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RESEARCH ARTICLE

The significance of oxidative stress in the pathophysiology of Long COVID and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

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ABSTRACT

Long COVID is now well accepted as an ongoing post-viral syndrome resulting from infection of a single virus, the pandemic SARS-CoV-2. It mirrors the post-viral fatigue syndrome, Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome, a global debilitating illness arising mainly from sporadic geographically-specific viral outbreaks, and from community endemic infections, but also from other stressors. Core symptoms of both syndromes are post-exertional malaise (a worsening of symptoms following mental or physical activity), pervasive fatigue, cognitive dysfunction (brain fog), and sleep disturbance. Long COVID patients frequently also suffer from shortness of breath, relating to the lung involvement of the SARS-CoV-2 virus. There is no universally accepted pathophysiology, or recognized biomarkers yet for Long COVID or indeed for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. Clinical case definitions with very similar characteristics for each have been defined. Chronic inflammation, immune dysfunction, and disrupted energy production in the peripheral system has been confirmed in Long COVID and has been well documented in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. Neuroinflammation occurs in the brain in Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome as shown from a small number of positron emission tomography and magnetic resonance spectroscopy studies, and has now been demonstrated for Long COVID. Oxidative stress, an increase in reactive oxygen and reactive nitrogen species, and free radicals, has long been suggested as a potential cause for many of the symptoms seen in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, resulting from both activation of the brain's immune system and dysregulation of mitochondrial function throughout the body. The brain as a high producer of energy may be particularly susceptible to oxidative stress. It has been shown in peripheral immune cells that the balanced production of proteins involved in regulation of the reactive oxygen species in mitochondria is disturbed in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. Fluctuations in the chronic low level neuroinflammation during the ongoing course of Long COVID as well as Myalgic Encephalomyelitis/Chronic Fatigue Syndrome have been proposed to cause the characteristic severe relapses in patients. This review explores oxidative stress as a likely significant contributor to the pathophysiology of Long COVID and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome, and the mechanisms by which oxidative stress could cause the symptoms seen in both syndromes. Treatments that could mitigate oxidative stress and thereby lessen the debilitating symptoms to improve the life of patients are discussed.

INTRODUCTION:

Long COVID and Myalgic Encephalomyelitis/ Chronic Fatigue Syndrome

Acute infections are often associated with ongoing chronic illnesses that affect a minority of patients, but these post-infectious disease syndromes are largely unexplained with poor understanding of their underlying mechanisms. [1] The current infectious disease outbreak from the SARS-CoV-2 virus is a classic example. The outbreak rapidly became a pandemic, now with ~600 million cases reported worldwide and has given rise to many millions of cases of a post-viral syndrome, known colloquially as Long COVID. This huge case load of the COVID19 pandemic has thrown the spotlight on the seriousness of post-viral fatigue syndromes, not only for the individuals affected, but for societies and economies worldwide. With an estimated incidence of 10-30% of cases of SARS-CoV-2 developing ongoing post-viral disease, [2] a high proportion of whom were suffering from a fatigue syndrome, means ~50-150 million cases are likely to be added worldwide to the estimated load of 20-40 million people with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). Given the name Post COVID-19 condition by the World Health Organization (WHO) in 2021, [3] Long COVID will be a significant burden on health and social systems of all countries. It has caught the universal attention collectively of clinicians, and of governments alike, leading to promises of large investment in research and patient care.

ME/CFS by contrast has always struggled to be recognised as a serious post-viral syndrome, despite 75 reports of small geographically isolated outbreaks of such an illness following viral infections. [4] More recently, it has also been appreciated about 20% of ME/CFS cases can arise from other stressors, such as exposure to toxic agricultural chemicals, other infectious agents, surgery or severe events that affect the physiology in susceptible people.[5] ME/CFS has become an umbrella term therefore for a physiological response in some people to a range of major stressors, most of which are viruses. Those affected by the chronic viral fatigue syndrome in the cases of the sporadic viral outbreaks number from only a few hundred or at most a few thousand infectious cases. For example, even the initial SARS-CoV-1 outbreak in 2003 had only a documented 8422 infectious cases worldwide, [6] but in 2011 a study documented a cohort from the outbreak suffering from a debilitating post-viral syndrome. [7] The sporadic nature of these infections has resulted in no widespread interest or significant investment in research or social services to support those chronically debilitated by what has proved to be a serious ongoing illness. An important report from the Institute of Medicine (now Academy of Medicine) of the United States Academy of Sciences in 2015 [8] concluded ME/CFS was a serious disease that warranted much more research investment and social support for both those affected and those who cared for them. It precipitated a needed change in how ME/CFS was perceived. The large promised investment into research into Long COVID is a positive development for ME/CFS sufferers who have been previously referred to as the 'Missing Millions', as they should benefit from this investment as well – some of whom have had their illnesses for 30 or 40 years.

Clinical case definitions:

WHO developed an iteratively-derived clinical case definition for the major symptoms of Long COVID, [3] but that likely reflects only the largest subgroup of those with Long COVID who have the classic post-viral fatigue syndrome. Initially, Long COVID following acute SARS-CoV-2 infection encompassed people with ongoing specific injury to the lungs, heart, kidney, and brain, as well as the classic post-viral fatigue condition. It was also proposed one subgroup might be suffering from post-traumatic stress disorder arising from their hospital treatment after being seriously compromised by COVID19. Nevertheless, the implication was that a considerable proportion of Long COVID sufferers had a very similar pathogenesis to that of ME/CFS. [9] The WHO clinical case definition for Long COVID [3] is very similar to the more recent refined versions of the clinical case definition for ME/CFS [10,11] suggesting the fatigue syndromes are very closely related. [12] It applies to individuals with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms that last for at least 2 months that cannot be explained by an alternative diagnosis. The most commonly experienced symptoms include fatigue, shortness of breath, cognitive dysfunction (brain fog), post-exertional malaise (PEM), health deterioration through physical or mental exertion), sleep disorders, which generally have an impact on everyday functioning. Apart from shortness of breath, which likely relates to the effects on the lungs from the specific SARS-CoV-2 virus, they are also the dominant symptoms of ME/CFS. Symptoms often present either following initial recovery from an acute COVID-19 episode, often very mild, or have persisted from the initial illness, and/or after

multiple infections with COVID19. As with ME/CFS symptoms, these are seen to fluctuate or relapse over time. One difference is that the condition has to persist for only 3 months before confirmation of diagnosis, whereas for ME/CFS it is defined after 6 months. It is this large subgroup of Long COVID patients with the post-viral fatigue syndrome that the discussion in this review encompasses, along with ME/CFS patients whose disease has resulted from multiple sources.

Understanding of ME/CFS has been hampered by its ill-defined pathophysiology and the lack of conclusive molecular biomarkers for a diagnostic laboratory test. ME/CFS has over 20 different clinical case definitions in the literature, The Fukuda 1994 diagnostic criterion has been commonly used by researchers and clinicians, [13] but more recent criteria like the Canadian Consensus Criteria (2003) [10] and its refinement in 2011 the International Consensus Criteria [11] - both developed by an international ME/CFS panel of experts - are now favoured. These criteria focus on core symptoms of post exertional malaise, fatigue, sleep dysfunction, and cognitive dysfunction. Additionally neurological and autonomic/neuroendocrine/immune symptom groups have been included. [10] The 2011 criteria included an emphasis on inflammation, neuropathology and energy impairments. [11] The number of research studies has gradually accelerated to reveal biological dysfunctions in many physiological systems. The known features of the pathogenesis of ME/CFS have recently been elegantly summarized and compared with what was known in the early stages of Long COVID - referred to then as Post-acute COVID-19 syndrome.[9] The authors speculated the pathogenesis of this illness may be similar to ME/CFS.

Search for a biomarker based diagnostic test

The most pressing need for Long COVID and ME/CFS is a simple specific diagnostic test, accessible to all clinicians to complement the clinical case definitions since the symptoms overlap with those of a number of other illnesses like multiple sclerosis, and fibromyalgia that have fatigue as a major component. [14] Exciting possibilities have emerged recently for ME/CFS. For example, nanoneedle bioarray technology developed by Professor Ron Davis and colleagues measures a unique impedance signature that can distinguish moderate to severe ME/CFS from healthy controls. It measures electrical impedance modulations from cellular or molecular interaction in response to high salt concentration utilizing patient peripheral blood mononuclear cells or plasma. Using supervised machine learning algorithms, the authors can identify new patients, an essential requirement for any robust diagnostic tool. [15] This is yet to be tested against other fatigue illnesses with overlapping symptoms to see whether it is specific for ME/CFS, but it also has potential to identify a substance in plasma of ME/CFS patients that is disease specific and itself would be a biomarker. [12] Another biomarker diagnosis based on three tests from Professor Paul Fisher's group measures a combination of lymphocyte death rate, mitochondrial respiratory function and a cell-sensing kinase (Target of Rapamycin Complex1), and gives high sensitivity and specificity for the accurate diagnosis of ME/CFS. [16] Unfortunately, despite its value and utility for selective confirmation of a diagnosis, because of the complexity of the assays it may not be so easy to adapt or develop as a simple universally available test in community diagnostic facilities. In our laboratory, a stress kinase, Protein kinase RNA activated (PKR), described as a 'universal immunological abnormality in ME/CFS [17] has been investigated. Activation of the kinase involves phosphorylation so the ratio of the active to the inactive form in peripheral blood mononuclear cells has potential for a biomarker test for ME/CFS. A pilot study showed no phosphorylation in healthy controls in contrast to ME/CFS patients. This test used immunological detection so it could have potential to be converted into a simple enzyme-linked immunosorbent assay (ELISA) assay. [5] A test for Long COVID will likely have similar biomarkers to that for ME/CFS but a feature uniquely arising from COVID 19 infection would be desirable so the two syndromes could be readily distinguished - effective therapies may be somewhat different for each of the two post-viral syndromes with their different origins.

Physiological characteristics of the ongoing post-viral illnesses

The key question posed in this review regards the importance of oxidative stress in the ongoing Long COVID and ME/CFS post-viral fatigue syndromes. Oxidative stress, resulting from an increase in reactive oxygen species (ROS), reactive nitrogen species (RNS), or free radicals has long been suggested as a potential cause for many of the symptoms seen in ME/CFS. Oxidative stress potentially can arise from both activation of the brain's immune system and dysregulation of mitochondrial function throughout the body, and has been linked to inflammation. [18] In this review the immune/inflammatory processes that lead to

oxidative stress in Long COVID and ME/CFS are discussed. The mechanisms causing oxidative stress that arise from dysfunctional mitochondria and ROS in the brain and in the periphery and how they may result in the symptoms of Long COVID and ME/CFS are outlined. The challenges of measuring oxidative stress in these post viral syndromes are considered, illustrated with examples from a study of ME/CFS subjects in response to exercise. Potential treatment therapies are highlighted along with the need for rigorous clinical trials with patients to assess their true potential.

Chronic inflammation

When the body's immune system is activated, either with a viral illness (for example, Epstein Barr for glandular fever or SARS-CoV2 for COVID19), or as a response to physiological stress, inflammatory cells are mobilized. The intensity and duration of this response determines whether the immune signaling results in healing or becomes destructive to normal physiology. [19] Brief controlled inflammatory responses are beneficial in removing a threat but if prolonged, as in ME/CFS and now suggested in Long COVID, inflammatory cells and cytokines continue to behave inappropriately despite there no longer being any outside danger. [20] Inflammation may be thought of as a grouping of immune cells in the extracellular area specific to a site of damage or infection, [20] for example in the lungs with SARS-CoV-2 infection. The brain and other components of the central nervous system (CNS) has its own immune response machinery that when activated can lead to neuroinflammation as a protective response. Just as in the systemic system, excessive or chronic neuroinflammation in the CNS can be damaging. Microglia, the immune cells of the CNS, when activated are spread through the brain and spinal cord, and may account for ~15% of its cells. [21] They play a key role in the innate immune response preventing invading pathogens from causing diseases in the brain, [22] and as the most plentiful immune cells in the CNS they also play a role in the development of neuronal networks. Primarily their function is to protect neurons, but any alteration in their function can conversely lead to neuronal damage. They have been implicated in many neurodegenerative diseases such as Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). [23-25] The role that microglia play in the pathophysiology of these diseases is intertwined with the production of ROS and oxidative stress. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and its various isoforms are the main enzymes responsible for ROS production in response to stimulus in acute events or in neurodegenerative disorders. [26,27] Amyloid beta plaques in Alzheimer's disease induce microglial activation, which then stimulate production of ROS through this pathway leading to neuronal damage and progression of disease. [27,28] Chronically-activated microglia promote inflammatory functions that give rise to ROS and lead to neurological dysfunction. [29] This is characteristic of what is seen in ME/CFS. A consequence of chronic inflammation in ME/CFS is a breakdown in homeostasis, and this neuroinflammation helps to maintain the *systemic* chronic inflammation and immune dysfunction. [4]

Trained immunity has recently emerged as a concept whereby the first line of defence against pathogens, the innate immune system, can unexpectedly provide a non-specific immune memory. This was thought previously to be a function of only the adaptive immune system, the immune subsystem that contains specialised systemic cells that provide memory so we are prepared to immediately fight reinfection at a later time. [30] While trained immunity could therefore give some lasting protection against the foreign organism as well by producing an enhanced response to a reinfection it is thought it may be relatively short term (months) unlike adaptive immunity. Nevertheless, trained immunity may be inadvertently contributing to the risk of developing ME/CFS by producing higher levels of proinflammatory cytokines thereby prolonging a chronic inflammatory state. Trained immunity, coupled with hyperactivation of the innate immune system as is found in ME/CFS [31] could promote a secondary response to sustain Long COVID and ME/CFS as ongoing illnesses with a low level chronic inflammatory state.

Evidence that Long COVID and ME/CFS are chronic inflammatory diseases

We have proposed, following activation of a systemic immune/inflammatory response to an infection or severe stress event, abnormal transport of signals or molecules into the CNS occurs through neurovascular pathways or a disrupted blood brain barrier. [4] If the initial stressor response is not resolved in susceptible people this leads to fluctuating chronic neuroinflammation that sustains and controls the complex neurological symptoms of Long COVID and ME/CFS, as evidenced from a comprehensive disruption to the cellular molecular biology and the body's physiological pathways. [4] In most people exposed

to viral infections or transient life stresses, inflammatory responses subside rapidly and post stress syndromes like Long COVID and ME/CFS do not develop. For some people, if the response becomes chronic and dysregulated as is the case with the two syndromes, then atypical signaling to the brain and central nervous system occurs. This will chronically activate and sustain the specific components of the microglial-mediated immunological/inflammatory response that results in chronic neuroinflammation.

A cycling of molecular "danger signaling" between the systemic innate immune system and brain's innate immune system may then be set up and persist. This might occur in the brain with damaged mitochondria acting as a signaling organelle, for example with leakage of the energy molecule, adenosine triphosphate (ATP) and subsequent purinergic signaling to activate microglia. [32] Here the chance of ROS becoming unregulated to precipitate ongoing oxidative stress is high. An overview model of the events that might lead to oxidative stress in Long COVID and ME/CFS is shown in Figure 1. It proposes following infection or major stress there is a proinflammatory response that if not resolved can lead to trained unregulated immunity and chronic inflammation. This, in turn, causes disruption of mitochondrial function and dysregulation of the control of ROS leading to oxidative stress. Communication to the CNS (see details in Tate et. al.[4]) can lead to chronic *neuro*inflammation and activated microglia with the production of ROS. The brain is an environment of high mitochondrial energy production and requires good regulation to prevent excessive production of these molecules. When dysregulated as in Long COVID and ME/CFS the brain may be the primary focus of oxidative stress in these illnesses.

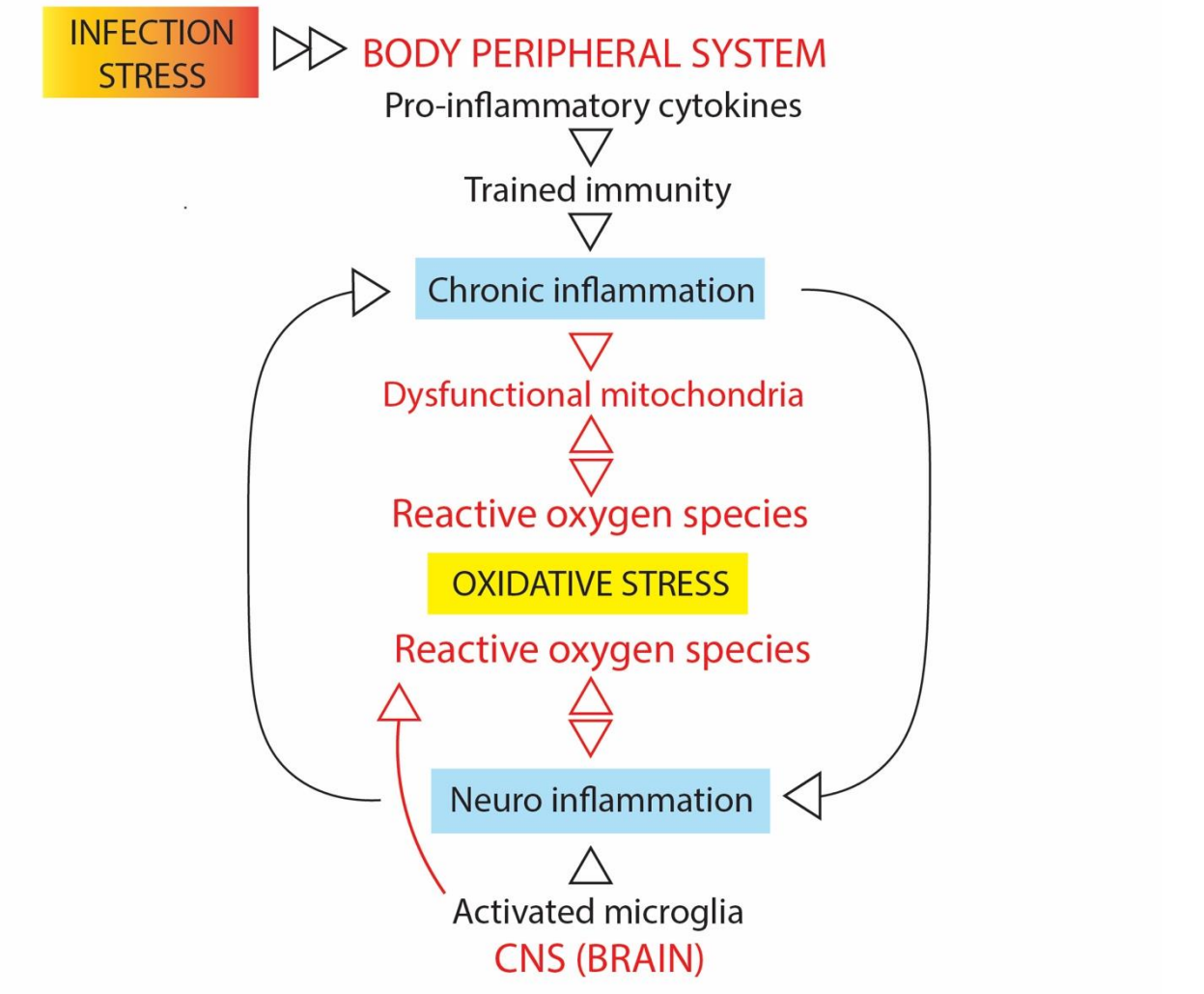

Figure 1. Pathways to oxidative stress in Long COVID and ME/CFS.

The paraventricular nucleus (PVN) of the hypothalamus is responsible for modulating the body's response to day-to-day stressors *via* the parvocellular neurosecretory cells that secrete the stress hormone corticotropin-releasing hormone (CRH), which is involved in autonomic and homeostatic regulation [33]. Release of CRH can lead to activation of the brain's immune cells (microglia, mast cells and T cells), from which the release of inflammatory mediators promote the chronic neuroinflammation. [34] The inability of the brain to manage stress in Long COVID and ME/CFS patients, *via* the stress centre in the PVN, means even minor stressful events would be a constant fuel stoking the immune response in the CNS.

Is there evidence in the periphery and the CNS for the ongoing inflammation as proposed in Figure 1? It is commonly stated the ME/CFS patients' peripheral immune systems are chronically activated or in "overdrive". [31] Proinflammatory cytokines make up 13 of 17 cytokines that correlate with the severity of ME/CFS and thereby are likely to be contributing significantly to the symptoms. [35] A distinct cytokine inflammatory signature associated with early disease has also been reported in ME/CFS. [36] Early ME/CFS cases have a prominent activation of both pro- and anti-inflammatory cytokines and dissociation of regulatory networks, signatures that were not found at later stages of the illness.

Our own research studies have examined the molecular biology of peripheral blood mononuclear cells for ongoing evidence of inflammatory dysfunction in patients in their chronic phase of ME/CFS. [37,38] Even in patients who have had their disease for a mean time of 10 years, there were 33 differentially expressed messenger RNA molecules (transcripts) between ME/CFS patients and healthy controls ($p<0.01$). The top three were interleukin-8 (*IL8*), nuclear factor kappa B subunit 1 inhibitor alpha (*NFKBIA*), and tumor necrosis factor alpha induced protein 3 (*TNFAIP3*), all members of inflammatory/anti-inflammatory pathways and early responders to tumor necrosis factor-induced nuclear factor kappa B (TNF-induced NF-kB) activation amplifying chronic inflammation. Of the other differentially expressed transcripts, ten reinforced significant immune and inflammatory over-activation and dysregulation as part of the ongoing pathology of ME/CFS. Pathway analysis highlighted the production of nitric oxide (NO) and ROS in macrophages. [37]

The peripheral blood mononuclear cell *proteome* exhibited 100 proteins that were differentially regulated in ME/CFS patients, 57 of which were immune-related. [38] The subfraction of the differentially regulated mitochondrial proteins involved in the *reactive oxygen species* stress response and in *energy production* are shown in Figure 2. Most are upregulated. This reflects a disturbance in the balance of proteins in the peripheral immune cells of patients in the chronic phase of their ME/CFS that has persisted for many years after the onset of their illness. Significantly it affects the complexes involved in mitochondrial energy production, and in the mitochondrial regulatory pathway controlling ROS. It is now well-recognized that inflammation, mitochondrial dysfunction, and oxidative stress, are intrinsically linked in neurological disorders. [18]

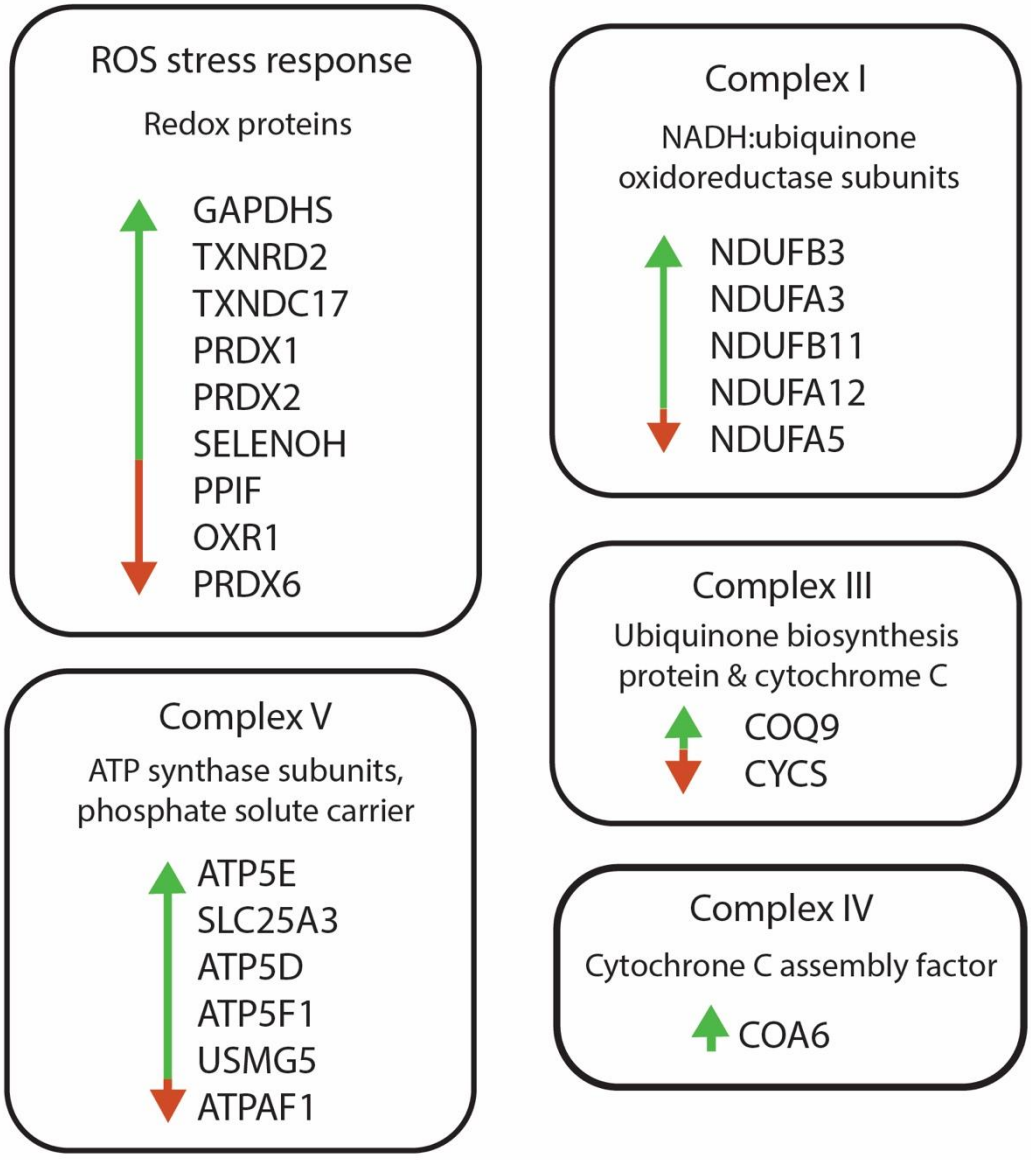

Figure 2. **Differentially regulated mitochondrial ROS stress response and energy production proteins in ME/CFS patients compared with healthy controls.** Peripheral blood mononuclear cells contained differentially regulated mitochondrial proteins ($p \leq 0.05$, fold change ≥ 1.3 and ≤ 0.75) involved in regulation of the ROS stress response (upper left), proteins of the ATP synthase energy production complex (Complex V)(lower left) and complexes of the electron transport chain (I, III & IV), leading to energy production. Green arrows are upregulation, red arrows downregulation. GAPDHS - Glyceralhehyde-3-phosphate dehydrogenase, testis specific; TXNRD2 - Thioredoxin reductase 2; TXNDC17 - Thioredoxin domain-containing protein-17; PRDX1, 2 or 6 - Peroxiredoxin 1, 2 or 6; SELENOH - Selenoprotein H; PPIF - Peptidyl-prolyl cis-trans isomerase F; OXR1 - Oxidation resistance protein 1; NDUF B3 or B11 - NADH:ubiquinone oxidoreductase beta subunits 3 or 11; NDUF A3, A5 or A12 NADH:ubiquinone oxidoreductase alpha subunits 3, 5 or 12; COQ9 - Ubiquinone biosynthesis protein COQ9; CYCS - Cytochrome c oxidase assembly factor homolog; ATP 5E, 5D or 5F1 - ATP synthase F1 subunit epsilon, delta or beta; ATPAF1 - ATP synthase mitochondrial F1 complex assembly factor 1; USMG5 - Up-regulated during skeletal muscle growth protein 5, COA6: cytochrome C oxidase assembly factor 6

Preliminary data from a pilot study in progress in our research comparing Long COVID and ME/CFS patients have shown the immune cell differentially-regulated proteomes of the two illnesses are closely

overlapping and well separated from age/gender matched healthy controls in a data-stratifying principal component analysis. Functional associations of the proteins identified in Long COVID patients, as in ME/CFS, cluster around immune dysregulation, inflammation, energy production, metabolism, and RNA biology. Upregulated inflammatory proteins are more prominent in Long COVID patients, perhaps because their ongoing post-viral illness is at an earlier stage (months from onset) compared with the ME/CFS patients (years from onset) (Tate unpublished).

Oxidative stress in ME/CFS in the periphery

Long before the advent of Long COVID, oxidative stress was suggested as a key part of the pathophysiology of ME/CFS. [39-43] Oxidative stress can be defined as an imbalance between pro-oxidant ROS and the anti-oxidant mechanisms that are meant to keep them in check. ROS are partially reduced metabolites of oxygen that possess strong oxidizing capabilities, important in health and disease. [44] Under normal physiological conditions ROS are involved with host defense, cell signaling and biosynthesis. 'Oxidative stress' is the pathological term for when high levels of ROS overwhelm the cellular antioxidant buffering capacity, resulting in damage to cellular macromolecules. [45] During the process of inflammation these molecular damaging properties of ROS are utilised as a defense mechanism against invading pathogens but after prolonged exposure to oxidative stress an excess of ROS can cause damage to lipids, proteins and carbohydrates of host systems; for example, the hydroxyl radical can react with DNA bases, altering transcription and ultimately protein function. [46] ROS can also induce lipid peroxidation damage to membranes causing cell death, a pathology that is universal to all respiratory diseases including asthma. [47] In the brain ROS activate microglia to stimulate neuroinflammation, a hallmark of neurodegenerative disease. [27] ROS are produced as a byproduct of metabolism and members include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO\cdot$) and the hydroxyl radical ($OH\cdot$). The superoxide anion ($O_2^{\cdot-}$) is generated during ATP energy production via the electron transport chain of mitochondria and during reduction of O_2 to $O_2^{\cdot-}$ through enzymatic catalysis by NADPH oxidase (NOX enzymes) or xanthine oxidase in various cells including phagocytes and endothelial cells. $O_2^{\cdot-}$ is converted to H_2O_2 and this interacts with a transitional metal ion (Fe^{2+}) to generate the highly reactive hydroxyl radical $OH^{\cdot}$ in the Haber–Weiss reaction. [48]

Oxidative stress can lead to nitrosative stress, which refers to a large increase in RNS such as peroxynitrite (ONOO-). Peroxynitrite is produced when nitric oxide (NO) and superoxide anions (O2-) react together. Increase in RNS, like ROS, can be damaging and as RNS species are produced when ROS are in excess, both are linked to oxidative stress. The presence of NO or ROS is not a pathological sign since normal endogenous levels are essential for cellular signaling, protection from pathogens, vasodilation and neurotransmission. Problems arise when there is either ROS in excess and/or natural antioxidants are reduced, thus creating an imbalance and inducing a state of oxidative stress, [49,50] Paul *et al.*[49] point out there are many biomarkers in ME/CFS that are indicators of oxidative stress resulting from redox imbalance (pro-oxidants vs anti-oxidants). In ME/CFS, increased levels of peroxides and superoxide causing oxidative stress have been shown to correlate with the severity of the symptoms [51]. Multiple biomarkers of nitrosative stress (NO, peroxynitrite and nitrate) have also been found in ME/CFS patients [50] but not healthy controls, and increases in such biomarkers have been documented following exercise [51]. Plasma metabolomics of ME/CFS patients have detected levels of metabolites that are consistent with a state of mitochondrial dysfunction and oxidative stress [52-54].

Mitochondria are intrinsically linked to ROS production and therefore oxidative stress. Mitochondria have often been considered to be at the core of many of the ME/CFS symptoms (and likely Long COVID symptoms) given that the dysfunction of the main energy producing organelles of the body would result in symptoms consistent with those experienced by these patients [40,55]. Oxygen plays a key role in accepting free electrons at the end of oxidative phosphorylation that produces the energy molecule, adenosine triphosphate (ATP), forming water. This prevents congestion of electrons in the associated electron chain complexes (I to V) on the inner mitochondrial membrane. However, in some instances when oxygen is reduced it can be converted to superoxide, rather than water. Superoxide will then be converted to hydrogen peroxide that is secreted into the cell leading to damage. Many studies have shown mitochondrial abnormalities or dysfunction in ME/CFS patients. [49,55,56] Reduction in ATP production has been shown in studies on neutrophils and lymphoblasts, and this has been claimed to be

a primary cause for ME/CFS symptoms. [57,58] Fusion of mitochondrial cristae has been observed that would negatively impact the ability of oxidative phosphorylation due to the reduced surface area for complexes. [59] Other studies on ME/CFS patients, however, have shown increases in ATP production and condensation of the cristae. [60] Nevertheless, mitochondrial abnormalities and dysfunction have been shown to lead to an increase in oxidative stress. [61]

Consistent with the above observations, many studies have now shown evidence of oxidative stress in the *peripheral system* of ME/CFS patients, with observations of decreases in antioxidant functions compared to healthy controls. [62] One study showed significant increases in markers for oxidative stress such as 8-hydroxy-2'deoxyguanosine (8-OHdG) and malondialdehyde (MDH) in ME/CFS patients compared to controls. Antioxidant enzyme activities of selenium-dependent peroxidase, glutathione transferase and soluble catalase (glutathione-independent H_2O_2 scavenging enzyme) were significantly increased in muscle biopsies compared to controls. [42] This is most likely a compensating response to cope with an increase in oxidative stress in ME/CFS. One study showed reduced levels of antioxidants such as dehydroepiandrosterone-sulphate (DHEA-S) and coenzyme-Q10 (CoQ_{10}). [63] This decrease in natural endogenous antioxidants could result from insufficient production or because they have been oxidized from increased ROS species. An interdependent relationship between ROS levels and mitochondrial function arises as ROS attack mitochondrial and nuclear DNA. These DNA mutations can lead to further abnormalities in oxidative phosphorylation therefore generating more ROS and creating a vicious cycle. [63] Two separate studies show plasma increases in isoprostanes, a marker of oxidative stress following free radical attack on cellular membranes of ME/CFS patients compared to healthy controls. [64,65]

Redox changes in the brain: evidence for oxidative stress and neuroinflammation

We await magnetic resonance spectroscopy (MRS) studies with Long COVID patients, but a recent MRS study of the anterior cingulate cortex in ME/CFS patients showed there were many abnormal levels of metabolites found in the brain. [66] Creatine, glutathione and myo-inositol were all found to be significantly decreased in comparison to healthy controls. Glutathione, the most abundant natural antioxidant, is an endogenous free radical scavenger. Reduced levels would therefore indicate the risk of having an increased susceptibility to oxidative stress. Myo-inositol is found in glial cells, and creatine acts as an energy reserve in the absence of sufficient ATP. Reductions in creatine may partially explain some of the fatigue in ME/CFS. [65]

Increases in lactate can indicate a raised level of oxidative stress. A study using MRS found increased ventricular lactate levels in ME/CFS patients. [67] Increased levels of ROS and oxidative stress were suggested as the primary mechanism for this increase, as oxidative stress can lead to cerebral hypoperfusion and/or secondary mitochondrial dysfunction in the brain, which suggest that increased lactate levels were a biproduct of anaerobic energy production. [67-69] If the glutathione level falls in the brain it can be the cause of a redox imbalance leading to oxidative stress. Indeed, reduced levels of occipital lobe glutathione have been reported in ME/CFS patients. [69] More importantly these levels were inversely correlated with increases in ventricular lactate indicating production of ATP by anaerobic metabolism independent of oxidative phosphorylation.

Another molecule important for protection against inflammation, and oxidative stress, that prevents oxidation of proteins is hydrogen sulfide (H_2S). [56] Some H_2S is produced in the brain and metabolism of this molecule has been found to be disrupted in many neurodegenerative disorders such as Huntington's, Parkinson's, and Alzheimer's diseases. [70,71] H_2S, based on its ability to inhibit mitochondrial oxygen use, may play a part in ME/CFS. [72] Potentially it could alter redox imbalance in the brain in ME/CFS.

How might oxidative stress manifest in the brain?

The production of ROS in the brain is essential for synaptic plasticity, neurotransmission and overall normal cognitive function. [49,73,74] The brain, although only weighing about 1400g, is highly metabolically active and consumes about 20% of the body's circulating oxygen. [75] Since ATP production by mitochondria is at such a high rate in the brain compared to other tissues there is more risk that small changes in the overall biochemistry may produce abnormal levels of ROS. Influxes of calcium, changes in glutamate levels or effects of endogenous neurotransmitters can all influence the level of ROS in the brain leading to oxidative stress. [73] The brain has fewer antioxidant systems and so increases in ROS are more likely to result in deleterious outcomes [76]. Throughout the body Fe^{2+} and Cu^{2+} are essential to the catalytic function of enzymes but the brain requires increased levels of

these transition metals to sustain function, energy production, myelin synthesis and neurotransmission. [77,78] Changes to iron and copper homeostasis in the rat are linked to increased oxidative stress in the brain. [79] The brain also has high levels of unsaturated lipids that correlate with an increased risk of oxidative stress. [73,80] Collectively, these observations imply much of the way the brain functions pre-disposes it to an increased risk of oxidative stress.

In an organ like the brain so susceptible to redox imbalance, therefore, how might oxidative stress arise in the brains of patients with Long COVID and ME/CFS when neuroinflammation may be key to the pathophysiology? [81,82] Activation of microglia is likely to be one of the primary causes of the neuroinflammation found in ME/CFS patients by the Positron Emission Tomography (PET) coupled with Magnetic Resonance Spectroscopy (MRS) imaging study [81], and now in Long COVID [82]. Indeed, a post-mortem case series on patients in Germany who died as a result of complications following acute SARS-CoV-2 infections, indicated extensive microglial activation, predominantly throughout the brainstem and cerebellum, indicating neuroinflammation is a significant feature in at least the acute stage of the disease [83]. The very recent study of two Long COVID patients shows widespread neuroinflammation that was not seen in controls [82]. Importantly, for both Long COVID and ME/CFS this is an indication of the presence of oxidative stress in the brain. Mitochondrial dysfunction, well documented in ME/CFS,[38,49,55-58] and now indicated for Long COVID,[84] is another route to generate oxidative stress. The question is how increased oxidative stress can lead to the symptoms of Long COVID and ME/CFS and that will be discussed below.

Challenges in measuring oxidative stress

Some studies on ME/CFS have found evidence of designated biomarkers for oxidative stress, but others have found no significant difference in the levels of such markers. It is not clear whether this reflects that there is indeed no significant difference between the patients and healthy controls, or because accurate measurement of oxidative stress biomarkers is very difficult. For this reason, measurement of natural levels of pro-oxidants and anti-oxidants in the body are also important when investigating susceptibility to oxidative stress. The ratio of the oxidised form of glutathione to the reduced form (the antioxidant) has been measured in relation to many diseases, [85,86] however, limitations of glutathione measurement are well known to those that study the molecule. In a study of cortical glutathione level in ME/CFS the authors point out limitations of magnetic resonance spectroscopy that led to other studies not finding significant changes in ME/CFS patients. [69] Measurement of some pro-oxidant ROS *in vivo* can be inaccurate due to their short half-lives and high reactivity, and therefore it is better to measure oxidative stress based on hydroperoxides as they are more stable oxygen metabolites. [62]

A commonly used approach to quantify oxidative stress is to measure modifications to proteins or DNA or lipid molecules that have resulted from excessive ROS. Blood and urine are typically used, although cerebrospinal fluid (CSF) samples or direct tissue samples have been used in other diseases. [87] Since levels of oxidative stress differ dependent on the sample source, comparing levels of oxidative stress in different diseases is difficult.

Modified lipid molecules commonly measured are Malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and isoprostanes. [88,89] HNE and MDA measurements are usually reliable but HNE measurement by gas chromatography coupled with mass spectroscopy (GC-MS) has been shown to be useful and accurate only in blood samples from patients with autoimmune disease. [87,90] MDA is usually measured by reacting it with thiobarbituric acid to produce a red adduct that can then be measured by a fluorometric or colourometric assay, but this is not specific and artefacts can be detected as well. [87,91] Isoprostanes, produced (as previously described) as a result of free radical damage on cellular membranes, are a reliable biomarker but accurate measurements are costly because a combination of expensive techniques is required.

DNA oxidation to give 8-OHdG is one of the most commonly assessed of the oxidative stress biomarkers [87,92] but also has its limitations. A study found that high-performance liquid chromatography (HPLC) coupled with GC-MS varied with GC-MS and gave different values. [93] Sample storage and handling such as freeze-drying before measurement can also contribute to artificial oxidation of deoxyguanosine contributing to misleading results. [92,93]

Protein carbonyls are formed following radical attack on protein side chains. They are a stable and sensitive marker of oxidative stress that can be easily stored and then measured using cost effective assays. [94-96] We have conducted an exploratory longitudinal study of oxidative stress using precision

medicine with individual ME/CFS patients following an exercise protocol. This was part of a study of the core symptom of Long COVID and ME/CFS, post-exertional malaise.

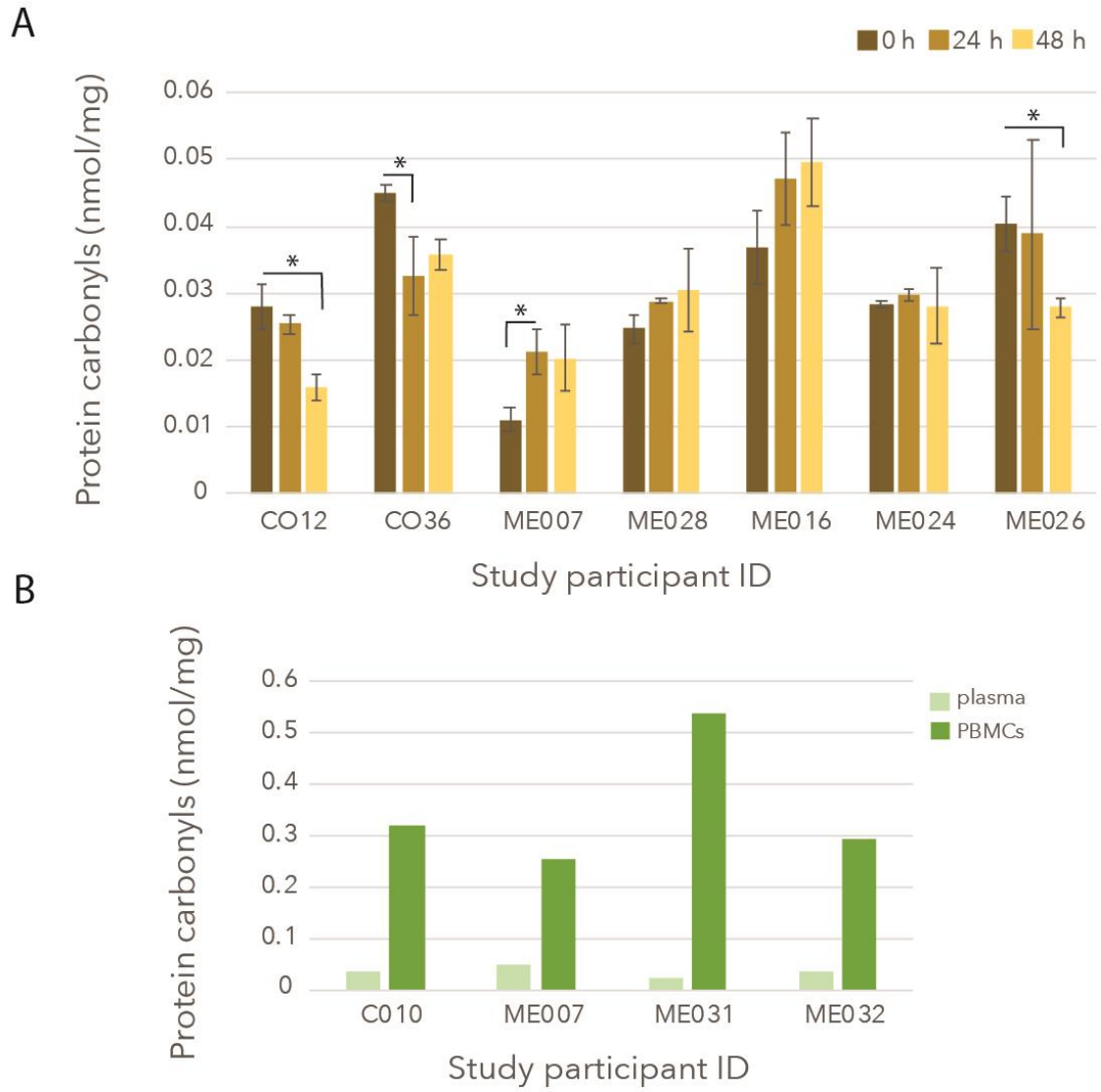

Figure 3. Protein carbonyls as a measure of Oxidative stress in ME/CFS patients following exercise. A. Precision medicine study of post-exertional malaise in individual ME/CFS patients (ME) and healthy controls (CO) assessed protein carbonyl modification to plasma proteins before and 24 h following each of two exercise sessions 24 h apart. CO12 & CO36 are controls, and ME007, ME028, ME016, ME024, and ME026 are ME/CFS patients. All participants were young women in their 20's. Error bars represent the SEM and a two-tailed students t-test determined significance of changes from before exercise in each case (* indicates a p-value ≤0.05). C012, C036 and ME026 showed significant reduction, ME007 a significant increase, while ME016 and ME028 trended towards significance increases. B. Protein carbonylation of proteins in plasma and from PBMC's from a control and three ME/CFS patients were compared and the n moles of carbonylation per mg of the PBMC proteins was an order of magnitude higher.

The study design

Five ME/CFS patients and two age gender matched healthy controls (all women in their 20's) took part in the study. Two exercise sessions were held 24 h apart on an exercycle in a specialised exercise physiology laboratory where cardiac physiology measurements could be made. Participants had blood taken for analysis before exercise, 24 h after a first exercise session, and then at 48 h, (24 h after a second exercise session). Cardiac physiology was

monitored under supervision during the exercise periods. Peripheral blood mononuclear cells (PBMCs) were isolated within 2h of blood collection in each case and used to determine mitochondrial function using a Seahorse analyser, and together with plasma were used for assessing biomarkers of oxidative stress. DNA was isolated from the PBMCs for DNA methylome and oxidative stress analyses. Detailed analyses of all aspects of the study are to be published together.

Specific individual patient responses were found with each of the parameters measured (cardiac physiology, mitochondrial function, oxidative stress). Here we show examples of the oxidative stress data in Figure 3A. The concentration of protein carbonyls in plasma samples detected using a Biocell Protein Carbonyl Assay Kit (Biocell Corporation Ltd, N.Z.) decreased in the two healthy controls and one patient across the exercise session, whereas it increased in three patients with ME/CFS, and one patient showed no change. This pilot study suggested oxidative stress occurs in some of the patients and a larger study is warranted.

Peripheral blood mononuclear cells (PBMCs) were evaluated as a comparison with plasma in ME/CFS patients and much higher concentrations of carbonylation were measured per standard amount of protein (seen in Figure 3B) suggesting studies with cellular proteins may be more informative.

Current methods to measure oxidative stress, therefore, although widely used, have limitations. Measurement of oxidative stress is almost always done on isolated samples and not in living tissues so that might limit accuracy. [97] If neuroinflammation in the brain is the main source of the oxidative stress in patients modified proteins will not pass through the blood brain barrier. Invasive sampling of CSF or imaging of the brain may be the only way forward to investigating the importance of oxidative stress in the brain.

Mechanisms by which oxidative stress causes symptoms

In 2018 Mackay and Tate proposed that fluctuating neuroinflammation in the brain and CNS could explain the many neurologically-based symptoms of the illness like fatigue, brain fog, unrefreshing sleep, hypersensitivity to sense stimuli (light, noise). These ideas were hypothesised to extend to patients with Long COVID, [98] and this has now been demonstrated as explained above [82]. To explain the relapses that frequently occur, it was postulated that the paraventricular nucleus encompassing the stress centre was not able to process even minor stresses leading them to be interpreted as though they were major acute events. This would likely link neuroinflammation to the well-established dysregulation of the Hypothalamus Pituitary Adrenal (HPA) axis in ME/CFS. [99] The hypothalamus connects the nervous system to the endocrine system, regulating both the HPA axis and homeostatic control of body temperature, fatigue, sleep, and circadian rhythms- all disturbed in ME/CFS.

As described, microglia, the innate immune cells of the CNS have a central role in mediating this neuroinflammation. Non-invasive imaging by Positron Emission Tomography (PET) coupled with Magnetic Resonance Spectroscopy (MRS) used radioactive ligands for a translocator protein that is expressed in activated glial cells. [81,82 252] ME/CFS and Long COVID patients were compared with healthy controls respectively in the two studies. An important outcome in the ME/CFS study was a correlation between the severity of the symptoms and the extent of activation of the microglia, that is the extent of the neuroinflammation. [81] This was largely in the limbic system but the brain stem has been highlighted as well as a probable key player in the neuroinflammation. [100] Many ME/CFS symptoms can be linked to the brain stem as 'the hub relaying information back and forward between the cerebral cortex and various parts of the body'. [101] An integrated evaluation of the brain stem studies on ME/CFS patients highlighted structural changes in the white and gray matter, abnormalities of functional connectivity between the brain stem and other regions, indicating neuroinflammation among other mechanisms that might partially explain a significant number of the symptoms of ME/CFS. [102] It has also been proposed that persistent brain stem dysfunction occurs in Long COVID that might explain the long-lasting nature of this post-viral illness. [103] Magnetic Resonance Spectroscopy (MRS) has also been used to measure brain metabolites (choline, myoinositol, lactate, and N-acetyl aspartate) linked to inflammation and to determine whether brain temperature is elevated in ME/CFS patients. Increased metabolic ratios over control subjects that correlated with fatigue were found in seven of forty seven brain regions evaluated and an increased temperature was observed in several brain regions, indicating possible neuroinflammation. [104]

Fatigue not alleviated by rest or sleep is one of the core underlying symptoms in Long COVID and ME/CFS. A review on inflammatory and non-

inflammatory conditions such as rheumatoid arthritis, Sjogren's syndrome, cancer and ME/CFS examined how levels of fatigue related to levels of IL-1. IL-1 has been shown to effect dopamine synthesis via oxidative stress. [105] While overall the results were contradictory there was some correlation between increased fatigue and IL-1 in many of the conditions. Altered energy metabolism may also present as "brain fog" another of the key symptoms seen in Long COVID and ME/CFS patients. [106]

There is also evidence of a link between neuroinflammation, the HPA axis and oxidative stress. [107-109] Encephalitis was shown to activate the HPA axis and lead to oxidative stress due to an increase in ROS and a decrease in endogenous antioxidants such as glutathione. Excess serotonin production has been shown to lead to increased levels of oxidative stress in humans due to ROS being a biproduct of serotonin breakdown. [110,111]

Treatments mitigating oxidative stress

Paul *et al*[49]. have speculated ' the symptoms of both Long COVID-19 and ME/CFS may stem from redox imbalance -which in turn is linked to inflammation and energy metabolic defects'. Currently there is no official treatment or cure for the symptoms of Long COVID or ME/CFS [63,112]. Based on studies done to date oxidative stress appears to be contributing to the pathophysiology of Long COVID and ME/CFS, and therefore recommending possible exploratory treatments targeting this redox imbalance may be appropriate. Figure 4 illustrates an investigation and treatment framework that may be helpful to clinicians.

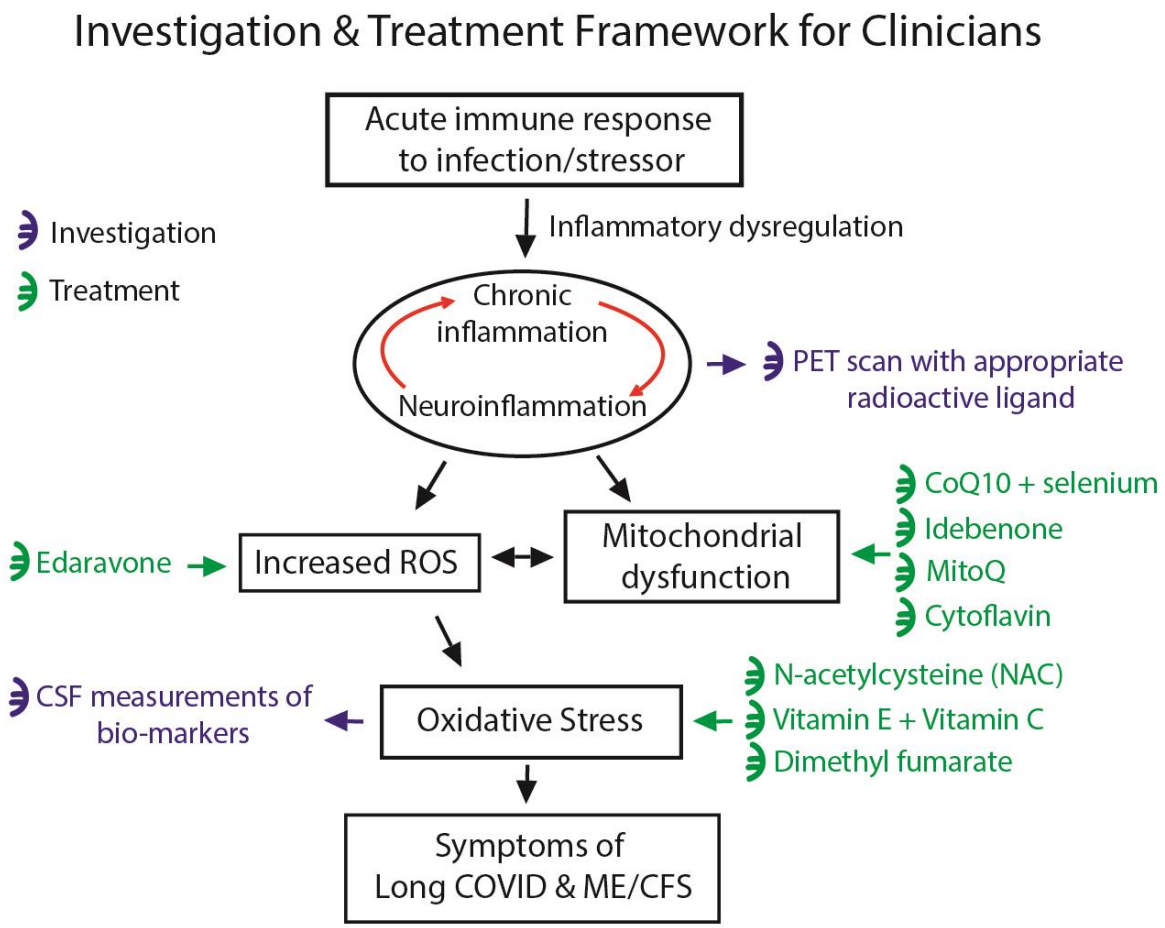

Figure 4. An investigation and treatment guide for clinicians. Promising investigative tools and drugs or natural antioxidants currently available are illustrated as potential pathways to help alleviate symptoms of patients with Long COVID and ME/CFS.

The body already has many endogenous defence mechanisms against oxidative stress. Vitamins A, C and E, natural antioxidants in food, and glutathione coupled with enzymes such as catalase (CAT),

glutathione peroxidase (GPX) and superoxide dismutase (SOD) are all important for managing ROS in the body and for mitigating the potential harms of oxidative stress. [87] Treatments to restore redox imbalance in the body may then involve stimulating these endogenous defence mechanisms or mimicking them to restore balance where it is needed.

As mitochondrial dysfunction is responsible for producing increased ROS and contributes to the symptoms of ME/CFS this organelle would be a key target for treatment. Studies on ME/CFS patients have shown a deficiency of Coenzyme Q10 (CoQ_{10}) responsible for carrying electrons between complexes I and II to facilitate ATP production within mitochondria. [112,113] CoQ_{10} deficiency leads to a reduction in energy production and also increased production of ROS. [114,115] A recent and promising study has targeted this deficiency by giving an oral combination of 400mg CoQ_{10} with 200µg selenium daily for 8 weeks. [116,117] Selenium was given in combination with CoQ_{10} as this coenzyme is reduced by a selenium dependent enzyme. There was a significant improvement in reported fatigue and quality of life, as well as in lipid profiles, pain scores and a reduction in inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, TNF-α). Idebenone (a CoQ_{10} analogue) has improved absorption and bioavailability. [118] While it was developed for use in dementia patients it has also shown therapeutic improvements in some cerebrovascular diseases such as stroke. Currently it is only indicated for Leber's Hereditary Optic Neuropathy in Europe. [119] Idebenone improved chronic fatigue in multiple sclerosis patients potentially meaning it could be used in Long COVID and ME/CFS patients. [120] Adverse effects reported are diarrhoea, back pain, nasopharyngitis and cough. MitoQ, an analogue of CoQ10 specifically targeted to mitochondria, also has promise as an antioxidant therapy for these conditions. [40] An early study designed by patients (via an online platform Mendus.org) showed MitoQ after 6 weeks reduced pain and improved memory in fibromyalgia patients, but not symptoms for ME/CFS compared to placebo. [121] A third group of ME/CFS patients on a lower dose of MitoQ (but without placebo) reported surprisingly significant increases in energy, sleep quality, mental clarity and activity at 6 weeks and greater at 3 months. Cytoflavin (a complement of inosine, nicotinamide, riboflavin, and succinic acid), a drug targeted at reversing mitochondrial dysfunction, reduced weakness, fatigue and breathlessness as well as improved patients ability to resume normal life in patients undergoing rehabilitation following SARS-CoV-2 infection compared to a comparison group. Due to cytoflavin's ability to potentially reduce mitochondrial dysfunction this drug should be trialled on Long COVID and ME/CFS patients to see if it can be beneficial. [122]

Other antioxidant drugs approved for use in neurological diseases consist of dimethyl fumarate (DMF) and edaravone. DMF is a nuclear factor erythroid 2-related factor 2 (NRF2) activator with immunomodulatory and antioxidant properties commonly used to reduce fatigue in relapsing multiple sclerosis. [123] It has been suggested it could have some therapeutic potential in Long COVID and ME/CFS patients. [124] Patients on this drug should have full blood count (FBC) every 6 months to monitor for lymphocytopenia, and should be warned of adverse gastrointestinal side effects such as nausea, vomiting, diarrhoea and abdominal pain. [125] Edaravone is a free radical scavenger originally developed for ischaemic stroke therapy but now indicated for amyotrophic lateral sclerosis (ALS). [126] Dosing of edaravone is recommended as 60mg IV infusion daily for 10 of 14 days followed by a 14 day break before a repeating cycle. [127] The main adverse events consisted of gait disturbance, contusions and allergic reactions. These three drugs, although never clinically trialled in Long COVID or ME/CFS patients, are powerful antioxidants that have shown therapeutic benefits in patients suffering from diseases with a redox imbalance pathophysiology. It should be noted therapies that focus on scavenging ROS are less effective than those that focus on inhibition of ROS generation meaning edaravone may not be as effective. [43,128] There are currently many other antioxidant drugs in clinical trials including sulforaphane (an NRF2 activator), ebselen (a glutathione peroxidase analogue) and GC4419 (a superoxide dismutase analogue). [128]

Natural and synthetic supplements also have been claimed to have benefits in improving redox imbalance. [129] Glutathione esters and N-acetylcysteine (NAC) have been used to increase levels of glutathione, a natural antioxidant. [130-132] Other NRF2 activators have been implicated as antioxidants including curcumin (turmeric extract), resveratrol and broccoli extract with ongoing trials. [133-135] Vitamin E and C have both been shown to reduce oxidative stress and can work synergistically. [136,137] 50µg of vitamin E a day has shown to reduce cognitive decline in patients with Alzheimer's disease compared to placebo. [90] Metal supplements selenium and vitamin B2 (riboflavin) are important in antioxidant enzymes and therefore

supplementation may be beneficial. [128] Caution should be taken regarding use of NRF2 activators, vitamin E and NAC in excess as some studies have shown an increased risk of malignancy. [138,139]

Conclusion:

Long Covid and ME/CFS are debilitating ongoing post-viral syndromes without effective current therapies. Chronic immune/inflammatory activation and mitochondrial dysfunction in the body's peripheral system is well documented in both syndromes. Evidence of neuroinflammation in both Long COVID and ME/CFS has been clearly established through PET scans and MRS imaging, and mitochondrial dysfunction in the brain has been deduced from metabolite profiles in ME/CFS. Inflammation in the periphery, and neuroinflammation in the CNS associated with activated microglia, as well as mitochondrial dysfunction in both are associated with production of excessive ROS. This disturbs the balance of pro-oxidants and anti-oxidants required for normal physiology and creates an imbalance in the redox status. This review indicates there is a need for larger studies to replicate the findings of increased oxidative stress and neuroinflammation to confirm they are widespread among Long COVID and ME/CFS patients. A range of antioxidants have promise as therapeutics to redress this redox imbalance and for ameliorating the distressing symptoms patients experience with these post-viral conditions. CoQ_{10} and its analogues (MitoQ and idebenone) as well as approved neurological drugs, dimethyl fumarate and edaravone, can control excessive ROS. Precursors to the body's most abundant antioxidant glutathione, like N acetyl cysteine can redress a deficit in the natural antioxidant that has been deduced to be deficient from some brain imaging studies. These antioxidants should be used in clinical trials with Long COVID and ME/CFS patients to determine if there is therapeutic effect. Even if clinical trials are not available patients should be encouraged to trial supplementation and antioxidants as long as they understand the risks involved and are monitored by their healthcare professional.

Conflicts of Interest: The authors have no conflicts of interest to declare

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