–	0	
	Negative	30	99.33	44	3.10	2	10.97		
VZV									
PLASMA	Positive	0	–	0	–	0	–	0	–
	Negative	184	143.58	4	7.54	1	10.76		
PBMC	Positive	0	–	0	–	0	–	0	
	Negative	74	126.92	3	8.79	0	–		
EBV (anti VCA IgG)									
PLASMA	Positive	0	–	0	–	0	–	0	HC (3), ME/CFS_MM (2), ME/CFS_SA (3)
	Negative	169	156.65	13	4.71	0	–		
PBMC	Positive	8	120.26	0	–	0	–	11.7	
	Negative	58	120.65	8	2.60	2	8.71		
EBV (anti EBNA-1 IgG)									
PLASMA	Positive	0	–	0	–	0	–	0	*HC (2), ME/CFS_MM (2), ME/CFS_SA (1)
	Negative	140	55.42	35	5.95	7	9.52		
PBMC	Positive	5*	47.93	2#	4.92	1~	8.58	11.7	#HC (1), ME/CFS_SA (1) ~ME/CFS_SA (1)
	Negative	50	50.56	13	5.68	5	11.25		
CMV									
PLASMA	Positive	0	–	0	–	0	–	0	HC (1)
	Negative	94	110.81	85	3.73	3	8.38		
PBMC	Positive	0	–	1	2.76	0	–	1.4	
	Negative	24	85.445	48	4.15	3	8.86		
HHV-6									
PLASMA HHV-6A	Positive	1**	100.33	0	–	0	–	0.5	**HC (1)
	Negative	180	59.15	6	6.475	2	11.85		+HC (1)
PBMC HHV-6A	Positive	1+	100.33	0	–	0	–	1.3	
	Negative	71	42.44	4	5.16	0	–		
PLASMA HHV-6B	Positive	2§	87.14	0	–	0	–	1.1	§HC (2)
	Negative	179	59.43	6	6.475	2	11.85		
PBMC HHV-6B	Positive	0	–	0	–	0	–	0	
	Negative	72	43.74	4	5.16	0	–		
HHV-7 (No serology data)^									
PLASMA	Positive	0						0	HC (7), ME/CFS_MM (9), ME/CFS_SA (9)
	Negative	182							
PBMC	Positive	25						49.0	
	Negative	51							

(Continued)

TABLE 1 | Continued

ddPCR		HHV serology						Prevalence ddPCR-positive (%)	Clinical Group of ddPCR positive samples (*n* =)
		Positive		Negative		Border			
		Number (*n* =)	IgG median (IU/ml)	Number (*n* =)	IgG median (IU/ml)	number (*n* =)	IgG median (IU/ml)		
HHV-8 (No serology data)^									
PLASMA	Positive	0						0	
	Negative	182							
PBMC	Positive	0							
	Negative	76						0	

^ *There were no commercial ELISA kits available for HHV-7 and HHV-8.*

HHV DNA Viral Concentration in Saliva in People Living With ME/CFS

The absolute HHV viral DNA concentration load in participants with ME/CFS was compared with that in HC for the four detectable HHVs across the 6-month sampling time frame (**Figures 3C–F**). There were no significant differences in absolute viral DNA load for HSV (**Figure 3C**) or EBV (**Figure 3D**) between clinical groups. HHV-6B viral DNA concentrations were on average 6.2-fold higher in ME/CFS_SA patients than in HC: the differences were significant at months 1 ($P = 0.039$), 3 ($P = 0.030$), and 5 ($P = 0.029$), with a trend toward higher viral load across all time points (**Figure 3E**). There was a trend toward higher HHV-6B DNA concentration in ME/CFS-MM participants than HC, with borderline significance at months 3 ($P = 0.073$) and 5 ($P = 0.080$). Finally, HHV-7 viral loads were significantly or borderline significantly higher in ME/CFS_MM [at months 1 ($P = 0.068$), 3 ($P = 0.054$), 4 ($P = 0.023$), and 5 ($P = 0.032$)] and ME/CFS_SA patients [at month 6 ($P = 0.073$)] compared to HCs, with a trend toward higher loads across all timepoints for both ME/CFS_MM and ME/CFS _SA groups, with an average 4.1-fold increase in ME/CFS-MM and 3.0-fold increase in ME/CFS_SA compared to HCs across the months (**Figure 3F**).

The Spearman correlation between plasma IgG concentration (in biobanked samples) and salivary HHV viral DNA load for each participant was analysed for EBV and HHV-6 (**Supplementary Figure 3**). There was no significant correlation between EBV viral load and either EBNA or VCA antibody concentration in people with ME/CFS or HC. Although, there was a significant negative correlation between anti HHV-6 IgG concentration and salivary HHV-6B DNA concentration in the HC group, this was largely driven by one individual and no correlation was seen in the ME/CFS participants. The low correlation might reflect the time interval between serum and saliva sampling.

There was no difference in salivary HHV viral load between participants who had been deemed as HHV DNA-positive or DNA-negative in the initial plasma/PBMC ddPCR screening assay (**Table 2**) although, among the HCs, those who were plasma/PBMC HHV DNA-positive were significantly younger than those who were plasma/PBMC HHV DNA negative (**Table 2**). One HC in whom HHV-6B was detected in plasma had very high concentrations of HHV-6B DNA in saliva but this was an outlier and may reflect a recent infection (**Supplementary Table 4**). All people living with ME/CFS in whom HHV-7 was detected in PBMC also had high concentrations of salivary HHV-7 DNA (**Table 2**).

When stratifying participants by age, the salivary HHV-6B DNA concentration was significantly higher in younger (<40 years) than older participants in the HC and ME/CFS_MM groups but not the ME/CFS_SA group (**Supplementary Table 5**) but there were no significant differences in HSV-1, EBV, or HHV-7 DNA concentrations between age groups.

Salivary HHV Viral DNA Concentration Through Time and During Acute Illness Episodes in People With ME/CFS

Salivary DNA concentrations of HSV-1, EBV, HHV-6B, and HHV-7 varied over time in individual participants (**Figure 4**). Concentrations of HSV-1 and EBV DNA fluctuated month by month in the majority of infected individuals with no obvious differences between clinical groups, whereas, there was less fluctuation in HHV-6B and HHV-7 DNA concentrations, with more participants with ME/CFS than HC exhibiting persistently higher viral loads (**Figure 4A**).

There was no discernible pattern of changing HHV DNA concentrations during periods of worsening disease symptoms in the seven ME/CFS participants (one MM case and 6 SA, one of whom had two episodes of worsening symptoms) who had an acute illness or worsening of disease symptoms (**Figure 4B**): rather there was fluctuation in detection of all four viruses during these episodes, with both increases and decreases in viral load during exacerbated disease (**Figure 4B** and **Supplementary Figure 4**).

HHV Co-Infections in People With ME/CFS Over Time

We investigated changes in detection of viral DNA from co-infecting HHV viruses over time in individual study participants (**Supplementary Figure 5** and **Supplementary Table 6**). At all

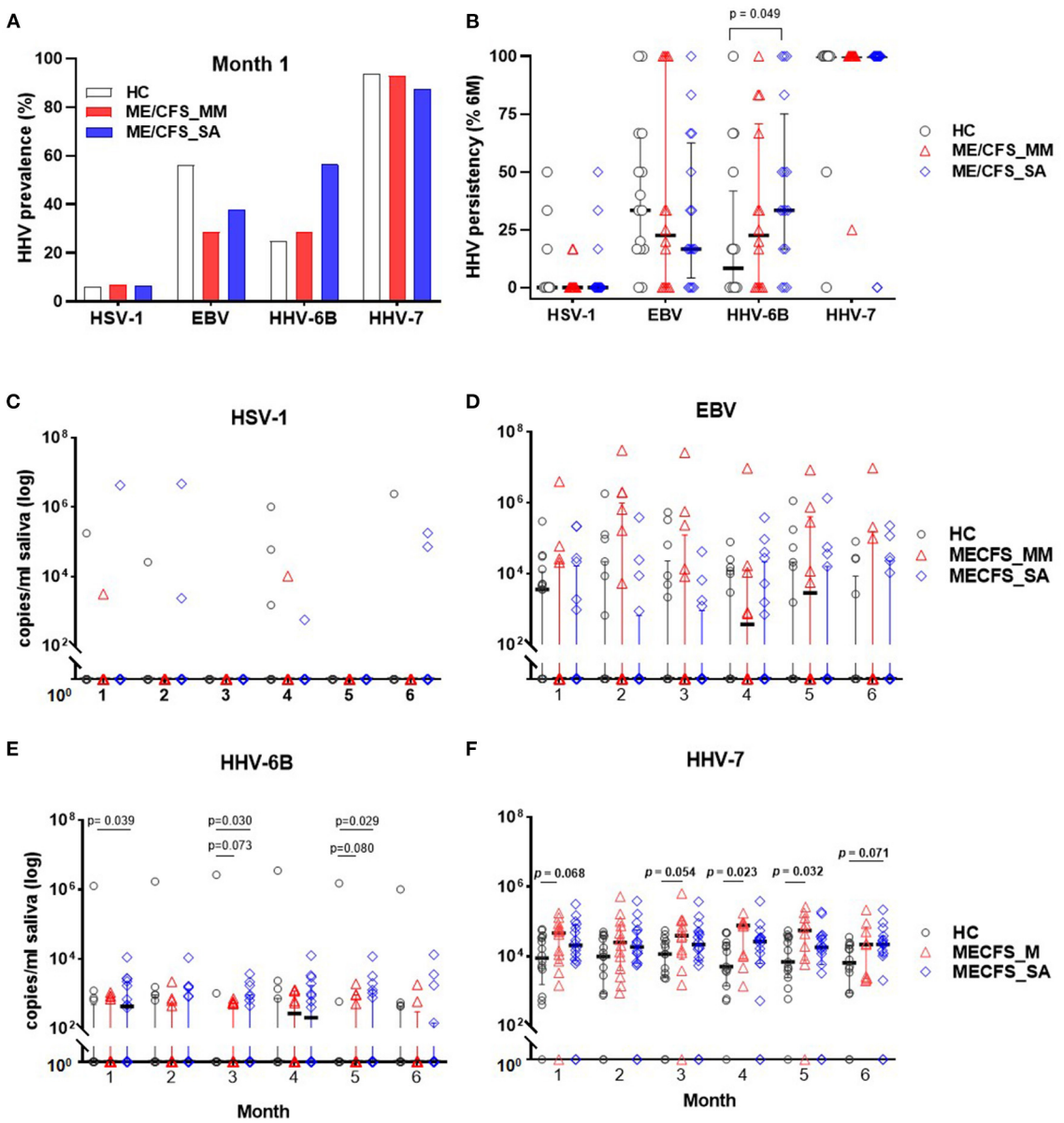

FIGURE 3 | Herpes virus DNA prevalence and concentration in saliva from people living with ME/CFS, measured by ddPCR. **(A)** Percentage HHV prevalence in HC ($n = 16$) and people with mild/moderate ME/CFS (ME/CFS_MM: $n = 14$) or severe symptoms (ME/CFS_SA: $n = 16$) at month 1. **(B)** Persistence of viral DNA positivity throughout 6 months. The persistency (%) was calculated for each participant as the number of times HHV was detected divided by the total number of samples collected over 6 months, multiplied by 100. HHV viral DNA concentration comparison between HC and ME/CFS patients. Each monthly viral DNA concentration in saliva from participants with mild/moderate (ME/CFS_MM) or severe (ME/CFS_SA) disease was compared to HC across the 6 months for **(C)** HSV-1, **(D)** EBV, **(E)** HHV-6B, and **(F)** HHV-7. Scatter dot plots show the median and the interquartile range. *P*-values < 0.1 (Mann-Whitney) are shown.

time points, HHV-7 was more frequently detected as the only HHV present in saliva than any other HHV; HHV-7 together with HHV-6B, and HHV-7 together with EBV, as dual combinations of HHV DNAs, and the triple combination of HHV-7 together with HHV-6B and EBV, were also frequently observed (**Supplementary Figure 5A**). The quadruple combination (HHV-7 with HHV-6B, EBV, and HSV-1) and other HHV combinations as dual or triple infections were detected only rarely (**Supplementary Table 6**). The HHV-7 with HHV-6B combination was significantly more frequently found in ME/CFS_SA than in HC (chi square $p < 0.05$ at months 1 and 5), in accordance with the higher frequency of HHV-6B DNA detection in severely affected ME/CFA patients. Dual detection of HHV-7 with EBV was more frequent in ME/CFS_MM than in ME/CFS_SA participants, with significant differences ($p < 0.05$) at months 2 and 4 and a trend in the other months.

TABLE 2 | HHV viral load in saliva of participants who were HHV DNA-positive or DNA-negative in the initial ddPCR screening assay of plasma and PBMC.

Month 1						Saliva ddPCR results Prevalence % Median viral DNA load (copies/ml saliva) (IQR)			
DNA positivity in plasma/PBMC at baseline		*N*	Median age (IQR)	*p*-value	Gender (*n*)	HSV-1	EBV	HHV-6B	HHV-7
HC	Negative	11	46 (37–51)	<0.05	F (8)/M (3)	0% 0 (0–0)	55% 3,600 (0–13,374)	18% 0 (0–0)	100% 16,308 (682–34,600)
	Positive	5	32 (20.5–43.0)		F (5)	0% 0 (0–0)	60% 4,500 (0–19,017)	20% 0 (0–63700)	80% 10,500 (2,400–41,167)
ME/CFS_MM	Negative	12	42 (37.0–49.8)		F (12)	0% 0 (0–0)	25% 0 (0–15,580)	33% 0 (0–805)	92% 46,500 (8,192–65,350)
	Positive	2	47.5 (42.0–53.0)		F (2)	0% 0 (0–0)	50% 30,075 (0–60,150)	0% 0 (0–0)	100% 43,653 (1,440–85,867)
ME/CFS_SA	Negative	11	49 (35–54)		F (7)/M (4)	0% 0 (0–0)	27% 0 (0–960)	45% 0 (0–690)	72% 11,400 (0–23,183)
	Positive	5	35 (25–56)		F (5)	20% 0 (0–2,137,500)	40% 0 (0–24,354)	60% 467.4 (0–2,512)	100% 48,300 (18,396–74,600)

People severely affected with ME/CFS could be broadly and evenly separated into two subgroups, dependent on how their combinations of salivary HHV DNA changed over time. People with pattern 1 showed varying combinations of one to four HHVs being detected over the 6-month time course, whereas, people with pattern 2 showed relatively simpler and more stable HHV positivity consisting of one to two HHVs detectable (**Supplementary Figure 5B**). The majority of people with mild/moderate ME/CFS symptoms (7/10) had the more stable HHV repertoire pattern with only three ME/CFS_MM participants fitting the fluctuating repertoire pattern (data not shown).

Correlation of ME/CFS Symptoms With Each Other and With Viral Load

To evaluate any association between disease symptoms and salivary detection of HHV DNA, the numerically graded responses to the clinical questions collected together with each saliva sample were categorised into seven domains (PEM, pain, sleep dysfunction, neurocognition, autonomic nervous system, neuroendocrine system, or immune system) to yield seven "symptom scores" for each sample. First, we compared symptom scores by group (**Figure 5A**). HC individuals had low scores across all symptom domains. People severely affected with ME/CFS had significantly higher PEM scores ($P = 0.003$) and a tendency toward greater autonomic dysfunction than the ME/CFS_MM group, whereas, other symptoms did not differ significantly between the two groups. We then examined the Spearman correlation of the symptom scores with each other: for all people with ME/CFS (i.e., ME/CFS_MM and ME/CFS_SA combined: $n = 30$), all symptom scores significantly correlated with each other (median of all $r = 0.508$, $P < 0.01$) (**Figure 5B**) and symptom scores were significantly ($P = 0.0007$) more highly correlated among patients who were mildly/moderately affected by ME/CFS than among those severely affected with ME/CFS (MM, median of $r = 0.604$; SA, median of $r = 0.385$, all $P < 0.05$ for ME/CFS_MM group comparisons, $P < 0.05$ for ME/CFS_SA group except Sleep dysfunction vs. PEM $P = 0.108$ and vs. Pain $P = 0.238$) (**Supplementary Figure 6**). Of the seven symptom domain scores, autonomic symptoms were highly correlated with neurocognition symptoms ($r = 0.70$) among all people with ME/CFS, and these two symptom domain scores were moderately correlated with other symptom scores (autonomic symptoms vs. all others, median $r = 0.586$; neurocognition symptoms vs. all others, median $r = 0.582$). In contrast, sleep dysfunction symptoms were only weakly correlated with other symptoms scores (median $r = 0.348$), except that among people severely affected with ME/CFS sleep dysfunction was highly correlated with neuroendocrine' symptoms ($r = 0.62$). Finally pain symptoms were highly correlated with neurocognition, autonomic and immune related symptoms ($r = 0.66$, 0.66, 0.60, respectively) in people severely affected with ME/CFS.

We investigated the correlation between salivary HHV DNA concentration and symptom scores across the 6 months of the study in people with ME/CFS (**Supplementary Figures 6A–C**) and healthy controls; overall, the extent of correlation was weak. Among people severely affected with ME/CFS, there were weak correlations between EBV DNA concentration and three symptom scores (pain, neurocognition, and autonomic: $r = 0.25$, 0.23, and 0.29, respectively, all $P < 0.05$), between HSV-1 DNA concentration and autonomic symptoms ($r = 0.24$, $P < 0.05$), and between HHV-7 DNA concentration and pain symptoms ($r = 0.25$, $P < 0.05$), and autonomic symptoms ($r = 0.24$, $P < 0.05$). EBV DNA concentration correlated with HSV-1 DNA concentration in people severely affected with ME/CFS ($r = 0.27$, $P < 0.01$), whereas, HHV-6B correlated with HHV-7 ($r = 0.22$, $P < 0.05$) (**Supplementary Figure 6B**). There were no significant correlations between salivary HHV DNA concentration and symptom scores among people mildly/moderately affected with ME/CFS. This group showed only weak negative correlation

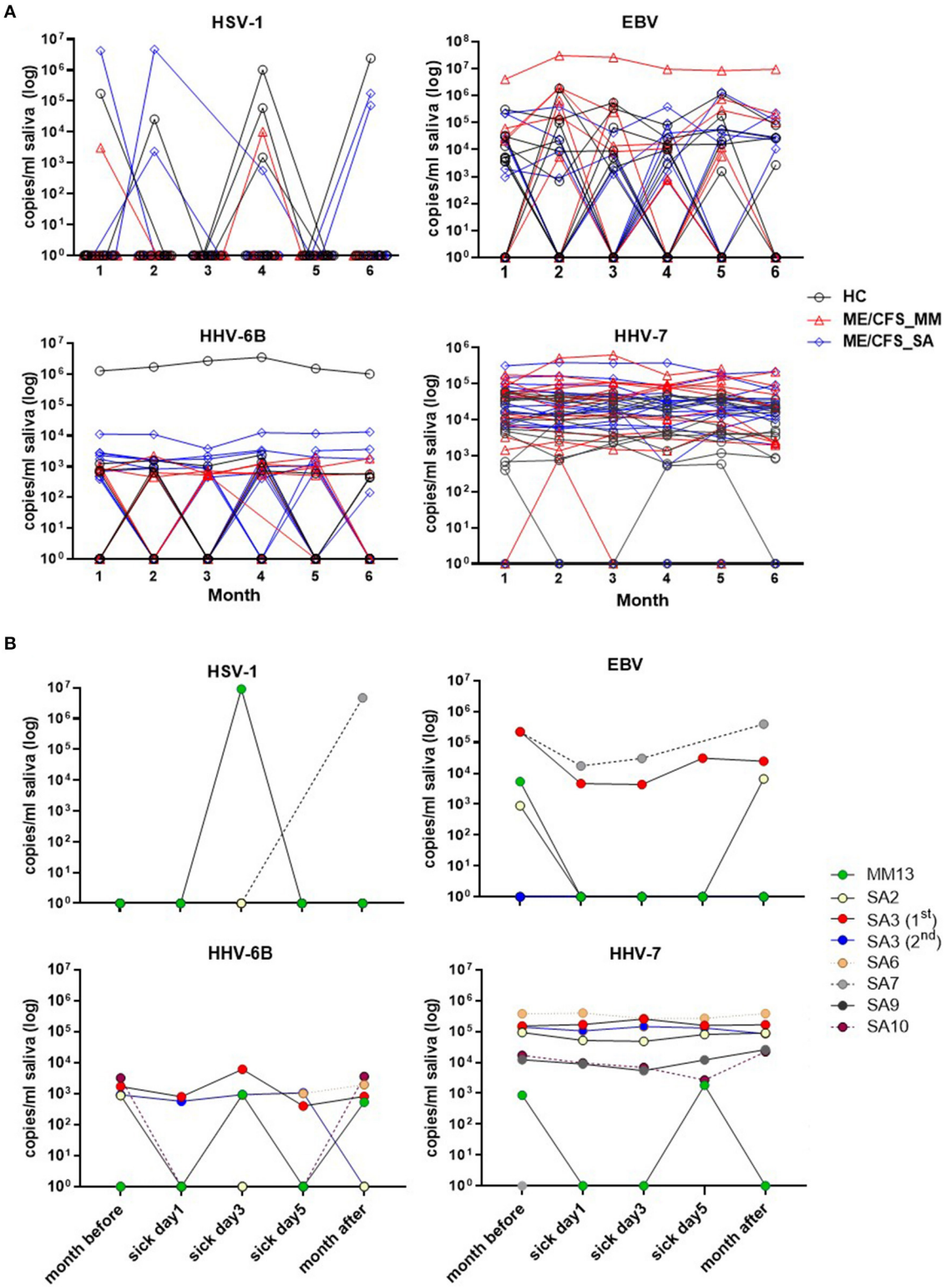

FIGURE 4 | Change in herpesvirus DNA concentration in saliva in individual participants over time. **(A)** The monthly viral load of HSV-1, EBV, HHV-6B, and HHV-7 detected in saliva from month 1 (Jan 2018) to month 6 (Jun 2018), measured by ddPCR assay, is shown for individual HC, ME/CFS_MM and ME/CFS_SA study participants. **(B)** The HHV viral load of HSV-1, EBV, HHV-6B, and HHV-7 in ME/CFS patients is shown on day 1, 3, and 5 of episodes when participants experienced an acute illness or worsening of disease symptoms.

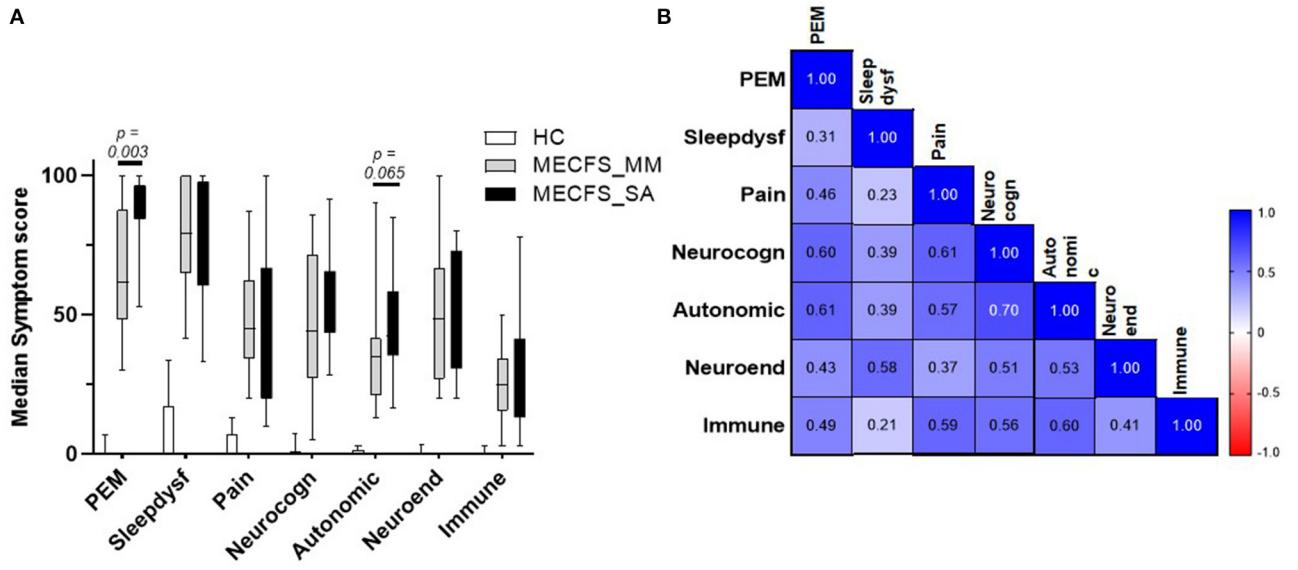

FIGURE 5 | Disease symptom and correlation in people living with ME/CFS and Healthy controls. **(A)** Median score and IQR of seven ME/CFS symptom domains across 6 months in people with mild/moderate ($n = 14$) or severe ME/CFS ($n = 16$) and HC ($n = 16$), based on questionnaires completed at the time of saliva sample collection by participants. **(B)** Correlation matrix between seven ME/CFS symptom domain scores in people with ME/CFS ($n = 30$).

between EBV and HHV-6B salivary DNA concentrations ($r = -0.25$, $P < 0.05$). Among the HC there were weak but statistically significant correlations between detectable HHV-6B DNA in saliva and neuroendocrine symptoms ($r = 0.21$, $P < 0.05$) and between HSV-1 DNA concentration and both autonomic and immunological symptoms ($r = 0.36$ and 0.22, respectively, all $P < 0.05$). This group also showed correlation between EBV or HHV-6B and HHV-7 salivary DNA concentrations ($r = 0.28$ and 0.22, respectively, all $P < 0.05$).

We next investigated the correlation between salivary HHV DNA concentration and symptom scores in the two subgroups of severely affected ME/CFS patients (pattern 1 and pattern 2, **Supplementary Figure 5B**) (**Figure 6**, **Supplementary Table 7**). In the pattern 1 ME/CFS_SA group (fluctuating levels of HHV DNA in saliva) there were strong and highly significant associations between HHV-6B DNA concentration and six symptom domain scores (sleep dysfunction, pain, neurocognition, autonomic, neuroendocrine and immune, $r = 0.47$, 0.52, 0.65, 0.50, 0.46, and 0.41, respectively, all $P \leq 0.01$). Three of these symptom domains were also correlated with HHV-7 DNA concentration (pain, neurocognition and autonomic, $r = 0.49$, 0.44, and 0.47, respectively, all $P = 0.002$ or < 0.001). In contrast, in the pattern 2 ME/CFS_SA group (stable HHV DNA in saliva), HHV-6B DNA concentration was negatively correlated with pain, neurocognition and neuroendocrine scores ($r = -0.59$, -0.43, and -0.42, respectively, all $P < 0.01$) whereas, EBV DNA concentration was positively associated with pain, neurocognition, and autonomic symptoms ($r = 0.48$, 0.40, and 0.39, respectively, $P = 0.001$, 0.008, and 0.010, respectively). Moreover, neuroendocrine and immune system scores were significantly higher in the pattern 1 group than the pattern 2 ($P < 0.01$) (data not shown). Finally, when people with ME/CFS had an acute illness episode, during their sick days, the HHV-6B DNA concentrations were positively correlated with sleep dysfunction and HHV-7 DNA concentration ($r = 0.454$, $P < 0.05$, $r = 0.548$, $P < 0.01$, respectively; **Supplementary Table 7**). PEM and Pain were negatively correlated with HHV-6B, $r = -0.647$, $P < 0.01$, $r = -0.426$, $P < 0.05$, respectively). Interestingly, in all seven patients reporting an acute illness or worsening of symptoms, participants, autonomic symptoms were markedly increased whereas, neuroendocrine symptoms were decreased (**Supplementary Table 7**).

DISCUSSION

Using duplex droplet digital PCR, we have analysed the prevalence and DNA load of human herpesviruses in the saliva of ME/CFS patients and looked for associations with severity of symptoms over time. In summary, HHV-7 was the most frequently detected HHV in saliva and salivary HHV-7 DNA concentrations were significantly higher in people with ME/CFS than in healthy controls; HHV-6B was more frequently detected, and with significantly higher viral loads, in people severely affected with ME/CFS than in healthy controls and those with mild/moderate ME/CFS, such that severely affected ME/CFS patients were more likely than other groups to be HHV-6B/HHV-7 double-positive; and HHV-6B and HHV-7 viral loads correlated with disease severity in a subgroup of people severely affected with ME/CFS whose salivary HHV repertoire fluctuated over the 6 months of the study, suggesting that HHV-6B and HHV-7 may trigger ME/CFS symptoms or be reactivated in concert with worsening symptoms. Importantly, although, HSV-1 and EBV were detected in some saliva samples, there were no consistent or

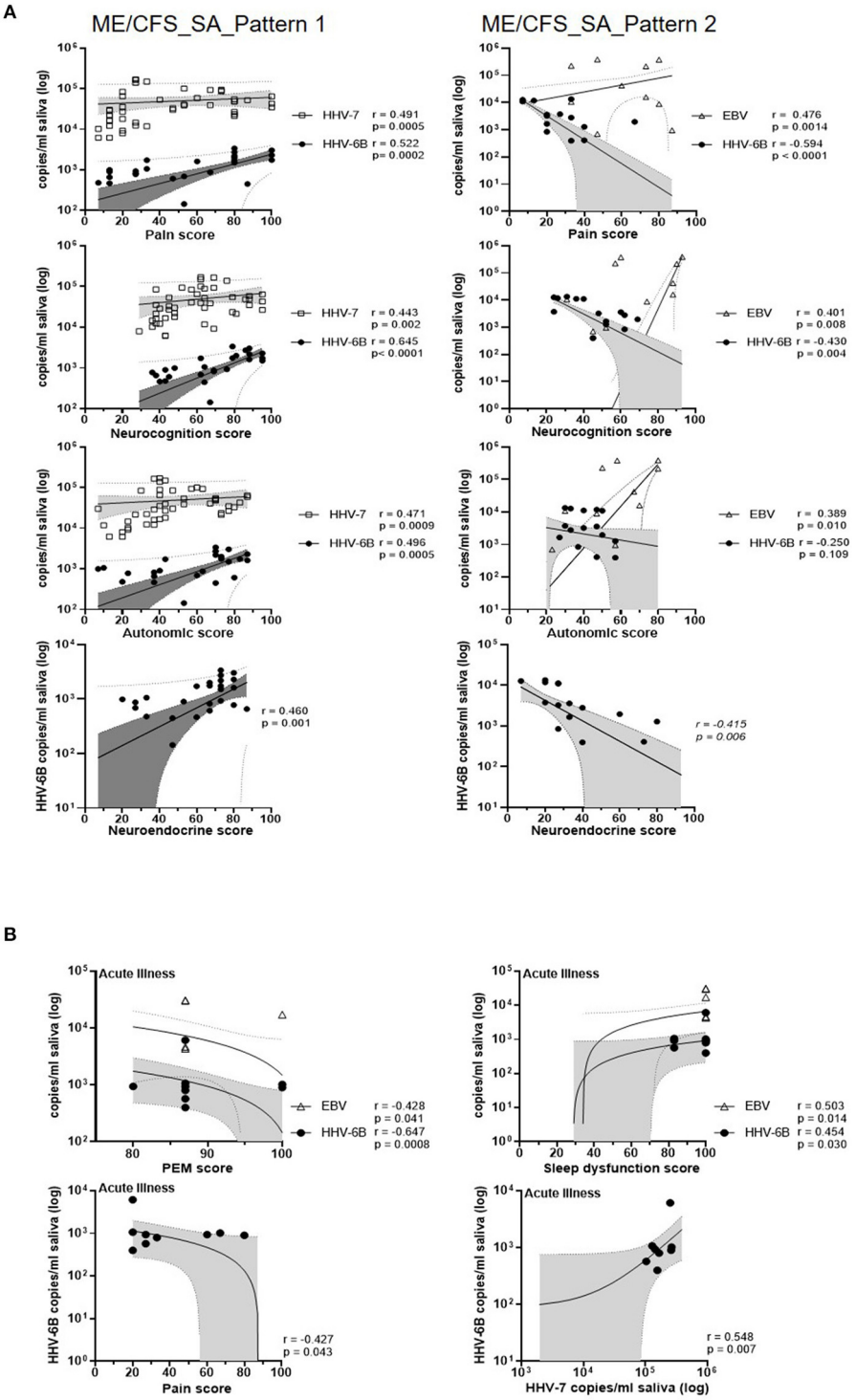

FIGURE 6 | Correlations of salivary HHV DNA concentration with disease symptoms scores. **(A)** Correlation between monthly symptoms score and monthly HHV viral DNA concentration in people with ME/CFS_SA (pattern 1, left panel; pattern 2, right panel). All participant's monthly HHV viral DNA concentration and symptoms score data were included in the correlation analysis. Each symbol represents an individual's monthly sample. NB: Zero values are not shown on the graph due to the logarithmic scale, but are included in the analysis. Spearman's coefficient is denoted as *r.* The best-fit regression line, 95% confidence interval (grey area) and prediction band (dotted line) are shown. **(B)** Correlation between symptom scores and EBV, HHV-6B, and HHV-7 DNA concentrations in saliva samples from participants experiencing an exacerbation of symptoms.

statistically significant differences in HSV-1 and EBV reactivation between people with ME/CFS and healthy controls.

These findings are consistent with, and extend, previous data linking EBV, HHV-6 and HHV-7 infection and reactivation to the clinical course of ME/CFS [reviewed in (30)]. Frequent HHV-6 and/or HHV-7 infection or reactivation has been reported in chronic fatigue syndrome patients (31–34) and has been linked to decreased cellular immune responses (31) and to ME/CFS symptoms including PEM and lymphadenopathy (32). The HHV-6A and HHV-6B primers used in our ddPCR assays allow the discrimination between HHV-6 subtypes which historically has not been possible serologically. Moreover, Chu et al. (35) reported that one third of patients had a documented acute HHV or B19 virus infection related to herpes viruses and B19 virus associated with ME/CFS onset. Although, the study reported here is of small scale, the results are in accordance with previous data, demonstrating that a large prospective epidemiological study is warranted to test conclusively the aetiological link between HHV reactivation and ME/CFS symptoms. Such a study should address potential confounding factors such as age and gender in relationship to ME/CFS development and presentation as well as immune control of HHVs. Recent data have shown reactivation of HHVs, including HHV-6, during Sars-CoV-2 infection (36), opening up the possible role of HHVs in so-called "Long COVID" in which symptoms in many cases overlap those presented by people with ME/CFS.

Saliva is a key transmission route for HHVs as the salivary gland is a particularly permissive site for HHV replication. We detected HSV-1, EBV, HHV-6, and HHV-7 DNA in saliva samples, either singly or in combinations of up to 4 HHVs, consistent with previous studies (37–40); HHV-7 has been reported to be almost universally detected in saliva (41). The prevalence of HSV-1, EBV, and HHV-7 in our healthy controls was broadly similar to a previous report Miller et al. (40) but the prevalence of HHV-6B (25%) was markedly lower than the 93.5% prevalence of HHV-6 reported previously; this difference may reflect the detection target (HHV-6B rather than the whole HHV-6 genome), methodology or genuine population differences. Furthermore, in all but one of our study participants, detection of HHV DNA in saliva (evidence of virus reactivation and shedding) was highly correlated with detection of viral DNA in previously collected (baseline) samples of PBMCs (where the virus may be latent) or plasma, indicating that these are longstanding, persistent infections rather than acute/recent infections. One of our healthy control participants had very high plasma and salivary concentrations of HHV-6B DNA which were positively correlated with immune-related and autonomic symptoms: it is possible that this participant had inherited chromosomally integrated HHV-6B which has been reported to lead to ME/CFS symptoms in some people (42). Another participant, with mild/moderate ME/CFS, had persistently high salivary EBV DNA. In the current study design it was not possible to test for active viral transcription or protein synthesis alongside viral DNA quantification. Future studies should investigate correlation between viral DNA concentration in contemporaneous plasma, PBMCs and saliva samples, as well as measurement of IgM and IgG to indicate recent or historic infection, and host DNA load, as well as direct measures of HHV reactivation (12, 43) and HHV epitope mapping studies (34) in order to more fully interpret the saliva data.

Herpesviruses maintain latent infections at different anatomical sites. HHV-6 and HHV-7 remain dormant inside leukocytes, mainly T cells, and their genomes integrate into host chromosomal telomeres providing a mechanism for viral reactivation *via* the release of telomeric circular DNA following T cell activation in response to a heterologous infection (44, 45). Furthermore, HHV-7 can reactivate HHV-6A/B while HHV-6 has been found to activate Epstein-Barr virus from latency (46–48). HHV-6 and HHV-7 latency within leukocytes leads to wide-ranging impacts on the immune system (4) including changes in host cell transcriptomes and metabolism (49), down-regulation of antigen presentation and modulation of cytokines and chemokines (50, 51), impairment of NK cell function (52), and enhancement of proinflammatory cytokine and chemokine responses to Toll-like receptor 9 signalling (53). HHV-6 reactivation in ME/CFS patients can also lead to mitochondrial fragmentation and severely compromised energy metabolism (54); this may be linked to reported interactions between the HHV-6B U95 early viral protein and the mitochondrial GRIM-19 protein (55) that results in reduced mitochondrial membrane potential and pronounced mitochondrial impairment. It has also been postulated that persistent HHV infection/reactivation during which effector T cells are controlled by regulatory T cells may also lead to ME/CFS symptoms (56). The weak but statistically significant correlations observed here between reactivation of HHV-6, HHV-7, and EBV, and the correlations between HHV viral loads and severity of disease symptoms in ME/CFS patients, may thus reflect T cell activation either by one of these HHVs or by another infectious agent; and/or HHV-induced dysfunction of various cells of the immune system; and/or direct or indirect effects of HHVs on mitochondrial function. Whilst we did not observe any significant association between HHV reactivation and episodes of disease exacerbation, this may be due to the low number of acute disease episodes in our study, and to the marked heterogeneity of ME/CFS patients. Larger and longer studies will be needed to properly address this hypothesis and identify potential triggers.

In summary, this pilot study demonstrates that it is possible to recruit people living with ME/CFS and to collect biologically relevant samples, longitudinally, even during periods of disease exacerbation, opening up the prospect of conducting large-scale studies to investigate the role of human herpes viruses in ME/CFS pathogenesis. Our data indicate that HHV6-B and HHV-7 are associated with ME/CFS disease severity. Herpesvirus reactivation might either be a cause or effect of ME/CFS manifestation: either a disturbance in immune system function caused by ME/CFS disease development enables a persistent HHV viral infection to become reactivated, or the reactivation of the virus might be the precipitating factor for the worsening of ME/CFS symptoms. Many factors, such as co-infection, long-term stress, immunosuppressive therapy, and autoimmune disease can lead to HHV reactivation, which would fit with heterogeneity seen in ME/CFS cohorts. Long-term, large-scale

studies are warranted to determine cause vs. effect for the role of HHVs in ME/CFS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the London School of Hygiene and Tropical Medicine (LSHTM) Ethics Committee (Ref. 6123) and the National Research Ethics Service (NRES) London-Bloomsbury Research Ethics Committee (REC ref. 11/10/1760, IRAS ID:77765). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ER, CR, EL, and LN devised the study and obtained funding. EL, CK, JN, and SO'B conducted the clinical assessments and collected samples. J-SL, CR, ER, and JC designed and implemented the laboratory studies. J-SL, JC, and LP analysed data or contributed to interpretation. J-SL, JC, EL, CK, LP, and ER wrote the manuscript. All authors revised the manuscript critically for important intellectual content and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.656692/full#supplementary-material

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A Natural History of Disease Framework for Improving the Prevention, Management, and Research on Post-viral Fatigue Syndrome and Other Forms of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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We propose a framework for the treatment, rehabilitation, and research into Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) using a natural history of disease approach to outline the distinct disease stages, with an emphasis on cases following infection to provide insights into prevention. Moving away from the method of subtyping patients based on the various phenotypic presentations and instead reframing along the lines of disease progression could help with defining the distinct stages of disease, each of which would benefit from large prospective cohort studies to accurately describe the pathological mechanisms taking place therein. With a better understanding of these mechanisms, management and research can be tailored specifically for each disease stage. Pre-disease and early disease stages call for management strategies that may decrease the risk of long-term morbidity, by focusing on avoidance of further insults, adequate rest to enable recovery, and pacing of activities. Later disease stages require a more holistic and tailored management approach, with treatment—as this becomes available—targeting the alleviation of symptoms and multi-systemic dysfunction. More stringent and standardised use of case definitions in research is critical to improve generalisability of results and to create the strong evidence-based policies for management that are currently lacking in ME/CFS.

Keywords: myalgic encephalomyelitis/chronic fatigue syndrome, chronic fatigue syndrome, ME/CFS, post-viral fatigue syndrome, chronic illness, management, research

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INTRODUCTION

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a complex disease of unknown aetiology with no diagnostic test or biomarker to enable accurate and timely identification of cases (1). Diagnosis is often delayed by years, due to factors including: (i) the marked heterogeneity of the disease, (ii) the extensive clinical investigations necessary to exclude alternative diagnoses, and (iii) the numerous case definitions available, which differ significantly (1). Without a specific diagnostic test, identification of ME/CFS cases largely relies on detailed clinical history and examination, together with the patients' description of current and past symptoms. Many cases are triggered by an infection (also known as post-infectious ME/CFS) and there is a growing body of literature that reports clusters of ME/CFS cases following viral infections, although it is often not possible to confirm the triggering infection (2–6). The UK ME/CFS Biobank (UKMEB) has a cohort of 306 consenting participants diagnosed with ME/CFS, of whom 68% (when recalling disease onset) reported having a viral infection prior to the start of symptoms; 35% of these participants reported that their viral infections were confirmed by laboratory tests.

The difficulties in diagnosing patients have a knock-on effect for both clinical management and research: the amalgamation of people with ME/CFS and those with chronic fatigue due to other causes (e.g., conditions such as diabetes or anaemia that are not adequately controlled with medication) contributes to a lack of specificity (7, 8), while the numerous case definitions and inconsistent subtyping exacerbate heterogeneity and non-generalisability of study results (1). In addition, retrospective study designs including participants with established disease inevitably neglect the crucial period of pre- or early disease (where disease is developing and progressing), and instead rely on patient recall in order to gather information for that period. Disease duration is critical for diagnosis; most current definitions require that symptoms be present for at least 6 months for a formal ME/CFS diagnosis to be considered (9, 10), and this, together with a lack of biomarkers, makes it nearly impossible to identify those people predisposed to, or in the early stages, of disease progression. Larger prospective cohort studies following acute infections are required to accurately describe disease progression, and to identify specific markers for each disease stage (11–13).

With this conceptual paper, we intend to outline the stages of this disease for the optimisation of treatment, rehabilitation, and research into ME/CFS by considering specific preventative measures, improving generalisability of results, and creating the strong evidence-based policies for management that are currently lacking in ME/CFS. This paper sets out how research and clinical management could be targeted to specific disease phases, with a focus on prevention and rehabilitation, to improve patient outcomes.

The Natural History of ME/CFS

In an earlier paper, we conceptualised the progression of ME/CFS using a natural history of disease framework (further summarised below) (14); a concept familiar to many other chronic diseases. By considering each distinct stage along a chronological development timeline, we can move away from the multitudes of ways patients have previously been characterised including, but not limited to, the following: symptom presentation (15); co-morbidities (16); genetic traits (17, 18); metabolomics (19); and disease duration (16, 20), enabling an initial alignment of disease stage, clinical phenotype and potential pathophysiological mechanisms (14). While ME/CFS aetiology and its pathophysiological mechanisms remain elusive at some of the stages, this proposed framing draws attention to the less defined pre-morbid phases, the understanding of which may be the key to identifying the early causes of ME/CFS, and where early intervention may be effective. The proposed stages are as follows:

Predisposition and Triggering of Disease (Onset)

This is the period before disease is initiated in the individual. Without a full understanding of disease aetiology, it remains unclear which individuals are predisposed, but there are certain well-accepted patterns including: gender- and age-specific factors (21–23); acute infection triggers, either sporadic or as part of outbreaks (24–28); and genetic heritability (29, 30). There are a number of other factors reported as triggers including stress, environmental causes and trauma (31–34). Most commonly, ME/CFS develops following an acute viral episode (of which various aetiologies have been noted) (3); other patients report a slower, more insidious onset with no obvious initiating factor (35). At these early stages, disease presentation is non-specific or related to the "triggering" insult. Current reports of chronic symptoms similar to those of ME/CFS have been described by people infected with SARS-CoV-2 (36–38).

Prodromal Period (0–4 Months)

A lack of research makes it difficult to substantiate exactly what happens during the prodromal (and early disease) stage, although the mechanisms involved in producing the first symptoms of ME/CFS likely result from the bi-directional interaction between the immune and the central nervous systems (CNS), pro-inflammatory cytokines and other mediators disrupt CNS function which, in turn, releases neurotransmitters and hormones affecting immune function (39–41). Consequently, the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS) are affected, interrupting the normal homeostatic processes in the body (42–45).

Early Disease (4–24 Months)

This stage represents the continued and increasing dysregulation initiated in the prodromal period, where physiological and homeostatic processes are unable to return to previous levels of equilibrium and instead settle into a new "aberrant" homeostatic rhythm: an alternative state of functioning at a less optimum level (46). Symptoms such as fatigue can be largely explained by local and systemic effects of cytokines (47) or toxins and systemic dysfunction (48–52). There is a shift towards conservation of energy for essential processes, and physiological responses and symptoms are modulated by the increased

production of anti-inflammatory mediators to balance out the pro-inflammatory stimuli.

Established ME/CFS (2 Years and Beyond)

The initial over-production of pro-inflammatory and neurotoxic factors and ongoing immune and CNS dysfunction leads to a prolonged state of low-grade neurological and systemic inflammation (31, 35, 39, 53–61). With time, there is a shift from a higher to a less active pro-inflammatory state (62), with possible changes to symptom severity. However, individuals may move between phases either upwards (i.e., towards homeostasis and better health status) or downwards (i.e., towards "aberrant homeostasis" and disease deterioration).

By considering the distinct disease stages in ME/CFS, management of symptoms will inevitably be improved, leading to an increased likelihood of recovery. This is because the distinct stage-specific mechanisms underpinning pathology, require differential measures that may help to restore normal functioning. Currently, the lack of research at the prodromal and early disease phases (compounded by the discrepant use of case definitions and subtyping described above) means treatment is limited to managing symptoms rather than tackling their cause. In the absence of recognised treatments, the best approach for people with ME/CFS would be preventative measures combined with symptoms management at each phase of disease.

METHODOLOGICAL APPROACH

In this theoretical paper, we have applied the natural history of ME/CFS framework to consider preventative measures, management and treatment of symptoms, and research targeted to specific stages of disease. We argue that the proposed framework will help to target the public health, clinical, and research efforts in ME/CFS in more effective ways, recognising that it will likely be improved by future research findings. **Table 1**, adapted from our previous conceptual paper on a proposed natural history of this disease (14), shows the putative stages of ME/CFS—from predisposition to established disease, which are correlated to clinical phenotypes (defined by symptoms)—and the possible prevention and disease management strategies. **Figure 1**, copied from Nacul et al., attempts to illustrates the key pathophysiological mechanisms operating in each stage of ME/CFS, based on current literature (14).

LEVELS OF PREVENTION, MANAGEMENT, AND RESEARCH BASED ON THE DISTINCT STAGES OF ME/CFS

Predisposition and Triggering of Disease (Onset)

Primary Prevention

While risk factors specific to ME/CFS remain ambiguous it is difficult to conceptualise, or even put into practise, evidence-based primary prevention strategies. It is reasonable to assume that, in the face of acute infections or other insults, individuals should avoid exposure to further stresses and prioritise periods of relative rest along with pacing activities (as appropriate) in order to facilitate recovery from acute illness. This requires support from employers, teachers (see section below for the role of "presenteeism"), and healthcare professionals. For cases that might be triggered by environmental contaminants (such as chemicals), environmental protection policies and regulation will play an important role (65).

Management

At this predisposition stage, potential management measures are quite limited, and would overlap with the primary preventative measures, if the triggers are identified as infection-related as described above.

Research

Any change in practise will result from a better understanding of risk factors specific to ME/CFS and from research with the design of a disease-specific strategy for ME/CFS prevention, which is currently lacking. This strategy should be considered within the context of wider determinants of health (66), using a model that applies to the prevention of chronic diseases in general and considers potential predisposing factors, including genetics (67–70). Knowledge of risk factors for ME/CFS, currently scarce, are essential for primary prevention and we therefore recommend research approaches used in the study of other chronic diseases to gain new insights into familial and individual risks, including genetic, environmental, and life-style factors (2). Examples of proxy models that could be used to further our understanding of risk factors and the immune response in ME/CFS include interferon-alpha (IFN-α) treatment for hepatitis C that was suggested to trigger chronic fatigue (71), cohort studies that follow fatigue after infection with Epstein-Barr Virus (EBV) (11), and more recently, SARS-CoV-2 (72).

In the first example, considering IFN-α as a "trigger" allows for observation and tracking of the disease profile prior to, during, and after the presence of the insult, and following cohorts of patients from an early stage further allows the identification of possible risk factors and biomarkers. While appreciating that distinct mechanisms may be at play in ME/CFS, it is reasonable to consider similar proxy models to seek better understanding of immune profiles and response to insults in other fatiguing diseases. Following disease progression in people infected with SARS-CoV-2 in the current pandemic using well-designed studies may answer some questions about the potential chronic responses to viral infections, including whether long-COVID can be defined as a similar or distinct disease (73). Furthermore, such studies would provide an opportunity for improved real-time characterisation of the natural history of disease.

Prodromal Period and Early Disease Phases

Secondary Prevention

Secondary prevention refers to early detection of a disease and to early intervention, with the aim of reducing morbidity and disability (74). In the case of ME/CFS, early diagnosis would have an impact on disease management, even in the absence of any specific treatment. In order to facilitate this, a provisional

TABLE 1 | Characterisation of ME/CFS progression on time, according to distinct stages from pre- to established, clinical phenotype, and levels of prevention.

Timing	No disease	Onset	0–4 months	4–24 months*	2 years+ †
Stage	Predisposition	Trigger and pre-illness	Prodromal period	Early disease	Established disease
Clinical phenotype	No symptoms	Non-specific or related to triggering "insult"	Fatigue-complex symptoms‡	Fatigue-complex symptoms with variable severity and progress	Mild, moderate, or severe and complicated disease
Prevention level	Primary prevention	Treatment of "insult" and primary prevention	Symptoms management and secondary prevention	Disease management and secondary prevention	Disease management and tertiary prevention

Table adapted from Nacul et al. (14).

*****3–6 months is commonly referred as the minimum period of symptoms before diagnosis is made in children and adults, respectively (35).*

†2 years has been used as a cut off to distinguish between short- and long-term duration of disease (63, 64), but its use as defining established disease is variable and depends on a range of factors, including individual response to early disease.*

‡Fatigue-complex symptoms: initially predominantly neuro-immune (prior to early disease) and progress to variable systemic symptoms in the established disease phase.*

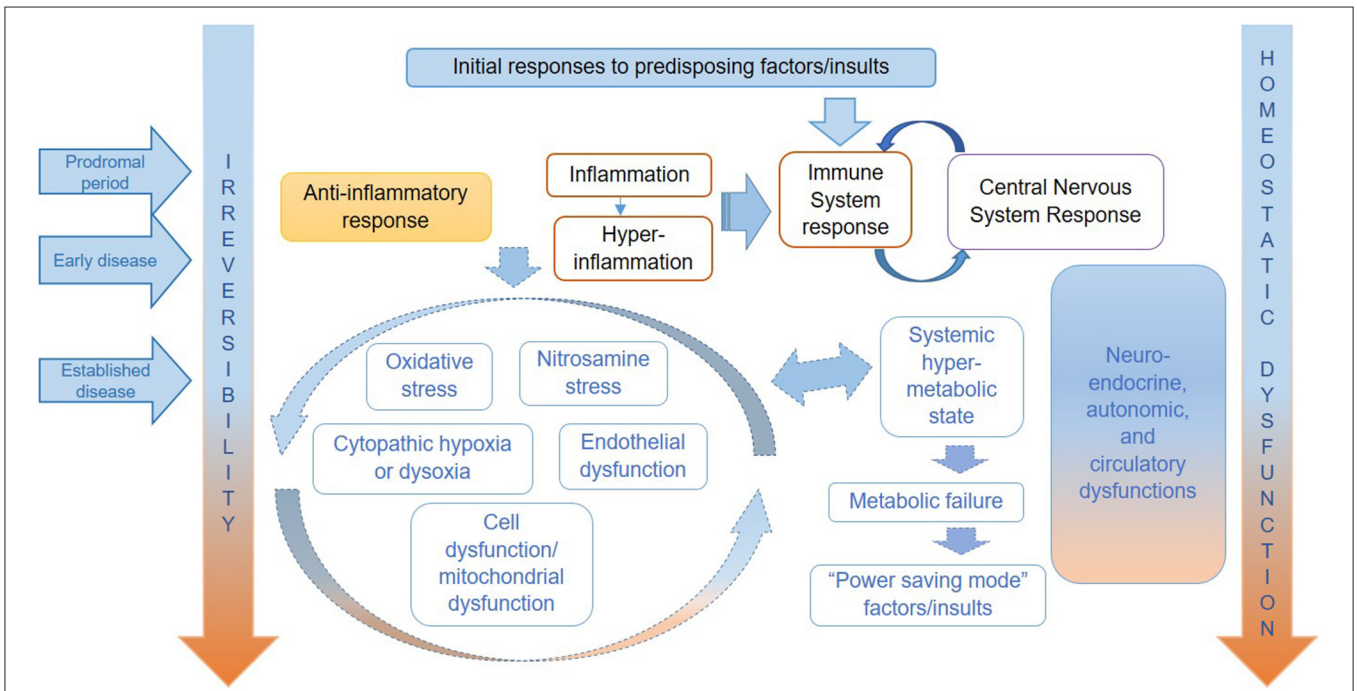

FIGURE 1 | Hypothesised key pathophysiological mechanisms for ME/CFS.

diagnosis of ME/CFS should be considered earlier (e.g., after 2 months of symptoms), with efforts made to regularly monitor patients until the disease can be confirmed at 4 to 6 months, after which regular reviews should continue (35).

Approaches towards early diagnosis require a major shift in perspective from both healthcare professionals and patients. For example, at the stage when a diagnosis of ME/CFS (or similar post-viral fatigue syndromes) is a possibility, we recommend a reduction in allostatic load including in activity levels (75–77), the avoidance of further stressors, and the treatment of the infection or triggering factor(s), when possible. Techniques such as pacing require substantial behaviour change in the patient firstly, to identify their energy threshold and, secondly, to adjust activity accordingly to avoid symptom exacerbation (i.e., keeping within the "energy envelope") (78, 79). Additionally, any specific therapies should be aimed at the correction of immune, CNS, and other dysfunction, alongside prevention of complications.

Strong support from educational institutions and workplaces is critical at this stage of the disease process. Accommodating the need for adequate recovery time away from, or with reduced time at, work or studies (80) is paramount, particularly with growing evidence of the higher costs of presenteeism (i.e., being physically at work, even if ill) compared to absenteeism, and the adverse effects on an individual's own health and productivity when turning up to work ill (81). Providing such support might require society as a whole to recognise the importance of the needs of the individual for adequate recovery time following acute illness and of taking the pressure off individuals to be productive or present

in their workplaces or classrooms at such times. This would avoid or minimise negative impacts on their health in the short- and long- term, as increased or persistent exertion (whether it is physical or mental) may result in the worsening of symptoms and a delay in recovery (35).

Management

Until there is conclusive evidence of specific pathophysiological mechanisms and effective treatments in ME/CFS, disease management at this stage should focus on reducing the severity of symptoms. Effective treatment plans rely on a strong health professional-patient relationship with regular follow-ups and a cautious degree of trial and error in treatment approaches (including starting any medications at low doses to monitor any sensitivities) (82, 83); such management should be informed by symptom characteristics and by awareness of changing symptoms which may reflect drug sensitivities or progression to later phase of disease. Although, not recommended specifically for the treatment of ME/CFS, a number of drugs have been shown in clinical practise to be helpful in some individuals for symptom management of pain (e.g., Low-dose naltrexone, Pregabalin, Gabapentin), orthostatic intolerance (e.g., Fludrocortisone, Midodrine), allergic/inflammatory reactions (e.g., antihistamines, sodium cromoglicate) and sleep (e.g., trazodone, Melatonin, tricyclics) (83). Non-pharmacological and behavioural approaches can also be helpful to relieve exacerbation of symptoms: acupuncture to assist with pain; support stockings and fluid/salt intake for orthostatic symptoms; memory aids and lists to help with cognitive issues; avoidance of specific foods and/or environmental factors (such as light, noise, touch etc.) (35, 83–86).

Management should be multidisciplinary, based on ongoing dialogue and partnership between professionals and patients extending to carers and family, with the involvement of the educational, occupational, and social sectors as appropriate.

With large numbers of the global population exposed to a potential viral trigger during this current SARS-CoV-2 pandemic, it is reasonable to suppose that a significant number of people with long-COVID may have, or will develop, ME/CFS. It is estimated that 1 in 10 people are experiencing persistent symptoms for over 12 weeks (87), often despite a relatively "mild" acute illness and reports of previously healthy lifestyles (88, 89), and are consequently diagnosed with long-COVID, post-COVID fatigue, or long-COVID fatigue syndrome (LCFS) (72, 73, 90).

There is much overlap in LCFS and ME/CFS symptom presentation (72), but it remains unclear whether they are the same condition. In the absence of that evidence, it would seem prescient to manage symptoms of LCFS by encouraging the use of recognised rehabilitation techniques long used for the management of ME/CFS (such as pacing) to reduce the likelihood of progression to post-COVID ME/CFS (91); while avoiding harmful treatments, such as the long-contested graded exercise therapy (92). Online long-COVID support groups report narratives similar to those of people with ME/CFS including being "dismissed" by healthcare professionals and labelled with "anxiety" (93). As healthcare workers themselves are diagnosed with long-COVID (94), such reports are being taken more seriously and there is growing awareness of the need for better recognition and management of post-COVID fatigue that is helping to drive change in the healthcare perspective (94, 95).

Research

In relation to secondary prevention, we believe that research should focus on the pathophysiology of early disease and early interventions at sub-clinical, acute, and early disease stages, and should target those factors that facilitate and hamper recovery from acute disease. Again, this would require prospective follow up of relatively large cohorts of individuals from exposure to an insult [such as an acute infection (11)], and thereafter in order to look at any differences between those who develop prolonged, chronic fatigue including ME/CFS and those who present no fatigue or with fatigue for shorter periods.

Within the field of ME/CFS research, studies using small participant numbers and a variety of diagnostic criteria are commonplace and lead to non-replicable or non-comparable results. The ongoing study of risk factors and potential biomarkers [of which a number of candidates have been considered (96, 97), although remain unconfirmed] may further benefit from existing large datasets [e.g., GP electronic health record databases for research (https://www.pcrd.purdue.edu/)]; or from general [e.g., UK Biobank (https://www.ukbiobank.ac.uk/)], and/or disease-specific databases and biobanks [e.g., UKMEB (https://cureme.lshtm.ac.uk/) and the SolveME/CFS Initiative (https://solvecfs.org/)].

Bioresources such as the UKMEB, which uses a strict protocol for the recruitment of participants and the collection and storage of biosamples (98), would serve to improve the replicability of studies by minimising the variation in sampling by the use of shared diagnostic criteria, and to provide samples for validation studies. Other opportunities include the application of life course epidemiology methods (99) on existing disease cohorts (that have well-documented medical histories) for diagnostic confirmation of ME/CFS, using retrospective or prospective longitudinal designs. Approaches could target individual, environmental, or genetic factors. Further genomic association studies could look at the association of candidate genes with disease, based on hypotheses from the evolving understanding of disease mechanisms at the molecular level; genetic family studies and large genome wide association studies could also contribute to identifying new susceptibility mechanisms.

Established Disease (More Than 2 Years)

Tertiary Prevention

Tertiary prevention refers to actions aimed at reducing the impact of long-term illness and resulting morbidity and disability (74), including through rehabilitative interventions. The absence of an evidence-based curative treatment should not detract from the main objective of supporting the individual and of managing symptoms and disability. A relatively new concept, quaternary prevention originally proposed by Jamoulle (100), has been conceptualised by Martins et al. (101) as "*the action taken to protect individuals (persons/patients) from medical interventions that are likely to cause more harm than good*", with the aim being "*to reduce over-medicalisation and iatrogenic harm*". It

is important that health professionals share decision-making with patients around the use of personalised treatments because of the wide range of often non-evidence-based therapies used for ME/CFS; these include alternative health practises (102) and behaviour-based therapies (103). Decision-making should be well-informed and acknowledge the availability (or lack of) evidence for the potential benefits and/or risks of treatments, while also considering the individual and the service costs of such treatments.

All rehabilitation strategies must be based on the understanding of the pathophysiology of severe and complicated disease and disability at individual, service, and societal levels. Tertiary prevention may improve with increased research focused on this specific stage of disease.

Management

Ideally, management of established ME/CFS should centre on the restoration of a healthier homeostatic balance through specific treatments and avoidance of aggravating factors, but the lack of sufficient treatment evidence necessitates limiting such management to life-style changes [including advice on planning and executing activities within the individual's energy limit levels (79)], and the use of symptomatic medications, such as analgesics and sleep medications (10), and the avoidance of other causes of neuro-immune overload, as described previously (83, 86). As evidence grows, treatments targeting multi-systemic abnormalities (such as those resulting from dysfunctions in the immune, neuro-endocrine, autonomic, circulatory, and neuro-muscular systems) will be critical for disease management; examples could include immune-based treatments, and those targeting oxidative stress and metabolic abnormalities.

Research

For established disease, research efforts should target the understanding of mechanisms that perpetuate abnormalities and the better understanding of pathways to recovery, including specific treatments targeted at various system and molecular abnormalities. Longitudinal studies are critical to address temporal pathophysiological changes in order to guide therapeutic approaches at different disease stages, and to investigate short- and long-term complications, including co-morbidities and mortality. Younger patients with a shorter duration of disease have been found to present with different phenotypes, in relation to autonomic nervous system manifestations (16), for example, and are therefore more likely to require specific treatment for postural orthostatic tachycardia syndrome (POTS) or postural hypotension symptoms. Description and/or comparisons of specific subtypes through longitudinal studies would help to determine differences in phenotype and encourage a more tailored approach to treatment management. Research on perceptions and attitudes to prolonged illnesses, from the individual, family, educational, occupational, and wider societal points of view, would help to change the way ME/CFS is managed, which would, in turn, help with secondary prevention.

Over 15 years ago, Bell wrote "*we need to change the focus of our telescope from looking at large organs to looking at single cells*" as he considered the search for evidence in ME/CFS (104). As we focus on the cellular level of molecular and systems medicine and transfer knowledge acquired from other conditions (such as acute severe injury), we should get closer to finding the real explanations for the various subgroups in ME/CFS. The "omics" technologies (e.g., transcriptomics, metabolomics, proteomics and genomics, including pharmacogenomics) are becoming increasingly accessible. Meaningful and translatable research outputs based on relevant research questions are now possible, as long as strong methodological approaches are applied. These should cover research design and case selection, sampling and management of bio-specimens, and appropriate application of technology and interpretation of findings.

As disease understanding evolves, we will move closer to personalised health care and medicine, and more specific strategies for prevention and treatment will become possible (105). Examples of such strategies include the targeting of high-risk individuals for screening, diagnosis and treatment; molecular diagnoses of subgroups, and targeted treatment according to molecular subtypes. It is imperative to balance the need of finding the best evidence with that of promoting well-being of patients while keeping in mind the importance of quaternary prevention (**Table 2**).

ADDITIONAL CONSIDERATIONS

General and Tailored Approaches

The nature of persistent dysfunctions, and whether they can be controlled or resolved, may be central to prognosis and treatment in ME/CFS. For example, in those with more severe post-exertional symptoms (more likely affected by autonomic, neuro-endocrine, and energy metabolism dysfunction), energy management, through pacing and sensible rest, are essential to allow the body to enact its recovery potential, combined with specific treatments to address systems dysfunctions. Those with long illness duration, but with milder symptoms which are improving, may become more tolerant to exertion, feel more energetic and have less cognitive dysfunction or "brain fog"; additionally, their post-exertional symptoms may be less pronounced or be limited to major activities. Such cases could benefit from a program of individually tailored, paced, stepwise and increasing exposure to activities. However, those in whom disease is progressing unfavourably may benefit from medical treatments targeted at specific dysfunctions alongside rehabilitation. As in many chronic debilitating diseases, psychological therapies have a role in supporting individuals through their chronic illness as part of an important supplementary component of holistic medical care that includes a personal approach to management and treatment.

Severe or Complicated ME/CFS

Approximately 25% of ME/CFS patients will develop a severe form of disease, rendering them house- or bed-bound (76). Difficulty accessing this particular portion of the patient community, in both clinical practise and research, further exacerbates inadequate access to specialist care (106), selection bias and non-generalisability of results (107, 108). Access to

TABLE 2 | Summary of prevention, management and research strategies for ME/CFS according to stage of disease.

Stage	Predisposition and triggering of disease (Disease onset)	Prodromal period and early disease	Established disease
Prevention	Primary: avoidance of further stressors; adequate rest; prioritisation of recovery of initial illness	Secondary: early detection; early intervention allowing recovery time	Tertiary: reduction of long-term morbidity/disability; rehabilitation
Management	Rest; Pacing activities	Early diagnosis; treatment of trigger infection; treatment of immune/CNS/other dysfunction; reduction in symptom severity (using drugs or non-pharmacological interventions); rest; pacing	Interventions aiming to restore homeostatic balance; symptomatic relief Personalised approach Future: treatments for multi-systemic dysfunctions
Research	Risk factor studies: genetic; environmental; life-style studies Cohort studies: following-up population after outbreaks (e.g., SARS-CoV-2), or proxy models (e.g., immune treatment for diseases that induce chronic fatigue)	Large scale longitudinal cohort studies focusing on early disease pathophysiology and interventions Use of electronic health record data Biobank samples and data, existing disease cohorts	Studies on perpetuation of systemic and molecular abnormalities Studies focusing on phenotypic subtypes: using "omics" technologies to aid personalised recovery

medical services is often limited, augmented by the lack of knowledge among healthcare practitioners due to both a lack of appropriate training in medical school and widespread scepticism concerning the disease (109); many healthcare professionals are reluctant to give an ME/CFS diagnosis, especially in the early stages of the disease.

Treatment for severe or complicated ME/CFS is more challenging, as patients may have achieved an advanced state of homeostatic dysregulation with increasing multi-system dysfunction and multi-system complications. The previous state of chronic inflammation may now be subdued, while the body may enter a hypometabolic state (48). This state includes the slowing of physiological pathways and reduction of energy output, with chronic cell and system malfunction.

In addition to the measures used in early and less severe cases, any treatment approach needs to consider specific mechanisms leading to and perpetuating cell dysfunction (e.g., those associated with endothelial dysfunction and cytopathic hypoxia). These treatments may include strategies aimed at reversing neuro-endocrine and metabolic abnormalities, and at rehabilitation. Examples of interventions include neurological rehabilitation (e.g., gentle or passive physiotherapy), nutritional rehabilitation (which might need to involve enteral feeding), and those targeting circulatory dysfunction (e.g., treatment of hypotension and postural tachycardia and other manifestations of orthostatic intolerance), and the various consequences of prolonged illness (e.g., screening for and treatment of osteoporosis). Severely affected patients have restricted activity, often struggling with self-care, and needing support from carers and from a multi-disciplinary health-team. For this sub-group of patients, effective input and support from social, educational and occupational health services may be even more important, alongside a range of rehabilitative interventions.

Treatment approaches targeting specific energy metabolic dysfunctions, as well as specific nutritional and hormonal supplements, may also play a restorative role; however, these still need development and validation before they can be used beyond individually tailored approaches.

Research at Different Disease Stages

Several research questions still need answers, requiring different strategies and ways of selecting research participants. Researchers should consider the advantages of restricting the study population of cases to those who meet diagnostic criteria with higher specificity (110) and of case stratification, including sub-grouping of cases into disease stages. Alternatively, research could focus on a specific stage. While there is no doubt that molecular research is essential to revealing disease pathways and for biomarker discovery, other types of research, such as clinical, epidemiological, environmental, health service, policy and education are essential for better disease recognition, prevention, diagnosis, and treatment while an emphasis on cross-cultural studies may encourage a more standardised view of ME/CFS internationally.

Refocusing to include pre-clinical or "invisible" stages of illness can be hugely beneficial for the study of disease. One such example is Alzheimer's where, over the past decade, a conceptual shift to consider the disease as a continuum has occurred and, along with the discovery of biomarkers, has re-focused the research agenda towards the pre-clinical stage and early intervention (111). Similarly, we suggest that a re-focus of the ME/CFS research agenda towards the pre-clinical stage (by way of larger prospective cohort studies), may contribute to revealing potential risk factors to support primary prevention efforts.

CONCLUSIONS

The conceptualisation of ME/CFS into disease stages helps to understand disease pathways, their operation, and interconnections along the disease course, and therefore to support the planning of public health and clinical interventions, as well as targeted research.

Discrepancies in the use of diagnostic criteria and sampling methods have led to much variation in research results in ME/CFS and this is mirrored in the care of those affected. As research is directed towards biomedical, systems and molecular

investigations, the need for better disease stratification becomes more evident, for both research purposes and clinical practise. It is important, therefore, to consider ME/CFS as a continuum and to examine the different stages patients go through throughout the course of their disease, their severity, and the presence and degree of complications as key parameters for stratification.

Pathophysiological patterns and changes along and across disease stages result in the expression of different, albeit overlapping, phenotypes and any approach to diagnosis, subgrouping, and clinical management will vary according to these phenotypes, as will research questions and the selection of patients for research. Loss of specificity caused by the amalgamation of people with ME/CFS with those with chronic fatigue due to other causes in observational and interventional studies is problematic. Similarly, ignoring different subgroups of ME/CFS, including those related to disease stage, will have an impact on research outputs and their interpretation when investigating disease mechanisms and pathways, including clinical trials.

The concepts of determinants of health and levels of intervention are useful as they provide a framework that can be used to guide disease prevention and management, as well as research direction. The recruitment of individuals for research at the pre-illness stage could be invaluable to understanding the biological mechanisms at play before, during, and after an insult. Longitudinal studies would help to determine where individuals are in terms of the natural course of the disease and to encourage the investigation of abnormalities and of treatments that take into account disease stage, here considered as an additional category for subtyping.

This paper seeks to re-focus research and treatment management efforts. While we wait for detailed mechanisms to be identified, acquired transferrable knowledge, and good health care are required to ensure safe, high quality care for those who are ill.

DATA AVAILABILITY STATEMENT

Data are available upon request from the authors.

AUTHOR CONTRIBUTIONS

LN and EL conceived the paper. SO'B contributed to drafting, referencing, and formatting. All authors contributed to drafting and to revising the manuscript and approved the final version to be published.

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