Oxidative and Nitrosative Stress and Immune-Inflammatory Pathways in Patients with Myalgic Encephalomyelitis (ME)/Chronic Fatigue Syndrome (CFS)

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Abstract: Myalgic Encephalomyelitis (ME) / Chronic Fatigue Syndrome (CFS) has been classified as a disease of the central nervous system by the WHO since 1969. Many patients carrying this diagnosis do demonstrate an almost bewildering array of biological abnormalities particularly the presence of oxidative and nitrosative stress (O&NS) and a chronically activated innate immune system. The proposal made herein is that once generated chronically activated O&NS and immune-inflammatory pathways conspire to generate a multitude of self-sustaining and self-amplifying pathological processes which are associated with the onset of ME/CFS. Sources of continuous activation of O&NS and immune-inflammatory pathways in ME/CFS are chronic, intermittent and opportunistic infections, bacterial translocation, autoimmune responses, mitochondrial dysfunctions, activation of the Toll-Like Receptor Radical Cycle, and decreased antioxidant levels. Consequences of chronically activated O&NS and immune-inflammatory pathways in ME/CFS are brain disorders, including neuroinflammation and brain hypometabolism / hypoperfusion, toxic effects of nitric oxide and peroxynitrite, lipid peroxidation and oxidative damage to DNA, secondary autoimmune responses directed against disrupted lipid membrane components and proteins, mitochondrial dysfunctions with a disruption of energy metabolism (e.g. compromised ATP production) and dysfunctional intracellular signaling pathways. The interplay between all of these factors leads to self-amplifying feed forward loops causing a chronic state of activated O&NS, immune-inflammatory and autoimmune pathways which may sustain the disease.

Keywords: Autoimmune, chronic fatigue syndrome, cytokines, inflammation, myalgic encephalomyelitis, nitrosative stress, oxidative.

1. INTRODUCTION

Myalgic Encephalomyelitis / chronic fatigue syndrome (ME/CFS) has been classified as a neurological disease by the WHO since 1969. In clinical practice ME/CFS people are classified as having ME/CFS merely because they present with pathological levels of fatigue accompanied by variable other symptoms whose origin cannot be determined by recourse to rudimentary biomedical measurements or whose origin is clearly psychiatric. However, there are patients who present with pathological levels of fatigue and muscle fatigueability accompanied by a wide range of neuro-cognitive and neuroimmune symptoms and biomedical abnormalities which become exacerbated following even minor increases in cognitive or physical activity [1,2]. The fatigue and intolerance to increased activity are associated with immune abnormalities consistent with activation of immuno-inflammatory and oxidative and nitrosative stress (O&NS) pathways [1].

This paper examines the development of a chronic illness initiated and maintained by self sustaining feed forward mechanisms based on initially prolonged elevations of systemic O&NS and immune-inflammatory pathways.

2. THE IMMUNE AND O&NS PATHOPHYSIOLOGY OF ME/CFS

2.1. Oxidative and Nitrosative Stress (O&NS)

Many studies involving peripheral blood measurements have demonstrated significant abnormalities related to increased O&NS in many patients with ME/CFS. These abnormalities include elevated concentrations of malondialdehyde (MDA), isoprostane, 8-OH-deoxyguanosine, 2,3 diphosphoglyceric acid, thiobutyrac acid, and protein carbonyls [3-18]. Inducible Nitric Oxide (NO) synthase (iNOS) levels are also significantly higher in patients with ME/CFS compared to healthy controls [19]. Unsurprisingly there is also evidence of excessive production of NO [3,20]. Levels of oxidative stress following exercise are prolonged and excessive compared to raised oxidative stress following exercise in healthy people [21-23]. Exercise induces striking changes in the excitability of muscle membranes in people with this illness [21]. Oxidative stress in skeletal muscles contributes to elevated fatigability in muscles [24]. Several authors have reported that O&NS measures demonstrate a
significant and positive correlation with symptom severity [3-6,8,13,15,25]. Miwa and Fujita [18] reported that the entry of patients into a state of remission correlated with a significant amelioration of oxidative stress.

Secondary IgM and IgG mediated autoimmune reactions occur in many patients with ME/CFS [3], testifying to the severity of oxidative stress which can occur in people suffering from this illness. These autoimmune responses are directed against neoepitopes, which are the result of severe damage by O&NS pathways to endogenous epitopes [26], including disrupted lipid membrane components (e.g. palmitic, myristic and oleic acid), residue molecules of lipid peroxidation (e.g. azelaid acid and malondialdehyde), anchorage molecules, such as S-farnesyl-L-cysteine, and nitrosylated amino-acids, such as nitro-tyrosine, nitrophenylalanine, nitro-tryptphan, nitro arginine and nitrocysteine [3,20,26]. These molecules have been damaged undergoing conformational change because of high levels of O&NS and have thus become immunogenic. It is worthy of note that the concentration of these damaged molecules displays a significant and positive correlation with the severity of the symptoms experienced by patients [3].

### 2.2. Activation of Immuno-Inflammatory Pathways in ME/CFS

Several groups have reported elevated pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF)α and interleukin (IL)-1β, in people diagnosed with ME/CFS [20,27,28]. Recent reports have challenged the previously dominant viewpoint that patients with ME/CFS display a chronically activated T helper (Th)2 biased immune system [1]. Pro-inflammatory and anti-inflammatory cytokines have been shown to co-occur in the same patients [29]. Nevertheless, in some ME/CFS patients pro-inflammatory cytokines are predominant [20,27,28,30,31]. Temporal changes in the cytokine population and concentrations have also been reported. Maes et al. [19] detected significantly increased levels of cyclooxygenase-2 (COX-2) and nuclear factor (NF)-κB in patients compared to healthy controls. Interestingly, they reported significant and positive correlations between the levels of COX-2 and NF-κB and the severity of selective symptoms.

### 2.3. Mitochondrial Dysfunction in ME/CFS

The weight of evidence implicates mitochondrial dysfunction, impaired oxidative phosphorylation and abnormally high lactate levels in the pathophysiology of ME/CFS [13,32-36]. Vermeulen et al. [32] reported that their patients displayed a decrease in ATP production and an increase in lactate concentration compared to healthy controls and that these relative abnormalities became even greater on repeat exercise tests. This echoed an earlier finding revealed by the use of NMR spectroscopy. Thus, Arnold et al. [37] and Kennedy et al. [13] reported elevated markers of oxidative stress in patients with ME/CFS and that the magnitude of the increase correlated positively and significantly with the degree of symptom worsening which followed increased energy expenditure. Behan [35] reported the presence of structural abnormalities in the mitochondria located in the skeletal muscle of ME/CFS patients. Lane et al. [38] also reported a significant increase in intracellular lactate and a lowered rate of ATP resynthesis stemming from impaired oxidative phosphorylation in the aftermath of exercise compared to controls. A number of other authors display a grossly abnormal increase in lactate levels following even trivial exercise and an excessively slow recovery from this state of affairs [32,36,39,40]. Muscle fatigue may actually originate as a result of an accumulation of ROS in muscle cells and a subsequent depletion of available ATP [41].

Mitochondrial DNA damage resulting from elevated O&NS may also result in the genesis of progeny cells which are mutated and dysfunctional [42-44]. This may go some way to explaining the lack of clinically significant or practically important benefit of exercise therapy in these patients [45] and indeed highlight its potential to do serious harm [5,46,47]. Patients with ME/CFS become exhausted much earlier than healthy controls engaged in the same level of exercise. This relatively earlier onset of exhaustion correlates with grossly reduced intracellular levels of ATP combined with an increased utilization of glycolysis compared to healthy people indicating a defect of oxidative phosphorylation [48]. Significantly raised levels of cerebro-ventricular lactate have been detected in patients diagnosed with ME/CFS indicating mitochondrial dysfunction and oxidative stress related abnormalities in the brain of people suffering from this illness [25,49,50].

### 2.4. Brain Dysfunctions in ME/CFS

Evidence supports the existence of neuroinflammatory processes in individuals with ME/CFS. The presence of activated microglia using [11c] PK 1195 positron emission tomography (PET) and the presence of dysfunctional astrocytes using magnetic resonance imaging (MRI) were reported by Nakatomi et al. [51] and Barnden et al. [52]. Puri et al. [53] carried out the first systematic MR spectroscopy (MRS) study of metabolite levels in the brains of people with ME/CFS. One of the major advantages of MRS as an investigative tool is that its use can detect the presence of pathology in brain tissue which is undetectable using conventional MRI [54]. Puri et al. [53] reported that the choline / creatine ratio was significantly elevated in patients compared to controls. These findings were essentially replicated using proton MRI by Chaudhuri and others [55] who also reported a significantly increased choline peak in patients compared to controls. These findings echo an earlier finding by Tomoda et al. [56]. The choline peak includes contributions from a range of cell membrane phospholipids containing choline. This molecule is normally found in an insoluble state hence an increased choline peak represents abnormal levels of choline motilility as a result of changes in myelin status, inflammation and general hypercellularity [57]. Elevated choline is held to be a reliable surrogate marker for the presence of gliosis [58] and is found in the brain tissue of people with Parkinson’s [59] and Alzheimer’s [60] disease and Multiple Sclerosis [61]. In the latter disease the presence of elevated choline peaks in normal appearing brain tissue is predictive of future lesion development [62,63]. The presence of significantly elevated ventricular lactate levels in patients with ME/CFS was reported by a team of researchers in three separate studies using 1H nuclear
magnetic resonance (NMR) spectroscopy [25,49,50]. Lactate levels were reported to be increased by over 380% compared to levels in healthy volunteers. When considering the abnormalities underpinning these findings these workers considered elevated oxidative stress, mitochondrial dysfunction as well as cerebral hypoperfusion, which have all been reported in patients with ME/CFS in numerous studies [2]. They ultimately concluded that elevated O&NS was the most likely explanation for their findings [25].

A large survey concluded that the most common abnormalities generating excess lactate in the cerebro-spinal fluid (CSF) were associated with mitochondrial dysfunction involving the electron transport chain [64]. The bidirectional relationship between elevated oxidative stress, mitochondrial dysfunction and neuroinflammation is well documented [65].

Studies examining the proteome in the CSF of patients with ME/CFS have reported signatures of an activated compliment cascade [66,67]. The latter authors also reported proteomic signatures indicative of protein misfolding, apoptosis and activated microglia. Gene studies have revealed evidence of chronic immune activation, mitochondrial dysfunction and abnormal neural function [68,69]. Regland and others [70] reported elevated levels of homocysteine in the CSF of people diagnosed with ME/CFS which correlated significantly with disease severity. Elevated levels of homocysteine are known to be a cause of blood brain barrier disruption [71,72] and neurotoxicity [73,74]. In addition to disruption of the blood brain barrier, homocysteine is likely to also contribute to neurotoxicity by activating N-Methyl-D-aspartate (NDMA) receptors in the brain [75].

3. SOURCES OF ACTIVATION OF SYSTEMIC INFLAMMATORY AND O&NS PATHWAYS IN ME/CFS

3.1. Infections

The vast majority of ME/CFS patients endure recurrent, persistent or subacute bacterial and viral infections [76-78]. The existence of these infections associates positively with the number and severity of symptoms, including the many neurological symptoms [79]. We previously described the processes involved in generating a chronic inflammatory state involving elevated levels of pro-inflammatory cytokines and O&NS. Briefly, acute, prolonged or persistent infections lead to chronic activation of the immune system and subsequent elevated levels of pro-inflammatory cytokines and O&NS. Briefly, acute, prolonged or persistent infections lead to chronic activation of the immune system and subsequent elevated levels of pro-inflammatory cytokines and O&NS. The inflammatory state may persist following pathogen clearance and is sustained and indeed amplified by the conspiratorial action of a number of autoimmune processes involving molecular mimicry, epitope spreading and bystander activation together with the corrosive actions of activated macrophages and dendritic cells. Prolonged and elevated O&NS leads to chronic immune activation via the activation of NF-kB. The increased levels of cytokines generated by NF-kB activation leads in turn to the increased transcription of iNOS and ultimately elevated NO and peroxynitrite leading to a self sustaining inflammatory environment as described elsewhere [1].

3.2. Bacterial Translocation

A number of studies have reported the existence of bacterial translocation from the lumen of the gut into the systemic circulation in people with ME/CFS [80-82]. In ME/CFS the severity of bacterial translocation is significantly associated with increased levels of pro-inflammatory cytokines and neopterin, suggesting that bacterial translocation drives at least in part the activation of immune-inflammatory pathways in ME/CFS [82]. Other studies have reported the presence of bacterial overgrowth in the gut of people diagnosed with ME/CFS [83-85]. Rao et al. [86] reported a disturbed gut interface in patients with ME/CFS either mediated by bacterial translocation or microbes within the gut. Increased bacterial overgrowth, particularly in the small intestine, may cause bacterial translocation [87,88]. Bacterial translocation is likely the cause of chronic immune activation in HIV positive people via the activation of Toll-Like Receptor (TLR)-4 receptors [89-91]. Circulating levels of bacterial lipopolysaccharides (LPS) disrupt the integrity of the blood brain barrier [92] and lead to the activation of microglia [93]. Microglia are responsible for activating and propagating the immune response in the central nervous system (CNS) following pathogen invasion [94,95]. Microglial and astrocytes are able to perceive pathogen signals via p75NTR recognition receptors. Binding of pathogens promotes recruitment and antigen specific activation of infiltrating leucocytes [95]. Activation of microglia and subsequent activation of astrocytes leads to profound neuropathology [96,97].

3.3. Activation of the Toll-like Receptor - Radical Cycle

TLRs are indispensable moderators of the innate and adaptive immune system and are highly expressed on professional antigen presentation cells such as macrophages [98]. TLRs activate immune responses when engaged by microbial pathogen associated molecular patterns (PAMPS), such as bacterial endotoxins, viral nucleic acids, and endogenous danger associated molecular patterns (DAMPS) liberated by stressed, damaged host cells or oxidized phospholipids [99]. Activation of the TLR-4 complex results in the upregulated transcription of NF-κB and the production of pro-inflammatory cytokines [99]. Elevated NF-κB also increases the transcription of iNOS, leading to the activation of NO, and genes encoding for COX-2 [19]. Increased bacterial translocation and raised concentrations of LPS in the periphery invokes a chronic pro-inflammatory state within the CNS via the engagement of TLR-4 receptors on microglia even in the absence of elevated cytokine levels [100-102]. TLR-4 expression is elevated in people with ME/CFS and may have a mediating role in this illness [103,104].

3.4. Secondary Autoimmune Responses

Prolonged elevation of O&NS species damages lipids, proteins, DNA and other structures to such an extent that they lose immunogenic tolerance and autoantibodies are formed against them. Details of immune responses generated against these O&NS modified neoepitopes can be found in Morris and Maes [1]. Phospholipids and polyunsaturated
fatty acids (PUFAs) are very vulnerable to chemical modifications induced by oxidation. These corrupted molecules located on lipoproteins and cellular membranes convert ubiquitous molecular structures into DAMPs, which engage with TLR-4 to instigate or amplify chronic immune activation and even cell death [99]. Proteins which have undergone redox mediated conformational change may also be perceived as DAMPs by the immune system [105,106]. Prolonged lipid peroxidation generates \(\alpha,\beta\)-unsaturated ketones and aldehydes, which form covalent bonds with a wide variety of proteins in the intracellular environment leading to the genesis of a wide variety of modified proteins, which function as DAMPs. These DAMPs are ligands of multiple proteins and their existence in large numbers lead to the chronic activation or upregulation of the adaptive and innate immune system [107,108].

Prolonged oxidative stress and elevated levels of reactive oxygen species produced as a result of chronic immune activation, inflammation or other stress signals can activate inflammasomes [109]. Inflammasomes are ultimately composed of multiple proteins located in the cytosol, which act as stress or pathogen sensors and are highly expressed in macrophages and other phagocytes such as dendritic cells. Once DAMPs or stress signals are detected the protein subunits oligomerise into a functional mature inflammasome unit leading to procaspase-1 cleavage generating active caspase-1, which in turn activates and secretes IL-1\(\beta\) and IL-18 [110,111]. This process produces a powerful inflammatory response via the release of pro-inflammatory cytokines, ATP or other pro-inflammatory mediators [112].

3.5. Lowered Omega-3 Polyunsaturated Fatty Acids (PUFAs)

PUFAs, such as arachidonic acid (AA) in the \(\omega\)6 series, and docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the \(\omega\)3 series have major roles in immune regulation and inflammation [113]. AA acts as a precursor molecule for the cyclooxygenase and lipoxygenase mediated synthesis of the eicosanoid family of inflammatory mediators such as prostaglandins and thromboxanes [113]. AA-generated eicosanoids are generally pro-inflammatory, whereas \(\omega\)3 PUFAs promote anti-inflammatory activities leading to a decrease in the production of inflammatory eicosanoids, pro-inflammatory cytokines and reactive oxygen species [114]. Maes et al. [113] reported increased levels of \(\omega\)6 PUFAs in patients with ME/CFS, while the \(\omega\)6/\(\omega\)3 ratios were significantly increased in patients than healthy controls and correlated negatively and significantly with the severity of the illness while the absolute levels of \(\omega\)6 PUFAs displayed a significant and positive correlation with the severity of symptoms [113]. Interestingly these authors reported a significant positive correlation between the \(\omega\)6/\(\omega\)3 ratio and the level of impairment in the expression of CD69 following the mitogen stimulation of a wide range of T cells. This observation indicates a defect in early T cell activation [113]. Behan and others [115] published the results of a controlled trial of \(\omega\)3 supplementation in 63 patients with ME/CFS over a three month period. These authors reported that \(\omega\)3 PUFA levels in red cell membranes of patients were abnormal at baseline and had been corrected by supplementation in patients who reported significant clinical improvement compared to patients in the placebo group. Puri et al. [116] reported clinical improvement in symptoms in all patients trialed on a \(\omega\)3 supplementation over a 12 week period compared to control. This finding supported the results of a case study by the same group [116], which noted structural changes in the brain of a patient with ME/CFS detected by NMR spectroscopy following a course of EPA supplementation. Thus, lowered levels of \(\omega\)3 PUFAs relative to increased \(\omega\)6 PUFAs levels could increase the inflammatory potential and thus activation of immune-inflammatory pathways in ME/CFS.

3.6. Lowered Antioxidant Levels

Oxidative stress results from an imbalance of O&NS species and antioxidants and can result from impaired cellular anti-oxidant defenses. Thus diminished activity of superoxide dismutase [117], zinc [118] and glutathione homeostasis [119,120] may contribute to increased O&NS. Prolonged O&NS is one of the major causes of mitochondrial dysfunction in neurological diseases [121]. Impaired antioxidant defenses and impaired glutathione homeostasis are found in Parkinson's [122], Alzheimer's [123] and Huntington's disease [124] and Amyotrophic Lateral Sclerosis [125]. We have reviewed elsewhere that ME/CFS is accompanied by lowered levels of key antioxidants, such as coenzyme Q10, zinc and glutathione [2].

4. CONSEQUENCES OF ACTIVATED INFLAMMATORY AND O&NS PATHWAYS

4.1. Neuroinflammation and Brain Disorders

Immunopathology in the periphery can result in central neuropathology via a number of different mechanisms. The simplest involves the disruption of endothelial tight junctions in the blood brain barrier and in the gut by elevated O&NS while more complex mechanisms all involve cytokine signaling between the peripheral immune system and the brain. We will deal with the simplest of these mechanisms first. Elevated levels of O&NS and TNF\(\alpha\) increase the permeability of endothelial tight junctions in the blood brain barrier [126,127] and the gut [128-131]. These O&NS and inflammatory pathways may not only facilitate neuroinflammation by causing disruption of the blood brain barrier and allowing encephalitogenic T cells to enter into the CNS [132], but also do so by increasing the permeability of tight junctions in the gut via activation of NF-kB [133] and allowing translocation of bacteria and LPS into the bloodstream. This increased bacterial translocation occurs in many people with ME/CFS [134,82]. The entry LPS into the systemic circulation or mesenteric lymph nodes provokes the activation of a range of immune-inflammatory responses [4,19,135].

Activation of peripheral innate immune cells as a result of pathogen invasion leads to the production of a range of pro-inflammatory cytokines such as IL-1\(\beta\), IL-6, and TNF\(\alpha\). This pro-inflammatory signal is transmitted across the blood brain barrier leading to the production of identical cytokines by glial cells in the brain. Glial cells (astrocytes and microglia) stimulated by the presence of cytokines also synthesise and release other pro-inflammatory molecules including prostaglandins [136,137] and NO [138]. The blood
brain barrier is responsible for conferring an immuno-privileged status on the brain and a number of mechanisms exist which enable a peripheral cytokine signal to overcome this formidable obstacle [139]. There are essentially three distinct pathways by which a pro-inflammatory cytokine message can transverse the blood brain barrier. Firstly, cytokines in the blood can simply diffuse into the brain through reasonably porous regions of the blood brain barrier which lie in close proximity to the circumventricular organs [140,141] and thus interact directly with astrocytes and microglia in the area of the brain called the glial limitans. A second major pathway involves interplay between cytokines and brain endothelial cells [142]. These specific endothelial cells possess receptors which are capable of transporting blood IL-1β into the brain or alternatively respond to engagement of receptors by manufacturing IL-1β de novo for release on the abluminal side [143]. The third route entails direct neural innervation by pro-inflammatory cytokines via the vagus nerve [144,145] and sympathetic nervous system circuits [146].

The first two routes of signal propagation considered above are dependent on the presence of elevated pro-inflammatory cytokines in the circulation. However, it has been demonstrated that inflammatory responses take place without the presence of detectable levels of cytokines in the blood [147]. This apparent paradox can be resolved by the fact that the afferent sensory fibres of the vagus nerve are able to detect minimally elevated levels of cytokines and relay the information indicating the presence of peripheral immune activation to the brain via ascending fibres [147,148]. Indeed, localized elevated levels of cytokines appear to be sufficient to activate afferent sensory fibres without the need for increased cytokine levels in the circulation. Interestingly, animal studies have revealed that a vagotomy may relieve fever because the brain no longer receives information indicating the presence of peripheral inflammation [148-150]. This explains why peripheral inflammation is often accompanied by microglial activation in the brain. Microglia have destructive capability by releasing a broad range of neurotoxins which includes pro-inflammatory cytokines [151-153], NO [154,155] and reactive oxygen species [156,155]. Upon activation, microglia rapidly release ATP and astrocytes act to amplify this release. Microglia are known to be regulators of neurotransmission in their own right as upstream partners of astrocytes. This is particularly relevant because activation of microglia and changes in patterns of neurotransmission are two early occurrences in the development of many brain diseases [156]. Microglia may also activate in response to the presence of elevated levels of LPS in the systemic circulation even in the absence of elevated pro-inflammatory cytokines [100]. The weight of evidence demonstrates that microglia are activated before astrocytes, and release ATP and other inflammatory messengers such as IL-1β, TNFα or prostaglandin (PGE)2, which consequently trigger activation of astrocytes [157,156]. Activated astrocytes in turn upregulate their synthesis and secretion of TNFα and/or PGE2 [158,159]. These multiple complex bidirectional interactions between microglia, astrocytes and neurons via O&NS, cytokines and other inflammatory and anti-inflammatory molecules underpin neuroinflammation [160]. For a more detailed description of the processes and players involved in the generation of neuroinflammation the reader is referred to [161]. At this juncture it would seem worthy of note that microglia may remain chronically activated long after the initial infection or other activating stimulus is past [162]. Once microglia have been activated by pathogen invasion for example they can remain activated when the initiating pathogen has been cleared by the immune system.

Activation of microglia is not the only trigger of reactive astrogliosis. Other triggering factors involve the presence of cytokines, PGE2 and O&NS. Mediators of innate immunity such as TLRs and LPS as well as certain neurotransmitters such as ATP, noradrenaline and glutamate also act as major activators of astrocytes [163,164]. In response to such indices of pathology, astrocytes abandon their role as maintainers of CNS homeostasis and acquire the status of immune cells and act to exacerbate the inflammatory milieu by releasing inflammatory mediators, such as IL-1β, TNFα, interferon-γ and PGE2 [165-168]. This intimate and conspiratorial relationship between the neuroglia may form the basis of another feedforward mechanism amplifying and sustaining chronic neuroinflammation, neuroprogression or overt neurodegeneration. Indeed once initiated, neuro-inflammation becomes a self-sustaining, self-amplifying process [160,169,170]. Moreover, the change in astrocyte phenotype means that the master control of CNS homeostasis is compromised and thus the function of the entire brain is placed in peril [171]. Neuroinflammatory processes underpin the pathophysiology in neurological illnesses [172] being a source of pathology in people with Parkinson’s disease [173,174], Alzheimer’s Disease [175,176], Amyotrophic Lateral Sclerosis [177,172], Huntington’s disease [160,178] and Multiple Sclerosis [179,180].

4.2. Toxic Effects of Increased NO Production

There are several lines of evidence demonstrating the existence of elevated NO in people with ME/CFS which were discussed in an earlier section. The formation of NO is catalyzed by an ubiquitous family of different NO synthase isoforms. Three members of the family are calcium ion / calmodium dependent and generate NO with a fleeting half life. One of these, i.e. iNOS, is expressed as a result of inflammation or immune activation [181]. This enzyme generates NO which may persist for hours or sometimes days [182]. A number of other stimuli can provoke the genesis of iNOS such as the activation of the tyrosine kinases, mitogen-activated protein kinases (MAPKs) and Janus Kinases. Tyrosine phosphatases on the other hand inhibit the transcription of iNOS. Hypermethylation is probably another mechanism which inhibits the transcription of this enzyme [183].

NO is a multifunctional molecule capable of acting as a vasorelaxant, a neurotransmitter and an effector molecule in the immune response. It is a thermodynamically unstable entity and is prone to react with other molecules, particularly proteins leading to their oxidation, nitrosylation or nitrination [181]. This NO-induced chemical modification can alter the structure and function of proteins and cause widespread disruption on signaling cascades [181]. NO can function directly as an intracellular molecule via the activation of.
guanylate cyclase. NO can also interact with MAPKs and proteins associated with the mitochondrial respiratory chain. It is also involved in post translational modification and cellular degradation of proteins [181].

At physiological concentrations, NO is a major player in neuromodulation. Endogenous NO regulates cholinergic transmission in the basal forebrain [184] and the rate of release of γ-aminobutyric acid (GABA) [185]. NO also plays an important role in managing the local levels of synaptic plasticity [181], and long term potentiation which is considered to be the neural mode of learning [186,187]. The concentration of NO also has a profound effect on glutamate release. Physiological levels inhibit the release of this neurotransmitter but at higher concentrations NO actually promotes the release of glutamate [188]. Indeed at higher concentrations NO ceases to be an essential and beneficial neuromodulator but instead becomes a promoter of neuropathology [181]. For example NO and NDMA receptors are jointly responsible for the regulation of noradrenaline release [189]. However NO together with peroxinitrite may deactivate the release of noradrenaline [190,191] and be an activator of NDMA mediated neuropathology [192].

4.3. Toxic Effects of Increased Peroxynitrite

Increased production of NO and superoxide elevates the generation of peroxynitrite anion, a very toxic product [193-195]. No enzyme is needed in the formation of peroxinitrite from superoxide and NO because no enzyme could possibly catalyze any reaction that occurs so rapidly. NO is produced at relatively high levels in vivo and reacts more readily with superoxide than superoxide dismutase and thus NO can overcome the antioxidant effect of this molecule. Peroxynitrite is significantly more reactive than O2 and NO despite not being a free radical [196-198]. The half-life of peroxynitrite is extremely short (of the order of 10-20 ms), but that is easily sufficient to diffuse across biological membranes over sufficient distance [199]. Peroxynitrite only reacts with a restricted number of functional groups, such as thiols and iron / sulfur centers [198]. The development and refinement of proteomic analyses confirms the highly selective nature of nitration and that this process is confined to a restricted range of tyrosine functional groups on a very small number of proteins [200-203]. Peroxynitrite can modify heme functional groups, e.g. hemoglobin [204], myoglobin [205] or cytochrome c [206], and inactivate enzymes such as iNOS by inducing oxidative modification of the heme group within its active site [207]. This reaction may be one of the prime mechanisms in the negative feedback mechanism designed to limit peroxinitrite levels in an inflammatory environment. Peroxynitrite reacts especially rapidly with iron-sulfur clusters, leading to the inactivation of enzymes normally involved in the regulation of crucial metabolic processes, such as mitochondrial aconitase [208] and phosphogluconate dehydratase [209], eNOS [210] and alcohol dehydrogenase [211]. Peroxynitrite reacts with various amino acids in a polypeptide chain inducing conformational changes affecting gross structure and therefore normal functioning. The most common reaction occurs with cysteine, so that thiol oxidation by peroxynitrite is a major cause of conformational change in protein molecules [212].

It is worthy of note that cysteine modification does not always result in the inactivation of an enzyme but can sometimes result in enzyme activation as evidenced for matrix metalloproteinases, now understood to be the mechanism underpinning peroxynitrite-related effects on heart disease [213-215] and stroke [216]. Peroxynitrite additionally inhibits superoxide dismutase [217-219], glutaredoxin [220] etc. Peroxynitrite directly oxidizes reduced glutathione and other thiols [221-223]. Therefore the sensitivity of cells to peroxynitrite is highly dependent on glutathione levels in the cells. Glutathione depletion exacerbates peroxynitrite induced pathology [224,222]. A number of authors have suggested that the relationship between diminished levels of glutathione and enhanced peroxynitrite toxicity is a major contributing factor underpinning the development of neurological illnesses such as Parkinson’s disease and Amyotrophic Lateral Sclerosis. The depletion of vital cellular antioxidants by peroxynitrite could induce a feedback loop of increased oxidants within cells and increased oxidative damage [222].

4.4. Protein Nitration

The majority of research teams have reported that nitration of tyrosine in proteins is associated with a loss of function. The inactivation of mitochondrial Mn-SOD is achieved by the nitration of a unitary tyrosine residue (Tyr-34) [218]. Nitrotyrosination suppresses tyrosine phosphorylation and hence disrupts many signaling pathways [225]. Moreover, nitrotyrosination has been observed in neurological illnesses connected to elevated levels of oxidative stress, such as Alzheimer’s [226,227] and Parkinson’s disease [228] and Amyotrophic Lateral Sclerosis [229]. Peroxynitrite also inactivates prostacyclin synthase by the nitration of a single tyrosine residue [Tyr-430] [230]. Tyrosine nitration modulates tyrosine kinase-dependent signaling and is responsible for the generation of neoepitopes on proteins, which are immunogenic. Studies have demonstrated cellular and humoral immune responses to a wide range of nitrotyrosine-carrying proteins [231,232] and nitrated proteins have a role in the pathophysiology of several autoimmune diseases, including Systemic Lupus Erythematosus [233,234].

4.5. Lipid Peroxidation and Modification of DNA

Major cytotoxic effects of peroxynitrite are lipid peroxidation in membranes [235], peroxidation of myelin lipids in the CNS [236-238] and damage to DNA by inducing oxidative changes in nucleoside bases [239,240] and guanine [241]. The nitration of guanine yields 8-nitroguanine ultimately generating basic sites susceptible to cleavage [239-241]. Peroxynitrite attacks may extract hydrogen atoms from deoxyribose molecules in the sugar phosphate backbone resulting in sugar ring opening once again leading to the genesis of DNA strand breaks [239].

4.6. O&NS Affect Energy Production

The peroxynitrite-induced oxidation of crucial cysteine groups inactivates several enzymes that play a role in
energetic processes, including creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, NADH dehydrogenase (complex 1), succinate dehydrogenase (complex 2), cytochrome c reductase (complex 3) and ATP synthase (complex 5) from the mitochondrial electron transport respiratory chain [242-249]. Oxidation of cysteine may inactivate tyrosine phosphatases [250,251]. Cytochrome C nitration of cytochrome C severely impairs its redox activity and hence this is another target of peroxynitrite toxicity. Notably, nitration of cytochrome C upregulates its peroxidatic activity, which increases the production of hydrogen peroxide causing more oxidative damage to mitochondria [252,253]. The inhibition of aconitase by disruption of its ferrous-sulphur active site is yet another mechanism by which peroxynitrite impairs energy production [208,254]. Nicotinamide nucleotide transhydrogenase, an enzyme which enables the reduction of NAD+, is another crucial mitochondrial enzyme inactivated by peroxynitrite mediated oxidation and nitrination [255]. The depletion of NADPH which follows such inactivation reduces the ability of mitochondria to regenerate reduced glutathione, which plays a major contribution in the augmentation of oxidative stress already existing within the organelle [256,257]. Peroxynitrite-mediated oxidation of cysteine-bound thiol is a possible cause of permeability transition pore opening observed in isolated mitochondria in an environment containing a moderately high concentration of this entity [257].

Finally from the perspective of the adverse effects of peroxynitrite on cellular energetics we consider the effect of peroxynitrite induced activation of poly [ADP-ribose] polymerase-1 (PARP-1) [258,181]. Chronic activation of PARP-1 diminishes cellular reserves of NAD+, which is an indispensable cofactor of the tricarboxylic acid cycle, glycolytic pathway, and the electron transport chain [258-261]. Therefore depletion of NAD+ results in marked decrease in cellular ATP stores causing cellular dysfunction or even programmed death via the necrotic pathway [262,263]. Reactive oxygen species not only exert oxidative effects, but also engage in signaling by influencing the activity of a number of transcription factors, such as MAPK, that are associated with inflammation [264-266].

4.7. Intracellular Signaling Networks

The foundation of signal transduction within cells is based on rapidly reversible protein phosphorylation, regulated by phosphorylating kinase enzymes described as serine / threonine kinases or tyrosine kinases and dephosphorylating phosphatase enzymes [267]. The weight of evidence now strongly indicates that peroxynitrite modulates phosphotyrosine signaling by modulating the activities of phosphorytosine phosphatases and phosphotyrosine kinases. Thus, peroxynitrite even at miniscule doses irreversibly inhibits phosphotyrosine phosphatases [250,268]. Peroxynitrite impairs tyrosine phosphorylation which may cause deregulation of vital cellular activities [269,270]. In T cells, nitration processes by peroxynitrite may block tyrosine phosphorylation which normally occurs in response to cell activation. This results in a diminished proliferative activity in activated T cells, hence impairing T cell responses [271]. The second mechanism resulting in phosphotyrosine upregulation is dependent on direct activation of phosphotyrosine kinases. Peroxynitrite promotes rather than suppresses tyrosine phosphorylation in several cell types [272-274] and strongly activates MAPKs, which are all transactivated as a result of double phosphorylation at a particular tripeptide enabled via a protein kinase cascade [275]. p38 MAPK and c-Jun NH2-terminal kinase (JNK) are collectively labeled as stress-activated protein kinases (SAPKs) [276]. The MAPKs have many targets, whose downstream activation modulates many cellular functions [277]. JNK activation by elevated levels of peroxynitrite has been repeatedly reported [278,279]. Peroxynitrite can also activate extracellular signal-regulated kinases (ERK) [280,281] with the consequence of increased neutrophil migration and superoxide synthesis in inflammatory conditions [282,274]. The Src family of kinases, which play a key role in mitogenesis and immune cell activation are also known to be activated by peroxynitrite [283-285] either by oxidation of cysteine residues or inhibition of tyrosine 527 binding to sulphhydryl groups [285,284].

Exposure to elevated O&NS leads to the oxidation, nitration and covalent modification of amino acid residues within the p53 molecule inducing conformational change resulting in an altered tertiary structure [286-288]. This change in structure impairs or eliminates the ability of the p53 protein to bind to DNA and thus carry out its normal functions [289,286]. Another result of prolonged excessively high levels of O&NS is the rapid activation of signal transducer and activator of transcription 3 (STAT-3) [290-292] and NF-kappa B [293]. Activated STAT3 also constitutively activates NF-kappa B directly [294]. STAT3 and NF-6β engage in mutual reciprocal regulation via feedback loops [295]. Moreover, a functional antagonism exists between NF-kappa B and p53. Initiation of NF-kappa B transcription of NF-kappa B within a cell leads to the termination of p53 transcription [296]. Loss of p53 allows the change to anaerobic glycolysis as an ATP source [297,298] resulting in diminished oxygen uptake and aerobic respiration in mitochondria. [299] leading to greatly diminished exercise capacity [300]. Loss of p53 not only leads to a reduced rate of mitochondrial respiration but also leads to greatly increased generation of free radicals in mitochondria [301].

Activation of NF-kappa B in response to lowered p53 activity instigates aerobic glycolysis also called the Warburg effect. This leads to a marked increase in the metabolism of glucose in an attempt to compensate for a fall in ATP production [302,303] and fosters glycolytic ATP generation [303,304]. A mechanistic explanation of the relapsing remitting pattern of ME/CFS based on interplay between p53 and NF-kappa B has been proposed [305]. p53 plays an indispensable role in coordinating metabolic activity within the cell to match energy requirements [306-308] and loss of its function would likely impair a person’s ability to raise energy production in response to the increased demands provoked by exercise.

5. CONCLUSIONS AND FUTURE DIRECTIONS

The proposal made herein is that once generated chronically increased levels of O&NS and immune-inflammatory substances in ME/CFS conspire to generate a multitude of self-sustaining and self-amplifying pathological...
processes which lead to the development of a chronic illness. The pathology stemming from chronically elevated levels of NO and peroxinitrite cannot readily be overstated. At high levels NO becomes a neurotoxin and a facilitator of glutamate-generated excitotoxicity and other NDMA receptor related pathology. Its role in modifying the activity of vital cellular enzymes and signaling pathways coupled with its ability to inhibit the performance of mitochondrial electron transport chain and compromise the production of ATP merely serves to magnify its importance as a source of pathology.

Peroxinitrite is a major regulator of cellular processes and elevated levels lead to chronically elevated inflammatory pathways and inactivation of vital regulatory enzymes responsible for the maintenance of other vital cell systems. The toxic effects of peroxinitrite on energy production are multiple, ranging from inhibition of enzymes within the mitochondrial respiratory chain, depletion or inactivation of vital players in the tricarboxylic acid cycle, disruption of the mitochondrial membrane potential and inactivation of p53. Loss of function of the latter molecule compromises the cells ability to respond to increasing energy demands and, via a range of processes, leads to loss of immune homeostasis. Compromised electron transfer chain function leads to increased production of O&NS species which in turn further compromise energy production both directly and indirectly and is a stark example of pathology which becomes self-sustaining and self-amplifying once initiated. It is held that the genesis of these self-sustaining pathological systems stems from prolonged and or intense pathogen invasion. Prolonged and or persistent infections, resulting in the development of autoimmune processes and bacterial translocation lead to chronic activation of the immune system and subsequent elevated levels of pro-inflammatory cytokines and O&NS leading in turn to O&NS damage to self epitopes and likely activation of the inflammasome which further acts to maintain chronic immune activation and increase the magnitude of inflammation.

The flame of chronic immune activation and elevated O&NS can be fanned by TLR engagement in the periphery and the CNS, lowered antioxidant defenses and a deficiency in o3PUFAs levels. The processes underpinning neuroinflammation involving conspirational interactions between astrocytes, microglia, O&NS, pro-inflammatory cytokines and other inflammatory mediators are also self-sustaining once initiated. The chronic activation of inflammatory pathways also makes a major contribution to disease persistence. It is also worthy of note that a pathogen invasion of sufficient intensity can leave microglia, and hence astrocytes, chronically active even once the initial invasion has been cleared. The interplay between all of these factors leads to the formation of self-sustaining, self-amplifying auto inflammatory feed forward loops leading to a state of elevated oxidative stress and chronic immune activation which sustains the disease.

Investigation into pathogens as causative agents need to be mindful of the experiences in Multiple Sclerosis where initial skepticism has now evaporated and the consensus view is that at least two pathogens are responsible for initiating and maintaining the disease in a significant proportion of patients. This consensus has developed following the realization that the blood compartment may reveal no association between viruses and disease causation while examination of other body compartments for the presence of virus reveal strong associations. Hence further research into virus causation in ME/CFS should focus on lymphoid and muscle tissue in the periphery and sampling CSF. The exploration of relationships between polymorphisms and the presence of actively replicating viruses in people with ME/CFS appears to be urgently required. There would also seem to be a great need for the investigation of intracellular signaling networks, such as NF-kappa B, MAPK, JAK-STAT, p53, the role of apoptosis and mitochondrial signaling cascades by means of high throughput methods analyzed by systems biology. Pharmaceuticals and nutraceuticals aimed at reducing O&NS levels are needed in order to treat individuals with ME/CFS. Endotherapia would seem to be a worthy candidate as its actions in ameliorating oxidative stress has provided objectively measureable improvements in patients with MS. Minocycline would be a candidate drug for reducing neuroinflammatory processes because of its nature as an inhibitor of microglia. Dietary supplementation with o3 PUFAs, zinc and other antioxidants has already demonstrated promise and supplementation with IV glutathione or its precursors such as N-acetyl cysteine should be considered.

AUTHORS' CONTRIBUTIONS
GM and MM participated in the design of this review. All authors contributed equally to this paper. All authors read and approved the final version.

COMPETING INTERESTS
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ABBREVIATIONS
ME/CFS = Myalgic Encephalomyelitis / chronic fatigue syndrome
O&NS = oxidative and nitrosative stress
MDA = malondialdehyde
iNOS = inducible Nitric Oxide (NO) synthase
TNF = tumor necrosis factor
IL-1 = interleukin-1
Th = T helper
COX-2 = cyclo-oxygenase-2
NF-kappa B = nuclear factor-kB
PET = positron emission tomography
MRI = magnetic resonance imaging
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