

NEW INSIGHTS IN THE MECHANISMS OF IMPAIRED REDOX SIGNALING AND ITS INTERPLAY WITH INFLAMMATION AND IMMUNITY IN MULTIPLE SCLEROSIS

Running title: Oxidative stress in multiple sclerosis

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Summary

Multiple sclerosis (MS) is an autoimmune neurological disease characterized by chronic inflammation of the central nervous system (CNS), leading to demyelination and axonal damage and resulting in a range of physical, mental or even psychiatric symptoms. Key role of oxidative stress (OS) in the pathogenesis of MS has been suggested, as indicated by the biochemical analysis of cerebrospinal fluid and blood samples, tissue homogenates, and animal models of multiple sclerosis. OS causes demyelination and neurodegeneration directly, by oxidation of lipids, proteins and DNA but also indirectly, by inducing a dysregulation of the immunity and favoring the state of pro-inflammatory response. In this review, we discuss the interrelated mechanisms of the impaired redox signaling, of which the most important are inflammation-induced production of free radicals by activated immune cells and growth factors, release of iron from myelin sheath during demyelination and mitochondrial dysfunction and consequent energy failure and impaired oxidative phosphorylation. Review also provides an overview of the interplay between inflammation, immunity and OS in MS. Finally, this review also points out new potential targets in MS regarding attenuation of OS and inflammatory response in MS.

Keywords: inflammation, iron metabolism, oxidative stress, immunity, autoimmune diseases, antioxidants, mitochondrial dysfunction

Introduction

Multiple sclerosis (MS) is an autoimmune neurological disease characterized by chronic inflammation of the central nervous system (CNS), leading to demyelination and axonal damage and resulting in a range of physical, mental or even psychiatric symptoms (van den Hoogen *et al*, 2017). The incidence of MS is on the rise: a global prevalence in 2013. was estimated to be at 2.3 million, constituting an increase of 0.2 million people from 5 years earlier (Ontaneda *et al*, 2017). About 85% of patients are diagnosed with relapsing–remitting (RR) MS, which is featured by alternating periods of neurological symptoms (relapses) and recovery (remissions). Approximately 70% develop secondary progressive MS 10–20 years after an initial RR course. Around 20% of patients are diagnosed with a primary progressive MS, which represents a progressive disease from onset (van den Hoogen *et al*, 2017, Ontaneda *et al*, 2017). Despite remarkable progress in the development of MS treatment in recent years, we are still far from finding a definitive drug for MS.

The etiology of MS is not fully understood, but it is believed to arise from a combination of genetic susceptibility, epigenetic and post-genomic events, and environmental factors such as viral pathogens, chemicals, smoking and diet (Grigoriadis *et al*, 2015, Penesová *et al*, 2018). MS is characterized by auto-reactive Th1 and Th17 effector cells and other cells of the immune system that infiltrate the CNS and attack the myelin sheath (Diebold, 2008). There is an emerging evidence that the pathogenesis of several neurodegenerative diseases, including MS, involves the excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) associated with mitochondrial dysfunction and energy deficit (Adamczyk *et al*, 2016, Fetisova *et al*, 2017, Mirshafiey *et al*, 2009, Neves Carvalho *et al*, 2017), indicated by the biochemical analysis of cerebrospinal fluid and blood samples, tissue homogenates, and animal models of MS (Haider, 2015, Neves Carvalho *et al*, 2017, Ohl *et al*, 2016). Oxidative stress (OS) causes demyelination and neurodegeneration directly, by oxidation of lipids,

proteins and DNA but also indirectly, by inducing a dysregulation of the immunity and favoring the state of pro-inflammatory response. In this review, we describe the mechanistic insights of the impaired redox signaling, as well as its complex interplay with immunity and inflammation in MS. We also point out potential targets of this delicate regulatory network which could be utilized in the treatment of MS, as a supplement to the established treatments.

Key players of redox milieu: reactive species and antioxidants

Reactive species. ROS and RNS are highly reactive small molecules which are formed as natural byproducts of the normal aerobic metabolism and processes involved in response to pathogens. To become more stable, these species donate or receive another electron, which leads to the formation of new free radicals, resulting in a chain reaction. At low concentrations, ROS and RNS play an important role as regulatory mediators in signaling processes, such as growth and apoptosis at the cellular level and contribute to complex functions, including cognitive function, and immune function at the system level (Di Meo *et al*, 2016, Brieger *et al*, 2012). This state is defined as oxidative eustress (Sies *et al*, 2017). On the other hand, at high concentrations, in the state of oxidative distress, these molecules may cause OS and become harmful for living organisms by damaging important biomolecules, such as deoxyribonucleic acid (DNA), proteins and lipids (AbdulSalam *et al*, 2016, Di Meo *et al*, 2016, Michalickova *et al*, 2018, Sies *et al*, 2017). This happens when neutralization of ROS and RNS through antioxidant protective mechanisms is overwhelmed by their generation. It is important to emphasize that a ROS level causing damage to one cell type may be within the normal range for another cell type that possesses higher antioxidant capacity (AbdulSalam *et al*, 2016). This implies that the concentrations of reactive species govern the shift from their beneficial to

deleterious effects, but the concentrations at which this shift happens are not generally known (Di Meo *et al*, 2016).

The free radical group includes compounds such as the superoxide anion radical ($O_2^{\bullet-}$), nitric oxide radical (NO^{\bullet}), nitric dioxide radical (NO_2^{\bullet}), hydroxyl radical (OH^{\bullet}), alkoxyl (RO^{\bullet}) and peroxy (RO_2^{\bullet}) radicals. Most typical non-radical reactive species relevant to biological systems are singlet oxygen (1O_2), ozone (O_3), hydrogen peroxide (H_2O_2), peroxyxynitrite (ONO_2^-), hypochlorous acid ($HOCl$), organic peroxides and aldehydes. Reactive species generation is mediated through various pathways including the direct interaction between redox-active metals and oxygen species (e.g. Fenton reaction), peroxisomal oxidation of fatty acids, oxidation by cytochrome P450 enzymes and oxidative burst in several immune cells (Neves Carvalho *et al*, 2017). Two principle intracellular sources of ROS are mitochondria and ROS-producing enzymes, such as lipoxygenases (LOX), cyclooxygenases (COX), myeloperoxidase (MPO), xanthine oxidoreductase (XOR) systems, NO synthase (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) (Adamczyk and Adamczyk-Sowa, 2016, Brieger *et al*, 2012, Neves Carvalho *et al*, 2017). Mitochondria's primary function is to provide cells with energy, in the form of adenosine triphosphate (ATP), through reactions of oxidative phosphorylation (OxPhos). OxPhos is carried out by five multisubunit electron transport complexes (ETCs), designated complexes I–V. Electrons are transported from NADH to NADH coenzyme Q reductase (complex I) to coenzyme Q, which also acquires electrons from succinate dehydrogenase (SDH/complex II). Coenzyme Q then transfers the electrons to cytochrome C oxidase (complex IV) via complex III (cytochrome bc1). Complex IV reduces molecular oxygen to water using these electrons (Paul *et al*, 2019). Beyond traditional roles of mitochondria in metabolism, mitochondria are also involved in ROS signaling, innate immunity and apoptosis (Shadel *et al*, 2015). ROS are formed as a by-product of OxPhos and up to 2% of the total cellular mitochondrial oxygen consumption may be related to the generation of ROS

under normal physiological condition (Kim *et al*, 2015, Neves Carvalho *et al*, 2017). Apart from mitochondria, other organelles, such as peroxisomes and the endoplasmic reticulum (ER) are also important intracellular sources of ROS (Kim *et al*, 2015, Neves Carvalho *et al*, 2017). The roles of mitochondria and ROS-producing enzymes in the pathology of MS are in detail described in the section *Redox signaling impairment in MS*.

Antioxidants. Antioxidant protection consists of enzymatic and non-enzymatic antioxidants, which generally act in two ways: they prevent the formation of free radicals or react with the free radicals before their reaction with the essential biomolecules. In this way, they hinder or reduce the damage to important biological systems. Non-enzymatic antioxidants are low molecular weight compounds and are synthesized in the body (glutathione, bilirubin, uric acid, glutathione, ferritin, ceruloplasmin) or ingested through foods (vitamins A, C, E, carotenoids, α -lipoic acid, flavonoids). Glutathione (GSH) is the main antioxidant in the brain, and plays a key role in the detoxification of reactive species (Carvalho *et al*, 2014). An important phenomenon in MS is an altered glutathione homeostasis, especially reduced glutathione reductase (GR) and glutathione peroxidase (GPx), although some researchers did not observe such abnormalities in their study subjects (Carvalho *et al*, 2014). Antioxidant enzymes catalyze the ROS neutralization reactions in the biological systems or the regeneration reaction, i.e. reduction of oxidized antioxidants. These enzymes include: superoxide dismutase (SOD), an enzyme that neutralizes the superoxide anion and represents the first line of defense against OS; glutathione peroxidase (GPX), which catalyzes the reduction of hydrogen peroxide and organic hydroperoxide in the presence of reduced GSH as an electron donor, while oxidized glutathione (GSSG) is formed at the same time; glutathione reductase (GR), which participates in regeneration of glutathione, i.e. reduction of oxidized glutathione in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADPH); catalase (CAT), an enzyme that has multiple functions, of which the most important is the elimination of hydrogen peroxide from

the biological system by decomposition into water and oxygen (Adamczyk and Adamczyk-Sowa, 2016, Michaličková *et al*, 2019).

The main regulator of the cellular antioxidant protective mechanisms is the transcription factor nuclear factor erythroid 2-related factor2 (Nrf2) is (Liddell, 2017, Neves Carvalho *et al*, 2017, Salim, 2017, Barancik *et al*, 2016). Under normal physiological conditions, Nrf2 is sequestered in the cytosol by its negative regulator, kelch-like ECH-associated protein 1 (Keap1), and constitutively targeted for ubiquitination and proteasomal degradation (Liddell, 2017, Zhang *et al*, 2004), Figure 1. This interaction keeps low basal expression of Nrf2 regulated genes. Oxidative or electrophilic challenges disrupt the complex between Nrf2 and Keap1, allowing Nrf2 to translocate to the nucleus, where it heterodimerises with small musculoaponeurotic fibrosarcoma (Maf) proteins (sMaf) and binds to antioxidant response elements (ARE) in the promoter region of target genes (Katsuoka *et al*, 2016, Liddell, 2017, Neves Carvalho *et al*, 2017, Zhang *et al*, 2004). Nrf2 controls the expression of a various cytoprotective proteins, including the vast majority of antioxidant enzymes, such NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1), peroxiredoxins, thioredoxins and enzymes that participate in the synthesis and metabolism of glutathione, such as γ -glutamyl-cysteine synthetase (GCS), glutathione-S-transferases, GR, GPX (Lee *et al*, 2003, Liddell, 2017, Neves Carvalho *et al*, 2017, Miao *et al*, 2019). In addition, Nrf2 also regulates the expression of antioxidant proteins like ferritin; several genes involved in autophagy, including SQSTM1 encoding p62 protein, enzymes with anti-inflammatory activity such as leukotriene B4 dehydrogenase; and proteins involved in protein quality control and degradation like heat-shock protein 70 (Hsp70) and subunits of the proteasome (Baird *et al*, 2011, Kwak *et al*, 2003, Neves Carvalho *et al*, 2017, Pajares *et al*, 2016). Emerging number of studies indicates a dysregulation of Nrf2 system in brains of individuals suffering from neurodegenerative diseases, such as Alzheimer's disease, parkinsonism and amyotrophic lateral sclerosis (Liddell, 2017, Jiang *et al*,

2018). A decrease in a transcription factor complex containing Nrf2 in human samples of MS grey matter has been reported, and this correlated with reduced expression of OxPhos genes and increased oxidative damage (Pandit *et al*, 2009). Moreover, Nrf2-deficient mice in the model of experimental autoimmune encephalomyelitis (EAE), the most used pre-clinical MS model, expressed a more severe phenotype and augmented microglial activation (Johnson *et al*, 2009, Lassmann *et al*, 2016). Importantly, it appears that Nrf2 expression level is cell-type specific. Higher expression of Nrf2 is found in astrocytes and infiltrating macrophages in active lesions (van Horssen *et al*, 2010), and in oligodendrocytes (OL) at lesion edges (Licht-Mayer *et al*, 2015). Nrf2 expression is however lower in neurons, even when surrounded by Nrf2-positive glia (van Horssen *et al*, 2010) and this may account for a limited capacity of neurons to cope with OS.

Nrf2 induction represents a promising intervention for the restoration of the cellular antioxidative response and attenuation of inflammation in MS. Currently, there is one drug approved by EMA for the treatment of RR MS, which activates Nrf2: it is dimethyl fumarate (DMF; trade name: Tecfidera). Up to date, there have been multiple preclinical studies and also several clinical trials evaluating potential of other Nrf2 activators in the treatment of MS. Noteworthy, these molecules also display anti-inflammatory effects through other mechanisms in addition to Nrf2 activation, such as SIRT1 activation, NF- κ B, c-jun N-terminal kinase (p-JNK), protein kinase RNA-like endoplasmic reticulum kinase (p-ERK), and phospho-p38 members of the mitogen-activated protein kinase (MAPK) pathway attenuation (Adamczyk and Adamczyk-Sowa, 2016, Miller *et al*, 2019, Saso *et al*, 2014). Based on preclinical studies, compounds which seem to have therapeutic potential are: sulforaphane, curcumin, melatonin, resveratrol, several flavonoids, such as quercetin, EGCG, naringenin (Long *et al*, 2018, Mohajeri *et al*, 2015, Li *et al*, 2013, Fonseca-Kelly *et al*, 2012, Muthian *et al*, 2004, Semnani *et al*, 2017). Results from the clinical trials are however less conclusive on this topic. Currently,

there are several clinical trials evaluating curcumin, melatonin, and EGCG. Two clinical trials (NCT03150966 and NCT01514370) examining curcumin efficacy in the patients with MS are still ongoing, and the results have not been published. Moreover, six clinical studies have been registered (NCT00525668, NCT02011451, NCT00799890, NCT00836719, NCT01451723, and NCT01417312) and three have posted the results. Majority of the clinical studies have examined the safety and the neuroprotective potential of EGCG by determination of levels of N-acetyl aspartate (NAA, which is utilized to assess the number and metabolism of neurons). The results of these studies are mixed; two small-scale clinical studies have shown no improvement in NAA level in the brain, with an enhancement of hepatotoxicity after 6-month supplementation with 800 mg EGCG extract (Lovera *et al*, 2015), whereas the other study (12 weeks, 600 mg EGCG extract) has shown some metabolic effects (decreased postprandial carbo-hydrates oxidation, adipose tissue perfusion, and glucose supply, and shift to a higher and more stable carbohydrate during exercise), but only in men and not in women (Mähler *et al*, 2015). Another study examining a single high dose of EGCG (750 mg) in healthy subjects has reported no impairment of COMT (Lorenz *et al*, 2014). Moreover, there have been several human studies evaluating melatonin in MS patients already treated with disease-modifying therapies (DMT, mitoxantrone, interferon beta, glatiramer acetate). Three registered studies are currently in progress or are finished, but did not post the results: NCT03150966 (phase II), NCT03498131 (phase I), NCT01279876 (phase II). No clinical studies have assessed the Keap1/Nrf2/ARE pathway expression in treated patients. Most of the studies did not include doubled blind randomized design, but rather healthy controls as a comparison to the melatonin-treated group of MS patients. Melatonin administration has improved several markers of OS and antioxidant protection (it has increased SOD and decreased MDA in serum) and inflammation RR MS patients, which in some cases coincided with the improvement in the quality of life of these patients. Melatonin was administered in wide range of doses (2 to 25 mg)

during the wide range of treatment duration (30 days to 12 months), so the future trials will have to provide information on the safe and effective dose. Details of therapeutic potential of these compounds are out of the scope of this review, but interested readers are referred to (Dinkova-Kostova *et al*, 2015, Dinkova-Kostova *et al*, 2018, Neves Carvalho *et al*, 2017, Shokeir *et al*, 2015).

Figure 1 should be placed here

Redox signaling impairment in MS

The central nervous system (CNS) is especially vulnerable to OS due to high oxygen utilization, suboptimal antioxidant defense mechanisms compared to peripheral tissues and high abundance of polyunsaturated fatty acids which making the CNS particularly susceptible to lipid peroxidation (Neves Carvalho *et al*, 2017, Paul and Snyder, 2019). Moreover, brain tissue contains significant amounts of metals ions, such as iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) which contribute to the generation of the highly toxic hydroxyl radicals (Sheykhansari *et al*, 2018). Finally, brain areas rich in catecholamines are particularly susceptible to OS, since catecholamines cause free radicals generation by auto-oxidation or through metabolism by monoamine oxidases (Neves Carvalho *et al*, 2017). However, the susceptibility to OS varies between different cells of the CNS, such as neurons or different glial cells. The level of energy demand, the dominant pathway of energy production, expression of antioxidant protective mechanisms, susceptibility for apoptosis induction and potential of handling the overload of divalent metal cations determine vulnerability of cells to OS (Lassmann and van Horssen, 2016, Stys, 2005). Neurons and OL are the cells most vulnerable to OS, while astrocytes and microglia appear to be more resistant (Lassmann and van Horssen, 2016). Neurons have a limited capacity to counteract OS due to low expression of the main

regulator of antioxidative defense Nrf2 (van Horssen *et al*, 2010) and a regulator of crucial mitochondrial biogenesis and antioxidant mechanisms, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) (Witte *et al*, 2013). High lipid content and iron accumulation might be responsible for high vulnerability to OS of myelin sheaths and OL to OS. On the other hand, astrocytes are better equipped with antioxidant protection and have a low tendency to iron accumulation, probably due to extensive expression of iron exporters (Zarruk *et al*, 2015, Lassmann and van Horssen, 2016, Nijland *et al*, 2014). Finally, microglia and macrophages, as the main cellular sources of ROS and RNS, have a considerable amount of antioxidant molecules and seem to be well protected from OS (Van Horssen *et al*, 2008), however, it should be mentioned that augmented iron accumulation happens in microglia in the ageing human brain (Hametner *et al*, 2013).

The main interrelated mechanisms responsible for generation of reactive oxygen and nitrogen species and oxidative injury in patients with MS are:

- A. inflammation-induced production of free radicals by activated immune cells and growth factors,
- B. release of iron from myelin sheath during demyelination and
- C. mitochondrial dysfunction and consequent energy failure and impaired oxidative phosphorylation (Haider, 2015).

Importantly, oxidative injury has been described in active and low grade, acute and chronic inflammatory lesions, in the white and gray matter, as well as in RR and progressive MS, but the mechanisms leading to oxidative injury in these conditions are not the same (Lassmann and van Horssen, 2016, Mahad *et al*, 2015, Witte *et al*, 2014). Initial acute inflammatory MS lesions are predominantly associated with inflammation and oxidative burst activation of microglia, which lead to severe mitochondrial dysfunction, whereas the progressive stages are mainly

characterized by amplification mechanisms, such as chronic mitochondrial dysfunction or iron accumulation in the brain and its liberation in demyelinating lesions (Lassmann and van Horssen, 2016). These mechanisms are described in the following section and presented in Figure 2.

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Inflammation

Key features of MS, chronic OS and dysregulation of inflammatory response, are closely related. Active inflammation and disruption of the blood-brain barrier (BBB) have been detected in the brains of patients with MS as gadolinium enhancing magnetic resonance imaging lesions (Filippi *et al*, 2019, Haider, 2015). Highly pro-inflammatory and autoreactive leukocytes (especially T-helper 1 (Th1) and T-helper 17 (Th17) cells) infiltrate into CNS, where they produce IFN- γ and IL-17A. The inflammatory reaction that follows further increases the permeability of the BBB and recruits other immune cells, such as B cells and monocytes, to the CNS (Adamczyk-Sowa *et al*, 2016, van den Hoogen *et al*, 2017). These processes are paralleled by continuous activation of resident macrophages/microglia, which produce pro-inflammatory cytokines and chemokines, as well as reactive oxidants, leading to OL death, axon damage, and ultimately neuronal loss. These immune cells in active MS lesions express enzymes involved in the oxidative burst, such as NADPH oxidase (NOX), iNOS, XOR and myeloperoxidase (MPO) (Fischer *et al*, 2012, Gray *et al*, 2008b). The initial source of oxidative injury in MS is an oxidative burst activation of macrophages and microglia. Another important source of reactive species during inflammation are the several receptors of growth factors, such as epidermal growth factor receptor (EGFR) (Meng *et al*, 2007) and platelet derived growth factor receptor (PDGFR) (Yang *et al*, 2016).

The main source of ROS in microglia is the family of NOX enzymes (for an excellent review on the role of NOX enzymes in the MS, see (Ma *et al*, 2017)). Pro-inflammatory cytokines, primarily tumor necrosis factor alpha (TNF- α) and IL1- β , represent the major inducers of both phagocytic and non-phagocytic NOX (Valacchi *et al*, 2018). NOX family of enzymes is responsible for superoxide production through the electron transfer from NADPH to oxygen (Kim *et al*, 2015). To date, seven NOX isoforms have been identified in mammalian cells, including NOX1 to NOX5 and dual oxidases (Duox1 and Duox2) (Ma *et al*, 2017). Each NOX isoform has a distinctive function, cellular localization, type of generated ROS and regulation (Ma *et al*, 2017, Neves Carvalho *et al*, 2017). NOX1, NOX2 and NOX4 are expressed in brain. NOX2 and NOX1 were reported to be upregulated in activated microglia in active demyelinating and chronic MS lesions in human MS patient brain tissue (Fischer *et al*, 2012). Additionally, NOX2 was found to be upregulated in activated microglia in pre-active MS lesions found throughout normal appearing white matter of MS patients. Since NOX is the only enzyme family that has an exclusive function to form ROS, its regulation might be particularly important in the context of MS pathology. Therefore, NOX, especially NOX2, represents an attractive therapeutic target in MS. NOX2 deletion in the model of EAE ameliorated OL loss, and reduced microglia reactivity (Li *et al*, 2011). Furthermore, NOX2 inhibitors, such as apocynin, celastrol, dextromethorphan and dietary phytol, have been reported to ameliorate clinical scores in EAE (Abdin *et al*, 2014, Blum *et al*, 2018, Choi *et al*, 2015, Yang *et al*, 2017, Chechneva *et al*, 2011). No clinical trials evaluating these compounds in clinical trials have been reported.

Another enzyme in phagocytes accountable for oxidative burst is MPO, a heme peroxidase responsible for the production of hypochlorous acid (HOCl) from hydrogen peroxide (H₂O₂) and chloride anion (Cl⁻) (Neves Carvalho *et al*, 2017). This enzyme was found to be predominantly expressed by macrophages and activated microglia within and in the vicinity of

MS plaques in white matter lesions, but also in a subtype of microglia surrounding cortical lesions (Gray *et al*, 2008a, Van der Veen *et al*, 2009). In the model of EAE, increased MPO activity in the CNS has been found (Forghani *et al*, 2012, Sajad *et al*, 2009). Interestingly, administration of non-toxic MPO inhibitor N-acetyl lysyltyrosylcysteine amide for 5 days starting at the peak of disease significantly attenuated EAE disease severity, reduced myeloid cell numbers and permeability of the BBB (Zhang *et al*, 2016).

Xanthine dehydrogenase (XDH) and xanthine oxidase (XO) are interconvertible forms of XOR, a system involved in the conversion of hypoxanthine to xanthine and the conversion of xanthine to uric acid (UA) (Meneshian *et al*, 2002). Apart from its role in the purine metabolism, XOR also plays a pathophysiological roles through the generation of various types of ROS, including superoxide, hydrogen peroxide and NO (Meneshian and Bulkley, 2002). Febuxostat, an inhibitor of XOR, was found to attenuate axonal loss and ameliorated both RR and secondary progressive EAE by preventing neurodegeneration in mice (Honorat *et al*, 2013, Honorat *et al*, 2017).

An additional hallmark of microglial over-activation is the high production of NO, however it should be stressed that NO production is much more relevant in rodents than in humans (Rojo *et al*, 2014, Genc *et al*, 2006). NOS is responsible for synthesis of NO in mammals though catalysis of a reaction of L-arginine and dioxygen that proceeds at the expense of NADPH oxidation. There are three major isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are constitutively expressed and require calcium and calmodulin for activation, whereas iNOS is largely calcium independent and induced in inflammatory conditions (Li *et al*, 2011). NO has a dual, both physiologic and pathophysiologic role in the CNS. NO acts as a messenger molecule between neurons, astrocytes and blood vessels (Bicker, 2001). NO has a protective role in the CNS, as it modulates signaling pathways which are crucial for survival and differentiation of neurons, such

as cAMP-responsive element-binding protein (CREB) and extracellular signal-regulated kinase (ERK) (Ockelford *et al*, 2016). Pathologic role in the CNS is displayed mainly through its reactive product peroxynitrite (ONOO⁻), which is formed in the presence of superoxide ion and hypoxic conditions (Haider, 2015, Lan *et al*, 2017). OL and oligodendroglial precursor cells (OPCs) are highly susceptible to NO/ONOO⁻. NO/ONOO⁻ cause OL mitochondrial dysfunction via inhibition of the respiratory chain, oxidation and disruption of mitochondrial DNA (mtDNA), and the impairment of the mitochondrial membrane (Jack *et al*, 2007, Mander *et al*, 2005). This leads to damage to the myelin structure, but also to impaired remyelination of axons (Pang *et al*, 2010). Recently, oxidoreductase glutaredoxin 2 (Grx2) was found to inhibit ONOO⁻ formation by conversion of NO to dinitrosyl-diglutathionyl-iron-complexes and this was associated with prevention of myelin damage in the EAE model (Lepka *et al*, 2017). Interestingly, glutaredoxin 2-mediated protection was not found to be related to its enzymatic activity as oxidoreductase, but to the disassembly of its coordinated iron-sulfur cluster using GSH as non-protein ligand (Lepka *et al*, 2017). This indicates that boosting Grx2 or other GSH coordinated iron-sulfur clusters leads to prevention of toxic ONOO⁻ generation and consequently leads to prevention of neuroinflammation. Another GSH-coordinated iron-sulfur cluster, $[[\text{Fe}_2\text{S}_2]^{2+}(\text{GS}^-)_4]^{2-}$ complex, that might be feasible in the condition of MS has been recently characterized (Qi *et al*, 2012).

Moreover, inhibition of iNOS could be a potential target in the treatment of MS (for an excellent review, see (Lan *et al*, 2017)). Up to date, several NOS inhibitors have been developed and examined in preclinical and clinical studies for conditions other than MS (Tejero *et al*, 2018). Selective iNOS inhibitors GW273629 and GW274150 have shown to be ineffective in clinical trials for migraine (NCT00242866; NCT00319137) (Høivik *et al*, 2010, Van der Schueren *et al*, 2009), but they have not been tested in EAE and MS. The pterin 4-amino-tetrahydrobiopterin (VAS203) is a non-selective NOS inhibitor that has shown efficacy in the treatment of traumatic

brain injury and has been used in phase II clinical trial (NCT02012582) (Stover *et al*, 2014). Additionally, edaravone, an inhibitor of iNOS, was shown to ameliorate the clinical severity of EAE and to reduce lymphocytes infiltration of lymphocytes to CNS (Moriya *et al*, 2008). This drug is currently approved for the treatment of amyotrophic lateral sclerosis (ALS) in the U.S. and Japan and additionally for the treatment of acute-phase cerebral infarction after two successful clinical trials (NCT00330681, NCT01492686).

Mitochondrial dysfunction

In addition to their primary function to provide cells with the energy, through the reactions of oxidative phosphorylation (OxPhos), mitochondria have a central role in the redox homeostasis, and the immune response regulation (Min *et al*, 2018). Mitochondrial dysfunction plays a central role in redox imbalance in MS (for comprehensive review see (Witte *et al*, 2014, Campbell *et al*, 2014)). Mitochondria uniquely possess residual mtDNA, which is responsible for regulation of 1200 proteins, including those that control the activity of OxPhos (Min *et al*, 2018). mtDNA, and generally mitochondrial biogenesis and homeostasis are under tight control of the nuclear DNA. Mitochondrial genome is smaller than the nuclear one, has only one DNA polymerase, and also lacks protective histones, which makes it highly susceptible to OS. ROS released during inflammatory response from the activated microglia and macrophages damage the components of the respiratory chain, through either functional inhibition or increased breakdown of the concerned proteins (Lassmann and van Horssen, 2016). Also NO competes with oxygen for binding sites of respiratory chain (Mander *et al*, 2005). In return, inefficient OxPhos further increases generation of reactive species, leading to a vicious circle of mitochondrial dysfunction and OS (Haider, 2015). Namely, in normal physiological conditions, only 1-2% of the electrons escape the OxPhos to the mitochondrial matrix (Halliwell *et al*,

1984), where they produce superoxide through the reaction with molecular oxygen (Cadenas *et al*, 1977). However, when ATP production is jeopardized, mitochondrial superoxide production is enhanced (Lassmann and van Horssen, 2016). Reactive species released during inflammatory response also damage mtDNA, causing mtDNA mutations and deletions (Campbell *et al*, 2011), which ultimately endanger OxPhos function, energy metabolism and ATP production (Lassmann and van Horssen, 2016, Mahad *et al*, 2008).

Over time and with cell aging, abnormal mitochondria are amplified in neuronal cell bodies through clonal expansion of mtDNA deletions and depleted transcripts (Campbell *et al*, 2014, Witte *et al*, 2014). Currently, it is not known why mitochondria with mutated mtDNA undergo clonal expansion (Haider, 2015). Increased number of defected mitochondria eventually leads to progressive mitochondrial dysfunction in neurons, which is especially featured in the progressive MS (Frischer *et al*, 2009, Witte *et al*, 2014). At certain point, mitochondrial dysfunction accumulates to such an extent that ATP production becomes insufficient for Na⁺/K⁺ ATPase to eliminate excess Na⁺ from the axons (Campbell *et al*, 2014, Lassmann and van Horssen, 2016, Witte *et al*, 2014). The increasing intra-axonal Na⁺ level leads to the reversal of the axolemmal Na⁺/Ca²⁺ exchanger, which in normal physiological conditions pumps Na⁺ in and Ca²⁺ out of the axon. Consequently, this leads to elevation of axonal Ca²⁺ concentration, which triggers a cascade of several deleterious events, eventually causing disruption of intra-axonal mitochondria and increased ROS production, and finally axonal degeneration (Campbell *et al*, 2014, Lassmann and van Horssen, 2016, Witte *et al*, 2014). Apart from energy deficiency, mitochondrial dysfunction can also give rise to neurodegeneration by other mechanisms (Lassmann and van Horssen, 2016). For example, release of cytochrome C and apoptosis inducing factor from mitochondrial stores induces apoptosis (Ow *et al*, 2008). This finding is important considering that apoptotic OL death can be seen as a primary mechanism of demyelination in MS lesions (Lassmann and van Horssen, 2016).

Furthermore, decreased expression of PGC-1 α , an important regulator of mitochondrial proteins and biogenesis, and the main regulator of energy metabolism, has been found in patients with progressive MS, even in normal appearing white matter (NAWM) (Nijland *et al*, 2014). NAWM is the tissue surrounding white matter inflammatory lesions and without any marked changes in myelin proteins expression. Decreased PGC-1 α expression seems to be an important contributor of defected energy metabolism (Nijland *et al*, 2014, Witte *et al*, 2014). However, it is still unknown what is the cause of this phenomenon, but it was hypothesized that continuous microglial inflammatory response might be crucial (Witte *et al*, 2014, Witte *et al*, 2013). Up to date, resveratrol (previously mentioned as a Nrf2 inducer) and several compounds belonging to group of thiazolidines have been reported to enhance PGC-1 α expression which has been associated with decrease in neuronal loss and improved axonal density in the SC (Shindler *et al*, 2010, Hondares *et al*, 2006). These results have to be corroborated in clinical trials.

Recently, multiparametric imaging technique for analysis of mitochondrial redox signals, assessing mitochondrial shape, redox state, pH, calcium levels and membrane potential has been developed (Breckwoldt *et al*, 2014). This technique has revealed that there are two kinds of axonal mitochondrial “contractions” (fluctuations in redox signals manifested through changes in mitochondrial shape, pH of mitochondrial matrix, calcium levels and membrane potential): transient and permanent. Transient fluctuations represent a mitochondrial response to physiological stress and are reversible, whereas permanent, which can become irreversible, are triggered by more severe axonal injuries, such as axotomy (Breckwoldt *et al*, 2016, Breckwoldt *et al*, 2014). Axotomy-induced permanent contractions were accompanied by transition pore opening and could not be prevented by ROS scavenging. However, transient contraction could be prevented by a class of mitochondria-targeted antioxidants (MTA). This groups includes mitoquinone (MitoQ), mitotocopherol (MitoVitE), skQ1, which are lipophilic cations that can pass through all biological membranes and accumulate within mitochondria more easily than

their non-targeted parent antioxidants. Many preclinical studies showed promising antioxidant and anti-inflammatory effects in EAE model and currently, MitoQ is in Phase 1 clinical trial for fatigue in MS (NCT03166800). Details on the therapeutic potential of MTA are out of the scope of this review, but interested reviewers are referred to a comprehensive review (Fetisova *et al*, 2017)).

Iron deposition

Dysregulation of iron metabolism in the brain is another mechanism contributing to OS within the pathogenesis of MS. For detailed review readers are referred to (Stephenson *et al*, 2014) and (Dusek *et al*, 2016). Excessive iron concentration was revealed in the white matter lesions and deep gray matter structures (Haider, 2015). Highest iron concentration was found in basal ganglia and was associated with increased disability and cognitive dysfunction (Schmalbrock *et al*, 2016, Hallgren *et al*, 1958). Conversely, reduced iron levels were revealed in the normal-appearing white matter (NAWM) and remyelinated plaques (Hametner *et al*, 2013), suggesting that the iron dysregulation associated with MS is not purely global iron accumulation, but rather a redistribution of iron levels between different areas of the brain (Stephenson *et al*, 2014). In the biological systems, iron can be found in the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states (Stephenson *et al*, 2014). In the human brain, iron is predominantly stored in the nontoxic trivalent (Fe^{3+}) form by ferritin in the myelin sheets, OLs and microglia (Haider, 2015, Hulet *et al*, 1999). It accumulates physiologically with age, plateauing at 40–50 years, depending on the anatomical area (Haider, 2015, Hallgren and Sourander, 1958). During myelin breakdown and subsequent phagocytosis of myelin debris which occur at active MS lesions, iron is liberated into the extracellular space. Subsequently, it undergoes conversion to the divalent ferrous form, which can increase the toxicity of ROS (Mahad *et al*, 2015). In Fenton reaction, Fe^{2+} is oxidized

to Fe^{3+} , while hydrogen peroxide (H_2O_2) is converted to highly toxic hydroxyl radical ($\cdot\text{OH}$) and hydroxyl anion (OH^-). Liberated iron is taken up by macrophages and microglia, which release and deposit iron during phagocytosis of damaged neurons (Neher *et al*, 2011). High iron load may even induce cell death, after which iron is further released initiating a second wave of OS. Additionally, activated macrophages release NO that can promote other iron release from ferritin (Neher *et al*, 2011). Finally, iron might also activate immune cells and affect their polarization (Stephenson *et al*, 2014). Diverse stimuli can shape macrophages and microglia respond to various stimuli by becoming polarized towards different phenotypes (Stephenson *et al*, 2014). The M1 phenotype of activated macrophages is characterized by proinflammatory immune response and promotion of type 1 T-helper cells differentiation. On the contrary, the M2 phenotype is related to increased debris clearance and is considered regulatory and anti-inflammatory (Durafour *et al*, 2012, Stephenson *et al*, 2014). Iron overload in macrophages promotes a pro-inflammatory M1 activation state (Durafour *et al*, 2012), leading to generation of reactive species.

Although preclinical studies showed that chelators ameliorate clinical severity in EAE, clinical studies had rather inconclusive and even disappointing results (Dusek *et al*, 2016). More research in the complex topic of iron metabolism in MS is needed before iron chelation can be recommended as beneficial therapeutic strategy.

Interplay between OS and inflammation: the state of “oxinflammation”

Recently, the term “oxinflammation” has been proposed to depict the vicious circle of chronic inflammation and OS, which is the hallmark of many chronic diseases, including MS (Valacchi *et al*, 2018). As previously described, pro-inflammatory mediators enhance generation of reactive species, but it is important to emphasize that reactive species in return also favor the

progression of inflammation. While in a redox balance state an increase in the reactive species level stimulates the endogenous antioxidant defense, during chronic impaired redox homeostasis, intracellular signaling pathways are altered and result in a loss of the regulation of signals transduction by the cells (Solleiro-Villavicencio *et al*, 2018). This occurs because of the activation of the phosphorylation pathways and inhibition of dephosphorylation enzymes (Solleiro-Villavicencio and Rivas-Arancibia, 2018). OS causes a dysregulation of immune response and favors the state of pro-inflammatory response. Moreover, reactive species have also been associated with the activity of matrix metalloproteinases (MMPs), proteins that have an important role in T cells trafficking into the CNS (Leppert *et al*, 1995). Reactive species can activate cells of immune system to induce protein kinase cascade (PKC, MAPKs etc.) and redox-sensitive transcription factors, most importantly AP-1 and nuclear factor kappa-light chain-enhancer of activated B cells (NF- κ B) (Valacchi *et al*, 2018). NF- κ B is a transcription factor, primarily involved in the modulation of inflammatory/immune responses, cell apoptosis and cellular growth. Upon activation, NF- κ B upregulates the expression of many genes involved in MS and EAE pathogenesis, including TNF- α , iNOS, IL-1 α/β , vascular adhesion molecules 1 and various growth factors (Winyard *et al*, 1996).

NOX2 enzymes are an excellent example of the interconnection between redox processes and inflammation in MS. As previously mentioned, NOX enzymes are the most important source of reactive species during acute inflammation response and the only enzyme family with an exclusive function to form ROS. Activation of NOX2 by pro-inflammatory mediators, such as TNF- α and IL-1 β , is followed by formation of ROS, which in turn elicit cascade of inflammatory events, and cause a formation of a number of pro-inflammatory mediators including those involved in the activation of NOX2 (Valacchi *et al*, 2018). The presence of a specific protein, termed negative regulator of ROS (NRROS), has been described (Noubade *et al*, 2014). This biomolecule directly interacts with the NADPH oxidase complex and facilitates

the degradation of NOX1 and NOX2 proteins, and consequently limits the ROS production from phagocytes during inflammation (Noubade *et al*, 2014). NRROS deficient phagocytes produce increased ROS upon inflammatory challenges: for example, mice lacking NRROS in the phagocytes exhibited elevated bactericidal activity against *Escherichia coli* and *Listeria monocytogenes*. On the other hand, these mice developed severe EAE due to excessive oxidative tissue damage in the CNS. Interestingly, NRROS expression in phagocytes was found to be repressed by inflammatory signals. Therefore, further understanding of this pathway may represent a novel therapeutic target for diminishing ROS production in various diseases, including MS.

Inflammasomes are another good example of mutual connection between OS and inflammation in MS. Inflammasomes are multiprotein complexes which detect pathogenic microorganisms and sterile stressors, and increase maturation of the pro-inflammatory cytokines IL-1 β and IL-18 or lead to cell death (Singhal *et al*, 2014). Inflammasomes can be found in the cytosol of several types of cells, including immune cells (such as T cells, B cells, dendritic cells, and macrophages), neural cells, microglia, and astrocytes, as well as pulmonary endothelial cells (Lang *et al*, 2018). The role of NLRP3 (nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3) inflammasome has been best described in the pathogenesis of MS. Two signals are required for NLRP3 inflammasome activation: a "priming" step that involves NF- κ B signaling, which is followed by activation of pro-caspase-1, a critical factor for maturation of cytokines (Singhal *et al*, 2014). Inflammasomes can also be activated experimentally by mitochondrial reactive species by the inhibitors of the respiratory chain, rotenone and antimycin (Bulua *et al*, 2011). Scavenging mitochondrial superoxide suppresses NLRP3 activation, whereas NLRP3 agonists elevate generation of reactive species (Zhou *et al*, 2011, Abais *et al*, 2015). However, the mechanisms of NLRP3 activation by ROS have not been elucidated (Fetisova *et al*, 2017).

It has been proven that the activation and differentiation of T lymphocytes, crucial drivers of MS development course, depends on redox conditions of the microenvironment (Solleiro-Villavicencio and Rivas-Arancibia, 2018, Ohl *et al*, 2016). Actually, ROS represent the third activation signal for T lymphocytes. Both amount and type of reactive species exert different effects upon T cells, which also show the ability to convert from one to another cell lineage under certain inflammatory conditions (Solleiro-Villavicencio and Rivas-Arancibia, 2018). Generally, chronic OS promotes differentiation toward pro-inflammatory phenotypes such as Th1 and Th17. For example, NOX-deficient mice showed a skewed Th17 response, while NOX-intact mice exhibited cytokine profile characteristic for Th1 response (Hubert *et al*, 2010). In another study, mitochondrial inhibitors of ROS N-acetylcysteine and mitoquinone reduced differentiation to Th17 phenotype in a mouse model of IEX-1 gene deficiency (this deficiency increases apoptosis) (Zhi *et al*, 2012). On the other side, it seems that OS exerts negative effects on Tregs, cells that represent an important peripheral mechanism of immune regulation. Although Treg cells are generally less sensitive to OS than CD4+ cells, due to better antioxidative equipment, high amount of ROS can induce apoptosis of Treg (Maj *et al*, 2017). Deleterious effects of OS on Treg function have mainly been ascribed to the inhibition of Foxp3 expression. Chronic OS stimulates an overproduction of IL-6, which inhibits Foxp3 expression during Treg differentiation (Yang *et al*, 2016). Additionally, OS can also promote the enhanced production of NO, mitochondrial hyperpolarization, and Ca²⁺ influx to the cell, resulting in overexpression of the mechanistic target of rapamycin complex 1 (mTORC1) (Solleiro-Villavicencio and Rivas-Arancibia, 2018). This protein complex inhibits the proliferation of Tregs, but also promotes the expansion of Th1 and Th17 pro-inflammatory lymphocytes (Perl, 2016).

Clinical implications: antioxidant therapy in MS

Considering all the above, there is an ample evidence that OS plays an important role in the pathogenesis of MS. This has given hope that attenuation of OS by antioxidant supplementation can be employed in therapy of MS. However, contrary to promising basic research and preclinical results, the results of clinical trials are still controversial (Adamczyk and Adamczyk-Sowa, 2016, Miller *et al*, 2019, Saso and Firuzi, 2014). Currently, a number of clinical trials are on way and hopefully more information will be available when these are completed.

Several explanations for failure of clinical studies examining broadly active antioxidants in MS have been proposed (Lepka *et al*, 2016, Neves Carvalho *et al*, 2017, Saso and Firuzi, 2014). First of all, it is important to note the difficulty of translation of preclinical results to clinical trials. Although EAE has been useful in elucidation of key pathological processes underlying neuroinflammation, it does not fully reproduce the extent of oxidative injury, neuronal loss and cortical demyelination seen in MS (Witte *et al*, 2014). Furthermore, many clinical trials have not used validated or appropriate biomarkers of OS, making interpretation of results inconclusive (Neves Carvalho *et al*, 2017, Saso and Firuzi, 2014). When making a design of a clinical trials this has to be considered, in addition to the appropriate dose, and length of supplementation. Bioavailability is another important factor which should be considered. Failure of antioxidants to reach CNS, that is, to cross BBB will lead to failure to attenuate OS in CNS. Moreover, many clinical trials have evaluated dietary antioxidants. It should be borne in mind that the majority of antioxidant capacity in the CNS is provided by endogenous enzymes (enzymes controlled by Nrf2: SOD, CAT, GPx etc.) and endogenous compounds (primarily GSH) and not by dietary antioxidants (Neves Carvalho *et al*, 2017). In addition, many dietary antioxidants do not cross the BBB. In case of mitochondria – targeted antioxidants, the inability to accumulate in mitochondria due to negative charge under physiological conditions, will result in lack of efficacy (Fetisova *et al*, 2017). Finally, it is plausible that the first attempts in use of antioxidant therapy have failed because specific ROS and RNS which are crucial for

the pathology of MS have been neglected (Lepka *et al*, 2016). In other words, attenuation of specific reactive species of importance to MS pathology, whether by inhibition of pathways responsible for their production or boosting of pathways liable for their scavenging, seems to be more efficient than unspecific application of broadly active antioxidants. In this context, this review highlights specific targets: Nrf2 induction, iNOS, MPO and NOX2 inhibition, scavenging of ONOO-, improving mitochondrial function, enhancing PGC-1 α activity in mitochondria.

Conclusion

In conclusion, there is an ample evidence that OS plays an important role in the pathogenesis of MS. As there is a strong mutual connection between inflammation, immunity and OS in the pathology of MS, fine modulation of this delicate regulatory network could be an appealing strategy in the treatment of MS.

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Conflict of interest

There is no conflict of interest.

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Figure legends

Figure 1. Cellular pathways driven by Nrf2 target genes. Oxidative or electrophilic challenges disrupt the complex between Nrf2 and Keap1 (kelch-like ECH-associated protein 1), allowing Nrf2 to translocate to the nucleus, where it heterodimerises with small musculoaponeurotic fibrosarcoma (sMaf) proteins and binds to antioxidant response elements (ARE) in the promoter region of target genes. This results in a cascade of reactions with ultimate anti-inflammatory and antioxidant effects.

Figure 2. Mechanisms of oxidative injury in multiple sclerosis. The time sequence and interrelation of redox events in MS are different in relapsing and progressive MS, acute and chronic MS, but also different in different stages of the disease and different patients. Therefore, this figure does not illustrate a timeline from events shown in (1) to those in (5). (1) The initial source of oxidative injury in MS is an oxidative burst activation of macrophages and microglia. The enzymes responsible for the oxidative burst are NADPH oxidase (NOX), iNOS, XOR and myeloperoxidase (MPO). (2) Reactive species released during inflammatory response from the activated microglia and macrophages damage the components of the respiratory chain, endangering energy metabolism and adenosine triphosphate (ATP) production. In the conditions of inefficient oxidative phosphorylation (OxPhos), respiratory chain itself also becomes an important source of OS. In addition, reactive species also damage mitochondrial DNA (mtDNA), causing mtDNA mutations and deletions. During time, defective mitochondria are amplified by the clonal expansion in neurons. (3) A combination of these events eventually results in energy failure and decreased ATP production. At certain point, mitochondrial dysfunction accumulates to such an extent that ATP production becomes insufficient for Na^+/K^+ ATPase to eliminate excess Na^+ from the axons. The increasing intra-axonal Na^+ level leads to the reversal of the axolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which in normal physiological conditions pumps Na^+ in and Ca^{2+} out of the axon. (4) This leads to elevation of axonal Ca^{2+} concentration,

which triggers a cascade of several deleterious events, eventually causing disruption of intra-axonal mitochondria and increased ROS production, and finally axonal degeneration. (5) Iron is physiologically stored within the myelin sheets; however, demyelination triggers its release into the extracellular space. Subsequently, it undergoes conversion to the divalent ferrous form, which can increase the toxicity of ROS. In Fenton reaction, Fe^{2+} is oxidized to Fe^{3+} , while hydrogen peroxide is converted to highly toxic hydroxyl radical and hydroxyl anion. Liberated iron is taken up by macrophages and microglia, which release and deposit iron during phagocytosis of damaged neurons. High iron load may even induce cell death, after which iron is further released initiating a second wave of OS and causing an amplification of oxidative injury.



