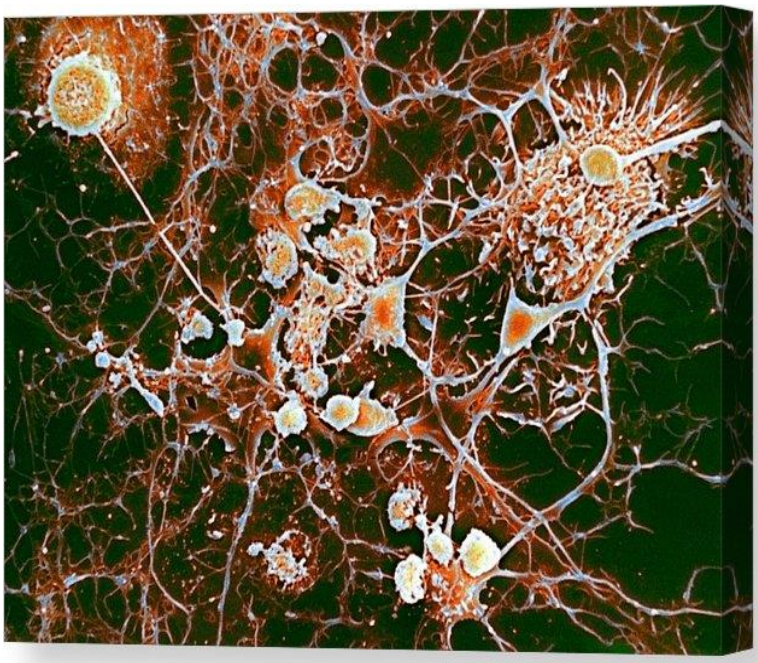

TROPHIC AND TOXIC EFFECTS OF IRON BALANCE IN MULTIPLE SCLEROSIS: FROM REMYELINATION TO NEURODEGENERATION

ABSTRACT

While iron is essential for physiological cellular brain functions such as normal mitochondrial function and remyelination, increased cerebral iron depositions have been linked to neurodegenerative disorders and particularly Multiple Sclerosis. Data from MRI and histological studies have found increased deposits of iron in the brain of patients with MS and specifically in macrophages and microglia, two types of central nervous system cells that are directly implicated in MS pathoetiology. This essay focuses on and discusses the role of iron balance in MS pathophysiology as well as on the physiologically significant role of iron within cells for oxidative metabolism and remyelination, thus offering insight into the trophic and toxic effects of brain iron levels.

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1. Introduction

Multiple Sclerosis (MS) is a chronic neurodegenerative inflammatory disease of the central nervous system, which primarily affects individuals aged between 20 and 40 years old. [4,5,6] Caucasian people and mainly white women of northern European ancestry, residing in cold/humid climate areas, are more susceptible to developing MS. [7]

From a clinical standpoint, MS is associated with the progressive loss of focal myelin surrounding the neuronal axons and is also characterized by damage of the oligodendrocytes, a large type of glial central nervous system cells, which are of vital importance in the production of myelin sheath insulating the afore mentioned neuronal axons. [1.2,3]

As far as MS epidemiology is concerned, more than 2.5 million people have MS globally [9] and around 80% of those patients diagnosed with MS, display symptoms related to relapsing-remitting multiple sclerosis (RRMS), which are defined as isolated episodes of decreased neurological functions (relapses) followed by periods of recovery and stability in between the relapses. (remission).

After the passage of 15 to 25 years, RRMS may transition into a new form of multiple sclerosis condition called secondary progressive multiple sclerosis (SPMS). SPMS is associated with major progressive neurological defects which are not showing any sign of remission. Lastly, 10-15% of the individuals enter this phase of MS within disease onset, which is defined as primary progressive multiple sclerosis (PPMS). [8,9]

The pathological mechanisms behind MS onset and progression remain unclear however a substantial number of factors are implicated in neurodegeneration leading to the onset and progression of MS. These factors include innate and adaptive auto-immune responses, molecules regulating inflammation, enzyme dysfunction such as proteases, glutamate excitotoxicity, mitochondrial abnormalities triggered by redox metals (such as iron and copper) leading to an excess production of oxidative stress, environmental exposure and genetic susceptibility.

1.1 Genetic and environmental factors affecting MS

Histopathological, immunological and epidemiological studies of patients with MS indicate that the immune system is an important factor mediating disease onset and progression.

Epidemiological genetic studies, focusing on patients with MS as well as family members of MS patients, have identified specific genetic risk factors associated with an increased susceptibility to developing MS. Various single nucleotide polymorphisms in genes such as the human leucocyte antigen (HLA) class II alleles DRB1*1501, DRB1*1303 DRB1*0301, that are expressed on cells of the innate

immune system, are related to an increased risk of developing MS. Interestingly, HLA class I allele A2 has been found to demonstrate a protective effect against MS.[10]

These set of genes are necessary for the recognition of antigens by CD4+ and CD8+ T lymphocytes.CD4+ T-lymphocytes recognize antigens complexed with MHC class II and assist in the control of immune reactions whereas CD8+ T- cells recognize antigens complexed with MHC class I and are responsible for cytotoxic activity.

Genome-wide association studies (GWAS) have also highlighted more than 100 single nucleotide polymorphisms in genes associated with multiple sclerosis, with the vast majority of these genes being responsible for adaptive immune system responses. [10,11,12,13]

These results show that MS is not a disease caused by a single gene defect but rather by the combined outcome of defects on several genes, which add to the increased risk of developing the condition.

In addition to that, a substantial percentage of gene defects (~22%) related to MS are also associated with other auto-immune diseases. However, this is not the case about the HLA allele DRB1*1501 which has been shown to have no association with other auto-immune conditions but only MS. It is safe to assume therefore, that this particular allele is linked to MS-specific auto-immune response, while the rest of the alleles associated with MS and other auto-immune diseases are regulating the immune response in a more general and non-specific way. [9,10]

Nonetheless a large number of gene defects linked to MS are related to genes which are essential for the differentiation of pathogenic T-cells as well as for modulating their functions. [10]

Epidemiological studies have also identified important environmental factors, that contribute greatly to an increased risk of developing MS. For instance, viral infection by the Epstein-Barr virus, smoking and low serum-concentrations of Vitamin D can lead to increased activation of the peripheral immune system and subsequent elevated risk for developing MS. [14,15,16,17]

The above mentioned scientific evidence support the hypothesis that the risk for onset of MS is primarily because of gene defects affecting the adaptive immune system, while environmental elements, affecting the peripheral immune's system response, have an add-on effect on the risk for the development and progression of the disease.

1.2 The role of immune system in MS

1.2.1 The adaptive immune system

Experimental evidence, mainly from MRIs and histopathological studies of the brain of MS patients, have identified widespread lesions in the grey and white matter of the central nervous system. White matter lesions in MS patients demonstrate an acute demyelinating effect, where the myelin breakdown is followed by increased infiltration of phagocytes, T-cells, B-cells, plasma cells and dendritic cells presenting antigens complexed with MHC class II molecules. [18] Astrocytes located in the periphery of the lesion show distinctive cellular damage, [19] while in acute lesions,

there is oligodendrocyte loss without infiltration of T and B lymphocytes. [20] When demyelination occurs, the level of inflammatory infiltration by T and B-lymphocytes is decreasing while astrogliosis remains in a substantial extent. Recovery and repair mechanisms such as remyelination continues to take place but often in a limited capacity leaving the process incomplete. [21,22]

Lesions of demyelinating nature have also been found in the grey matter of central nervous system in MS patients, a manifestation linked specifically to MS and no other neurodegenerative diseases. [23,24] Grey matter lesions are more often found throughout the early onset of MS but the demyelination effect present in grey matter lesions does not correlate with the extended effect of demyelination observed in white matter lesions. Moreover, grey matter lesions manifest less damage to the blood-brain barrier, accompanied by decreased level of T and B lymphocytes infiltration with regard to white matter lesions. [25]

Investigative animal models have elucidated the inflammatory processes of the adaptive immune system involved in the pathogenesis of MS. Demyelination in experimental auto-immune encephalomyelitis rodent models have shown that the rate of demyelination is contingent on the activation of CD4+ T-cells that respond to specific myelin proteins such as the myelin oligodendrocyte glycoprotein (MOG) and the myelin basic protein.[26,27] The induction of experimental auto-immune encephalomyelitis in animal models is achieved through the transfer of activated CNS antigen specific CD4+ T- lymphocytes to immunocompetent rodents.[28]

Opposed to CD4+ T-cells, CD8+ T-cells are seldom found in lesions of experimental autoimmune encephalomyelitis. Nevertheless, CD8+ T-cell specific MHC class I epitopes were found in myelin antigens that were used to induce experimental autoimmune encephalomyelitis, implying that CD8+ T cells also contribute to lesion progression in the brain. [29,30]

1.2.2 The innate immune system

The role of the innate immune system in the pathogenesis of MS has been highlighted in results from in-vivo microscopy studies performed on animal models of experimental autoimmune encephalomyelitis. Microglia and macrophages are present in MS lesions, with the phagocytes driving the damage and removal of the myelin sheaths and the extent of this damage can be monitored by the amount of myelin degradation products in phagocytes. [31] Moreover, phagocytes are often located very close to damaged neuronal axons and axonal damage is induced where phagocytes are accumulating. [32] Activated microglia and macrophages in MS lesions are also likely to initiate the production of reactive oxygen species, a mechanism that potentially affects the development of lesions in grey and white matter. [33,34,35]

1.2.3 Immunological mechanisms and hypotheses

The afore-mentioned scientific evidence clearly suggest that MS is neurodegenerative disorder where the immune system is actively involved in the onset and development of MS lesion in the brain. This observation has also led to the shaping of two distinct

postulations, regarding the role of the adaptive and innate immune system in the development of MS lesions in the grey and white matter of patients with MS.

The first theory is supported by evidence based on animal models of experimental auto-immune encephalomyelitis and suggests that the pathogenesis of MS commences with a central nervous system antigen-specific activation of the immune system in the periphery and then this immunization process is being transferred to the central nervous system, with ensuing detrimental effects.[36,37] The activation of autoreactive T-cells in the peripheral network, for example in intestine and lungs, is followed by the relocation of these cells to distinct lymph nodes. A small number of these antigen-specific activated T-cells infiltrate the central nervous system in what is determined as prephagocytic phase of lesion development. [18]

CD4+ cells invade and surround the perivascular space of the brain, a fluid-filled space around certain blood vessels, releasing cytokines disturbing astrocytes' end feet and interrupting the physiological functions of astrocytes and oligodendrocytes. Differentiated types of B-cells, known as plasma cells, emerge in the periphery and finally invade the brain where they accumulate and discharge antibodies targeting myelin sheaths and astroglia. Further release of inflammatory intermediates increases blood-brain barrier permeability, allowing the incursion of phagocytic monocytes into the brain promoting the development of the phagocytic lesion. This hypothesis suggests that the adaptive immune system acts as an initializer for the pathogenesis of the phagocytic lesion in the brain of MS patients, whereas the innate immune system has a contributing cause to the formation of the lesion. [18,36,37]

In the context of this hypothesis, MS could be considered as an organ-specific auto-immune disease with immune-mediated neurodegenerative damage. However, in order for a condition to be classified as auto-immune, an autoantigen must be detected in MS patients with an established immune response against it. Identification of a specific autoantigen in MS patients is still missing.

Additionally, the detected autoantibodies against the afore-mentioned autoantigen should be identified within a lesion or serum of patients in association with the level of disease activity or noticeable clinical improvement after relevant treatment. [44] Extensive research efforts have been conducted to investigate the identification and quantification of antibodies against myelin oligodendrocyte glycoprotein and non-myelin antigens in patients with MS, but these studies have yielded inconsistent results. [45,46,47,48,49]

This absence of a confirmed auto-immune response that is capable of producing antibodies targeting a specific autoantigen in the central nervous system of MS patients contend the above-mentioned hypothesis supporting that MS is primarily an autoimmune disease.

The second theory associated with the role of the immune response in the formation of MS lesions states that a major event within the central nervous system (for instance, a genetic mutation or a viral infection) initiates oligodendrocyte death and the activation of resident microglia, the brain-resident immune cells, a process that can subsequently lead to microglia-mediated neuroinflammation and augmentation of

the already ongoing immune response. Antigens arising from this primary immune response leak out of the central nervous system into cervical lymph nodes, prompting a secondary response of the peripheral adaptive immune system. The antigens then invade neighboring tissues where they are picked up by dendritic cells and transfer them to lymph nodes, initiating the T-cell migration to the target tissue and the subsequent release of cytokines. [38,39,40]

The possibility of antigens being drained out of the central nervous system is low with evidence showing that the central spine fluid could serve as a sink for antigens considered residual waste. [41] However, it still remains unclear whether and most importantly, in what way, these antigens are able to elicit an adaptive auto-immune response in the periphery. Furthermore, it is not known how a genetic mutation or a viral infection occurrence in the central nervous system can trigger oligodendrocyte loss. Initial damage to oligodendrocytes and subsequent oligodendrocyte death is not supported by the results from studies in MS patients. In addition to that, the inability of present scientific studies to identify a viral agent in the brain of MS patients, which could justify oligodendrocyte damage, means this hypothesis is highly unlikely. [42,43]

1.3 Iron accumulation in neurodegenerative diseases

Iron accumulation emerges as a key factor in the pathogenesis of various neurodegenerative diseases. On the one hand, iron is necessary for physiological brain processes where it acts as a cofactor for normal cellular processes, including enzymatic reactions and proper mitochondrial function. Iron accumulation is normal in the brain of individuals and iron is gradually increasing as a natural outcome of aging. [50]

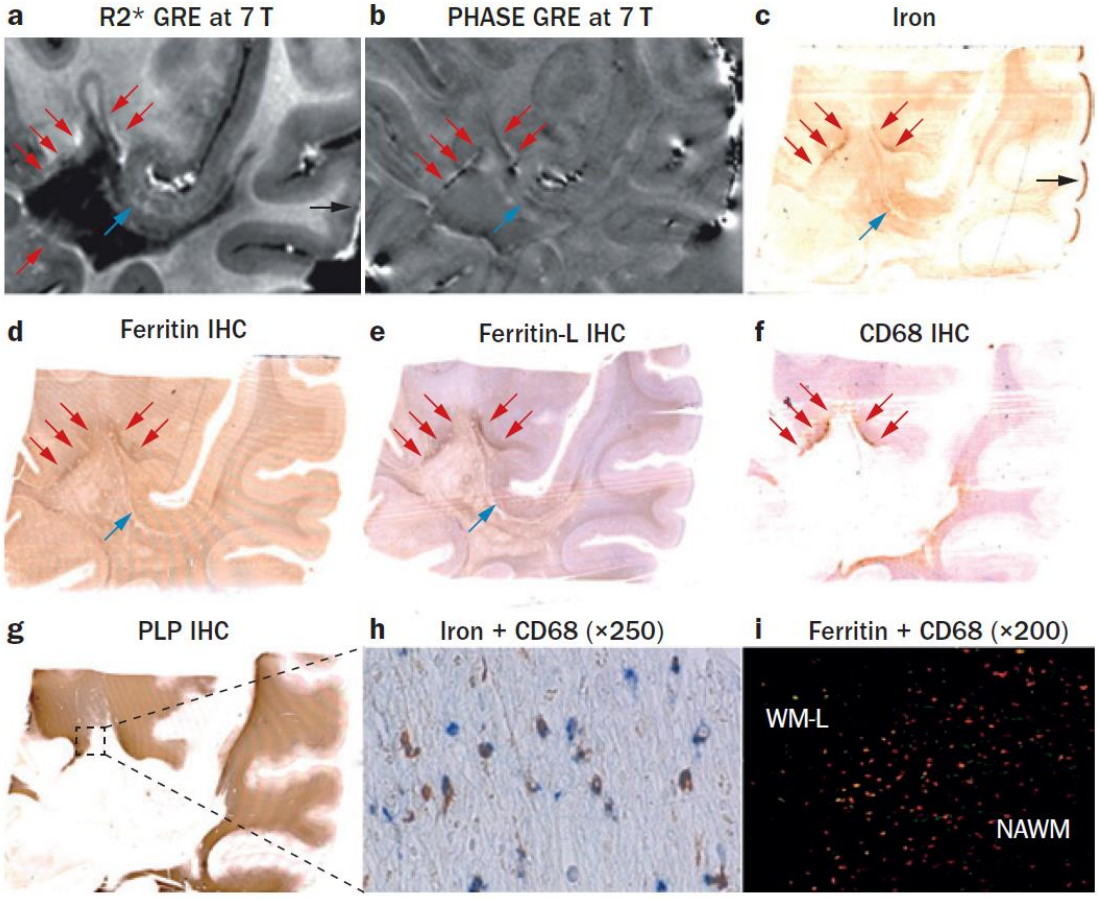
On the other hand, interruption of iron homeostasis can lead to production of reactive oxygen species (ROS) and oxidative stress, a process that can have toxic effects on the brain as well as to other organs and tissues. [51]

Moreover, impaired antioxidant defenses and mitochondrial dysfunction, a natural consequence of aging can disorganize the iron homeostasis and cause iron accumulation. [52] Iron excess and accumulation have been found to be major risk factors in neurodegenerative diseases that are more prevalent with age. Iron depositions surrounding neurons and microglia can be observed in the substantia

nigra, where the majority of dopaminergic neurons are located, in patients with Parkinson's disease while excessive iron has been also found in amyloid plaques and neurofibrillary tangles in Alzheimer's disease patients [53,54,55,61] Iron accumulation has also been detected in the brain of patients suffering from amyotrophic lateral sclerosis, [57] Huntington's disease, [58] Friedreich's ataxia, [59] and traumatic brain injury. [60,62]

Recently abnormal regulation of iron metabolism and subsequent iron distribution in the brain has been gradually implicated in MS by a growing body of scientific data reporting abnormally high iron levels in multiple sclerosis patients [3,4,6,7,56]

Magnetic resonance imaging (MRI) and histopathological studies have demonstrated major changes in the iron levels of the brain and central nervous system of MS patients as it can be seen in Figure 1 below.



Bagnato, F. *et al.* Tracking iron in multiple sclerosis: a combined imaging and histopathological study at 7 Tesla. *Brain* (2011), 134 (12), 3599–3612 [95]

Figure 1: Increased iron depositions around a white matter MS lesion (red arrows) in a patient with secondary progressive MS. **a-b; post-mortem susceptibility MRI, c-f; tissue staining for iron and ferritin with colocalised staining for microglia and macrophages. The blue arrows indicate a local area of iron deposits in the perivascular space, g; the same white matter lesion under PLP staining reveals moderate degrees of demyelination, h; iron (brown) and macrophages/microglia(blue) present overlapping in demyelinated areas, I; double immunofluorescence shows colocalisation (yellow) of ferritin (green) and CD68$^+$ cells (red)** [95]

Free iron, in excess, can lead to accumulation of iron in the brain and particularly in macrophages and microglia, resulting in toxic effects such as pro-inflammatory macrophage-type-1 activation and inflammation. Nevertheless, iron contributes to the integrity, proliferation and differentiation of oligodendrocytes and myelin, facilitating their regeneration after injury or inflammation. In light of the above mentioned neurotoxic and neuroprotective effects of iron in the brain of MS patients, this essay investigates the delicate role of iron in MS pathophysiology by presenting research findings, models and scientific techniques from the literature and then discusses the

possible trophic and toxic effects of iron balance in the brain, from adequate levels of iron vital to remyelination and repair mechanisms to excessive iron accumulation, resulting in neurodegeneration and damage.

2. Iron distribution in the healthy brain

Metals are necessary elements which take part in numerous physiological functions of intercellular as well as intracellular processes. Iron is an essential cofactor for enzymes and a fundamental stabilizing element for biomolecules. In the central nervous system, iron exhibits an important role as it participates in the metabolism of the brain and is essential for neurotransmitter synthesis, oxygen transport, myelin production, glucose metabolism and nerve transition [50,53]. Moreover, iron engages in physiological processes within oligodendrocytes for the production and maintenance of myelin, where enzymes utilize iron. [3] Understandably, iron homeostasis is of vital importance for the optimal functioning of the brain.

Brain iron levels are low at birth but after two decades of life, iron accumulates rapidly. After the span of two decades' brain iron levels increase with a slower rate reaching a plateau state around the age of 40 to 50 years old. [73]

Iron in high concentrations is normally found in the globus pallidus, caudate nucleus, putamen, dentate nucleus, red nucleus, and substantia nigra. [74]

A major portion of iron components is found as iron, non-bound to heme, in the oligodendrocytes and in the myelin sheaths. Basal ganglia also exhibit high iron contents and the increase of iron deposition in basal ganglia is positively correlated with the process of ageing. [75]

Oligodendrocytes are the brain cells that synthesize transferrin controlling intracellular iron transport. In contrast, microglia express DMT1, amyloid precursor protein and ferritin, which enable neurons to preserve iron hemostasis in the brain and also protect normal neuron function by iron regulation.

The ability of iron to act as a transition metal and its ability to transfer electrons makes it vital for the mitochondrial enzymatic processes. However, due to the afore-mentioned ability, iron can react with oxygen and hydrogen peroxide working as a catalyst for the formation of superoxide and hydroxyl radicals, which in turn, can further react with lipids and macromolecules leading to cellular oxidative stress and cell death. These potential catastrophic effects are prevented by adequate iron storage in the brain, a process controlled by ferritin, hemosiderin and neuromelanin.

2.1 Cellular transport of iron

Iron is found in two alternate ionic states: an insoluble ferric (Fe^{3+}) or soluble ferrous (Fe^{2+}) state that takes part primarily in mitochondrial enzymatic processes like electron transfer and redox reactions (Figure 2), which are of outmost importance for physiological cellular function and oxygen transport but also against the production of reactive oxygen species and induced cellular toxicity. [66]

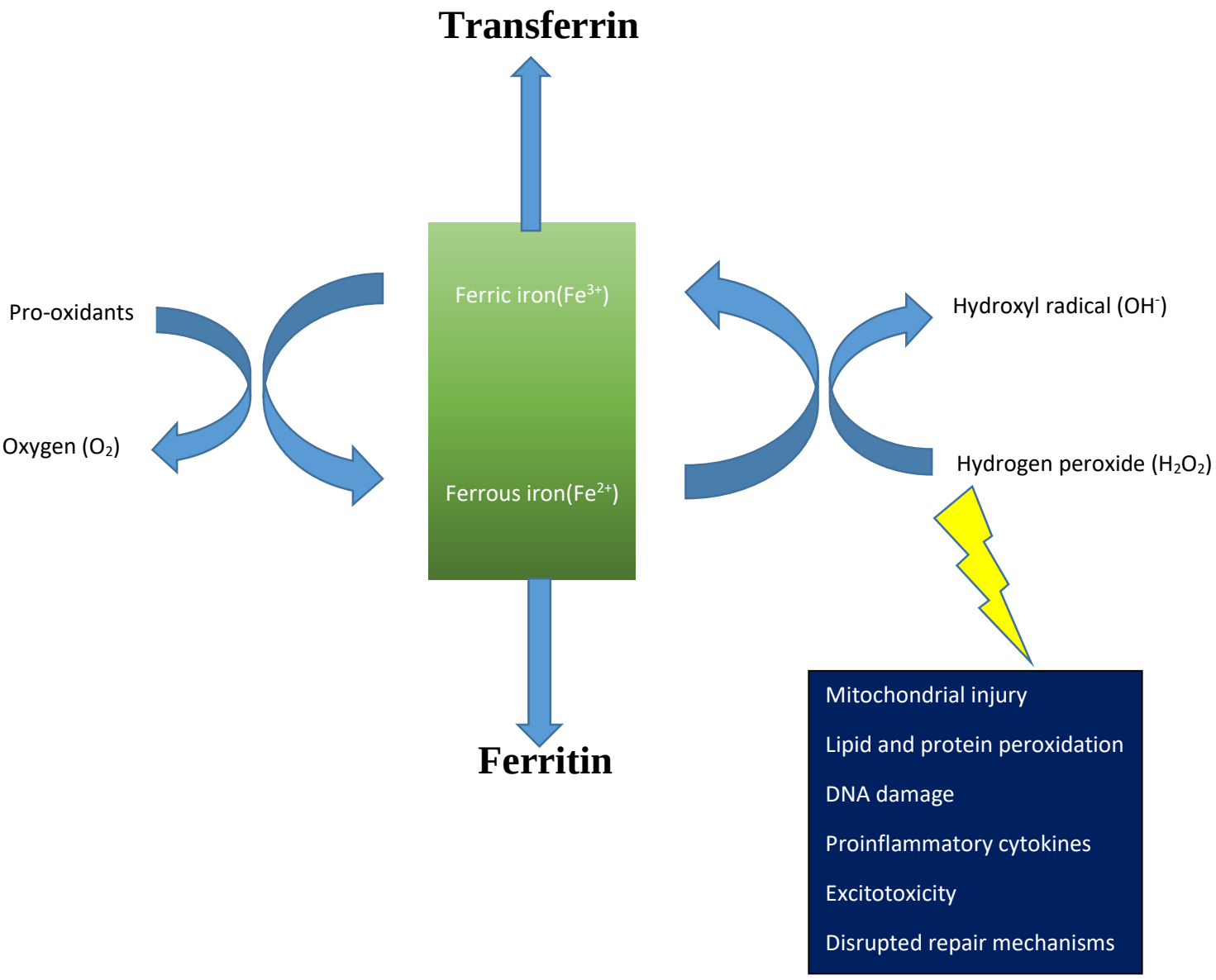
The iron balance in the human organism is maintained and regulated by liver enzymes and proteins such as ceruloplasmin and hepcidin, while the cellular uptake of iron is mediated by iron transporters, namely transferrin and divalent metal transporter 1 (DMT1). [50] Ceruloplasmin is expressed by astrocytes and endothelial cells and after the bonding of iron to transferrin, this activity is being increased to regulate physiological iron distribution. Non-transferrin bound iron (NTBI) is normally synthesized in the cytosol and is the iron pool for oligodendrocytes and astrocytes when transferrin is highly saturated by iron [71,72]

Iron circulating in the bloodstream is in the ionic state of ferric iron (Fe^{3+}) and bound to transferrin. Fe^{3+} enters the cellular space upon binding with the membrane protein transferrin receptor 1 (TFR1). After this ligation, ferric iron relocates in the endosome. In the endosome, metalloreductase STEAP3 catalyzes the reduction of insoluble ferric iron to its soluble form, ferrous iron. Ultimately, divalent metal transporter 1 (DMT1) promotes the release of Fe^{2+} located in the endosome into a labile iron pool (LIP) in the cytoplasm. Excess iron is stored in ferritin, a protein complex constituting of ferritin light chain (FTL) and ferritin heavy chain 1 (FTH1). Iron release form the intracellular to the extracellular space is managed by the ferroportin, a membrane located protein, which catalyzes the oxidation of Fe^{2+} back to Fe^{3+}. [67] Ferroportin is mainly expressed in astrocytes and endothelial cells, in order to export excessive iron and maintain their proper functions. The stabilization of ferroportin is managed by the amyloid precursor protein (APP), which enables iron export in neurons, and its activity has been shown to be controlled by iron [53]. In turn, the regulation and maintenance of physiological cellular iron levels is achieved through iron regulatory proteins (IRP) and iron response elements (IRE). IRP and IRE are controlling the expression of iron transporters and their respective receptors, whenever the cellular iron levels are too high or too low. [68]

Another protein involved in the regulation of iron in the brain is Lipocalin-2 (LCN 2). LCN 2 is an acute-phase protein which is generated and secreted as a physiological reaction to inflammatory and pathological events. LCN 2 in the brain is mainly found in astrocytes located in the majority of regions within the healthy adult brain, apart from the olfactory bulb, brainstem and the cerebellum, where astrocytes' LCN 2 levels are considerably higher than the afore-mentioned regions. Moreover, LCN 2 is produced by the brain endothelium and by astrocytes whereas its receptor, brain type organic cation transporter 24p3R is also located in neurons and brain endothelial cells apart from astrocytes. Although the role of LCN 2 has not been fully understood yet, the secretion of LCN 2 involves neuronal proliferation and homeostasis via iron transportation by LCN 2. [125,126] Evidence from a scientific study demonstrated the capacity of LCN 2 in iron-binding, a capability that increases dendritic spine density

and morphology. Similarly, disruption of iron-binding properties of LCN2 or increasing its iron-free form led to opposite toxic effects. [128] Evidence from another scientific study has described the capability of 24p3R receptor of LCN 2 to bind iron and deliver it to the intracellular space. After the binding, the 24p3R-iron complex enters the intracellular space via endocytosis and the bound iron is then released, leading to increased levels of intracellular iron. Further investigation revealed that iron-loaded 24p3R receptor increased iron levels within the cells, down-regulated the pro-apoptotic protein BIM and exerted anti-apoptotic effects while iron-lacking 24p3R receptor greatly reduced intracellular iron load, an event that leads to the activation of the pro-apoptotic protein BIM and ultimately to apoptotic cell death.[127] Consequently, LCN 2 regulates cell proliferation and/or apoptotic pathways as a result to its iron-binding and transport capabilities, suggesting that LCN 2 holds a key role in the maintenance of intracellular iron homeostasis.

Figure 2: Iron redox cycling and metabolism via the Fenton reaction.



3. Cytotoxic effects of iron overload in MS

The potential for iron to exchange electrons and swap between the two transition states, that of ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), renders iron a vital element for the completion of important cellular biochemical actions. This is called the redox switching reaction or Fenton reaction, which enables mitochondria to complete their respiratory burst activity and provide other cells and hence the human organism with the required energy and metabolic activity to sustain a healthy and physiological function.

However, this ability of iron to transit between the two states means that it can also take part in reactions which can produce reactive oxygen species, with detrimental results affecting the cellular components. As mentioned above ferrous iron acts as a catalytic element and assists the conversion of hydrogen peroxide, via the Fenton reaction, to hydroxyl radical. The hydroxyl radical is extremely reactive and if it's not converted to less reactive radicals, it can damage membrane lipids, DNA and increase intracellular calcium leading to cell damage and death. This is achieved with the oxidation of ferrous iron to ferric iron. The redox cycling of iron then takes place, when a reducing analog, such as superoxide reacts with ferric iron to reproduce redox-active iron. [76,77]

3.1 Ferroptosis

The iron-mediated formation of reactive oxygen species described above can directly lead to ferroptosis, a unique form of cell death which is characterized by increased intracellular iron levels. Ferroptosis is characterized by the presence of smaller than normal mitochondria that exhibit unique morphological abnormalities. On the biochemical level, ferroptosis is characterized by accumulation of lipid peroxidation byproducts that arise from iron-catalyzed oxidation of polyunsaturated fatty acids, which results into lipophilic iron-catalyzed reactive oxygen species that cause damage to lipids and excessive depletion of the plasma membrane unsaturated fatty acids (PUFAs).

Ferroptotic death is associated with a number of key factors including cysteine availability, biosynthesis of glutathione (GSH), intracellular iron overload and enzymatic or non-enzymatic lipoxygenase (LOX) mediated lipid peroxidation.

The first upstream regulator in the ferroptotic signaling pathway is the cysteine-glutamate antiporter system X_c^-. The X_c^- system is a heterodimeric amino acid transporter system that its role involves exchanging extracellular cysteine for intracellular glutamate in a molecular ratio of 1-1. When extracellular cysteine enters the cell, it is reduced by GSH or thioredoxin reductase 1 to cysteine, for the synthesis of GSH. This reaction is catalyzed by two different enzymes, γ-glutamylcysteine

synthetase and glutathione synthase. Pharmacological inhibition of either of these two enzymes can cause depletion of the intracellular glutathione reserves and therefore ferroptotic cell death. This observation highlights the importance of GSH as an anti-oxidant which counteracts the lipid peroxidation by the reactive oxygen species caused by iron overload within cells. Furthermore, GSH acts as a substrate for the glutathione peroxidase 4 (GPX 4) that is believed to have a central role in the ferroptotic death pathway. GPX 4 is an anti-oxidant enzyme that contains selenium and its role is to metabolically inactivate phospholipid and cholesterol hydroperoxides (-LOOH) by breaking them down to their respective alcohols (-OH) using two molecules of GSH as electron donors. Production of phospholipid and cholesterol hydroperoxides in cells is mediated through enzymatic and non-enzymatic pathways involving lipoxygenase (LOX) -mediated oxidation and radical-mediated autoxidation respectively. Upon binding of GSG to GPX4, oxidized glutathione (GSSG) is generated by the GPX4-GSH complex and GSSG is then reduced back to GSH by glutathione reductase (GR) in an enzymatic reaction requiring NADPH. NADPH in turn is produced by glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD) and malic enzyme. Ferroptosis occurs when the GPX4 catalyzed reduction of phospholipid and cholesterol hydroperoxides is insufficient to prevent the iron-mediated generation of lipid hydroperoxides. Lipid hydroperoxides which are natural byproducts of the iron-mediated lipid metabolism accumulate and directly cause peroxidation of PUFAs and subsequent ferroptotic cell death. [117, 118]

Peroxidation of PUFAs also plays a key part in the induction of ferroptosis. Oxidation of PUFAs by LOXs can lead to increased levels of lipid peroxidation breakdown products than subsequently trigger ferroptosis. The accumulation of these lipid peroxide radicals is commonly referred to as the lethal lipid signal. Evidence arising from recent lipidomics studies have led to the discovery that Acyl-CoA synthetase long-chain family member 4 (ACSL4) promotes ferroptotic cell death through the accumulation of oxidized cellular membrane phospholipids. Specifically, oxidized phosphatidylethanolamines (PEs), produced via the ACSL4 enzyme, were identified as the lethal lipid species that cause ferroptosis. PEs that contain two fatty acyls, namely arachidonic (AA) and adrenic acid (AdA) are explicitly produced and activated by ACSL4, particularly in the mitochondria. ACSL4 catalyzes the ligation of AA and AdA to generate AA or AdA acyl Co-A derivatives. After the formation of AA or AdA acyl Co-A derivatives by ACSL4 the derivatives are esterified into AA-PEs and AdA-PEs by the lysophosphatidylcholine acyltransferase 3 (LPCTA3). The oxidation of AA-PEs and AdA-PEs is ultimately catalyzed by 15-LOX in order to generate lipid hydroperoxides, the main mediators of ferroptosis. Under physiological cellular conditions, the toxic lipid hydroperoxides are reduced by GPX4. [120,121,122]

The role of iron in association with ferroptosis was revealed when iron chelators such as deferoxamine inhibited ferroptosis, induced by the pharmacological agent erastin. In erastin-induced ferroptosis, iron regulatory protein 2 (IRP 2) increases the cellular levels of iron to up-regulate ferroptotic cell death. IRP 2 is bound to iron responsive elements (IREs) located on the 5’ side-chain of ferritin and ferroportin and in the 3’-side chain of TFR1 and DMT1.IRPs and IREs are responsible for the maintenance and regulation of intracellular iron homeostasis. Knockdown of the IRP 2 in mice

significantly increased the up-regulation of the expression of genes associated in iron metabolism (F-box and leucine-rich repeat protein 5, iron-sulfur cluster assembly enzyme and ferritin) and effectively tackled erastin-induced ferroptosis.[117] Data from another study with cells artificially sensitized to ferroptosis after Ras-genes mutation, indicated that the ferroptosis-sensitized cells demonstrated increased TFR1 and decreased ferritin expression in comparison to ferroptosis-resistant cells.[123] This observation leads to the conclusion that increased iron uptake, mediated by the over-expression of TFR1, and impaired iron storage by down-regulation of the expression of iron-storage protein ferritin can promote iron accumulation during ferroptosis. Therefore, one could suggest that intracellular systems involved in iron uptake and iron storage are contributing to the initiation of ferroptotic effects,

Another important aspect of ferroptosis is the cellular autophagic process of ferritin degradation a term commonly referred to as ferritinophagy. |As mentioned before ferritin is an iron-storage protein responsible for physiological iron homeostasis. When intracellular iron levels are low, nuclear receptor co-activator 4 (NCOA 4) binds to the 1-heavy chain of ferritin (FTH 1) in the autophagosome and the complex is relocated to the lysosome where the process of degradation takes place. This process is essential when iron levels are too low to meet systemic iron requirements. In contrast, when the iron levels within the cell are too high, NCOA 4 ligates selectively with the HECT and RLD domain containing E3 ubiquitin protein ligase (HERC 2) in order to trigger its ubiquitination and subsequent degradation [124]. Scientific evidence demonstrated that upon initiation of ferroptosis, the pathway of ferritinophagy is activated and therefore the cleavage of ferritin increases the iron levels of LIP, an event that triggers ROS generation and accumulation, ultimately leading to ferroptotic cell death. In support of this observation, silencing the NCOA 4 gene showed a repression of the ferritin breakdown and subsequent suppression of ferroptosis, whereas the over-expression of the NCOA 4 gene yielded the opposite results and consequently sensitized cells to ferroptosis. [119]

3.2 Lipocalin 2

Lipocalin -2 has also been implicated as a critical mediator in the onset and progression of MS.LCN 2 as mentioned previously is important in regulating iron levels within the cells and is found in abundance mainly in astrocytes. Therefore, alteration of the brain levels of LCN 2 can influence brain iron concentration and neuroinflammatory responses caused by the increased iron load.

LCN 2 was found in high levels in activated astrocytes in the brain of experimental auto-immune encephalitis (EAE) mice, in brain regions typically affected in patients suffering from MS. Moreover, evidence from the same study indicated that LCN 2 is also present in the cerebro spinal fluid (CSF) of the EAE mice probably due to the increased generation of LCN 2 by the activated astrocytes. [129]

Moreover, the secretion of LCN 2 results in the activation of resting microglia and astrocytes towards the M1 phenotype, which is known to induce pro-inflammatory signals and neuroinflammation, thus leading to neurotoxicity. LCN2 can lead to the

increased expression of chemokine (C-X-C motif) ligand 10 (CXCL10) which induces cell migration and in particular migration of astrocytes and microglia. This process involves partly activation of JAK2/STAT3 and IKK/NF-B signaling pathways. LCN2 also up-regulates the expression of glial fibrillary acidic protein (GFAP) that leads to changes in the structure of reactive astrocytes. [125,126]

LCN2 is also present in high levels in the spinal cord and secondary lymphoid tissues during the initiation of EAE in mice .Data arising from scientific research indicated that LCN 2 knockdown leads to reduced EAE severity, lower levels of CNS infiltration by activated microglia and astrocytes, reduced degree of expression of proinflammatory cytokines and decreased demyelination effects in the spinal cord .Upon treatment with recombinant LCN 2 protein, the expression of IL-17a, IFN-γ Rorc and Tbet ,chemokines produced by autoreactive T-cells was increased. Moreover, MMP-9 was up-regulated, the production of which is related to neurotoxic effects in the EAE mouse model. [130] In support of this observation, the authors of another study demonstrated that increased levels of LCN 2 in the brain parenchyma, particularly in activated astrocytes and infiltrating neutrophils is positively correlated with the active phase of EAE progression. Upon treatment with natalizumab the levels of LCN 2 were suppressed and clinical characteristics of the disease were attenuated. The authors of the same study also investigated CSF levels of LCN 2 in MS patients and in healthy individuals, revealing that MS patients had significantly higher levels of LCN 2 that the healthy controls. [129] This finding suggests that LCN 2 is directly involved in the pathogenesis of MS and that LCN 2 levels can be further utilized as an important biomarker to identify MS onset and progression during the different phases of the disease.

Transcription factors such as nuclear factor B (NF-B) and CCAAT-enhancer-binding proteins (C/EBPs) also play an important part in the transcriptional regulation of Lcn2 gene expression following inflammatory events. Proinflammatory cytokines such as TNF-α, IL-1, IL-3, IL-6, IL-17, and IFN-γ affect LCN 2 expression and secretion under inflammatory responses. [125,126]

The synthesis and secretion of LCN2 protein is triggered after the up-regulation of the LCN 2 gene transcription The LCN2 protein binds to its cell surface receptor (24p3R) and involves a number of biological effects including apoptosis, morphological changes, cell migration, and promotion of inflammatory responses. For instance, LCN2 promotes NO release that induces apoptosis by up-regulation of the integrin-beta 3 (ITGB3) expression. [125,126]

3.3 Mitochondrial damage

Mitochondria are also a prime target for damage by reactive oxygen species given the fact that mitochondria are the natural environment where reactive oxygen species are normally being produced to sustain the metabolic and energy requirements of cells. The ongoing oxidative metabolism is mitochondria is assisted by iron via the redox cycling and Fenton reaction and understandably damaged mitochondria can be the source of extensive electron leakage and excessive production of reactive oxygen

species. Taking into account that mitochondrial DNA is more prone to damage from oxidative stress than nuclear DNA, the effects of excessive oxidative stress can lead to increased mitochondrial toxicity and rapid cell death. [79,80]

MRI scans from lesions found in the brain of MS patients reveal high mitochondrial damage and consequently iron leakage from damaged mitochondria can cause further production of free radicals and impede the repair mechanisms of the already damaged mitochondria. [50,81,82]

Therefore, it is reasonable to consider the natural sites where iron reaction and hydroxyl radical formation takes place, as potential biomarkers for assessing cytotoxic effects.

Iron deposits and the subsequent production of oxidative stress has deleterious effects on cultures of oligodendrocytes and neurons but not particularly to astrocytes. This observation might be attributed to the fact that astrocytes have the remarkable ability to upregulate certain antioxidants, like glutathione (GSH) which decrease the amount of toxic free radicals and their metabolites [83,84,85].

3.4 Glutamate release

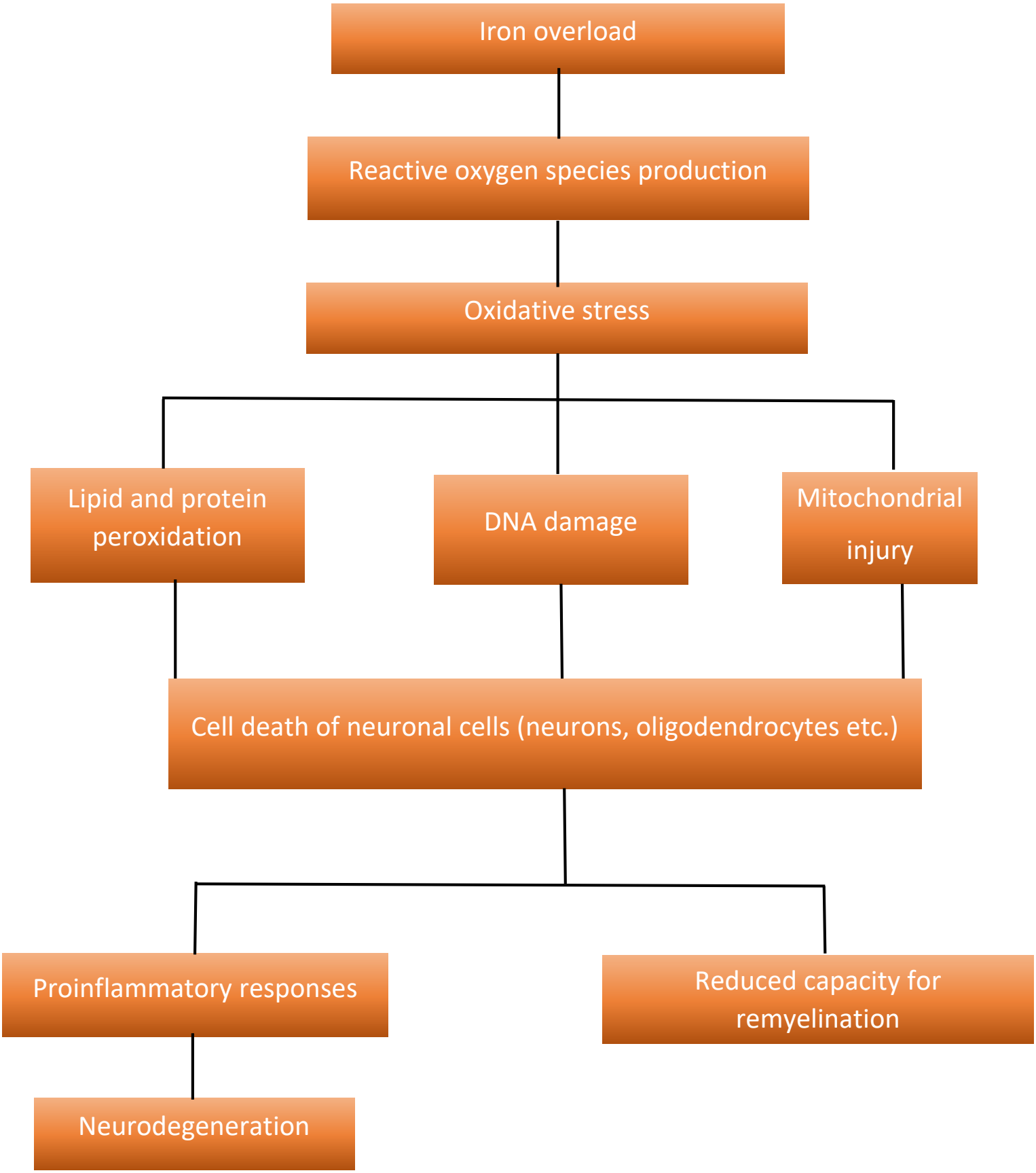
Iron is known to stimulate the release of glutamate, an excitatory neurotransmitter binding to N-methyl-d-aspartate (NMDA) and non-NMDA glutamate receptors on oligodendrocytes. This release of glutamate can cause over activation of the above-mentioned receptors resulting in excitotoxicity, a pathological process than can damage neurons and cause extensive cell death. [90]

Apart from oligodendrocytes, cells that participate in the process of remyelination in MS called oligodendrocyte progenitor cells, (OPCs) are also prone to damage caused from iron-induced oxidative stress which results in the poor performance of repair mechanisms and further exacerbates the already existing effects of myelin loss. [91]

Myelin accumulation in activated macrophages and microglia might also contribute to the inability of these cells to store iron. In demyelinating MS lesions, the leakage of myelin is rapidly growing and this myelin can be taken up by M1 macrophages reducing their ability for iron uptake. Consequently, the reduced capacity of these cells to accumulate iron, leads to iron overload in the extracellular space which can then be taken up by other cells and amplify cytotoxicity.

A summarized overview of the cytotoxic effects of iron overload in MS is presented below (Figure 3).

Figure 3: The role of iron overload in MS and its toxic effects in the central nervous system



3.5 Sources of iron overload in MS

As mentioned previously, oligodendrocytes are the brain cells with the highest metabolic activity and iron load mainly because of the abundancy of iron-requiring enzymes and their role in lipid and myelin synthesis needed for the formation of the myelin sheaths around neuronal axons. Taking into account the above-mentioned characteristics of oligodendrocytes, the decay of oligodendrocytes and the loss of myelin sheaths seen in MRI scans of active MS lesions is one apparent reason for the iron accumulation observed in activated microglia and macrophages surrounding the edges of the lesion.

Dysregulation of iron regulatory proteins and iron transporters is another obvious candidate for the observed iron accumulation in MS lesions. Monocytes and macrophages with high contents of iron have been found around the rim of MS lesions. These cells are involved in physiological iron homeostasis and are responsible for regulating iron in the peripheral systems as a response to environmental stimuli. Their infiltration in the central nervous system during MS onset and progression might indicate that these cells might constitute another possible for source for the increased iron levels in MS. [3,94,95]

Lastly, damage to blood vessels in the brain causing the cell death of erythrocytes and subsequent degradation of hemoglobin. Hemoglobin is an iron-containing metalloprotein responsible for the transport of oxygen and this degradation of hemoglobin can be another potential source of iron accumulation in MS patients. Indeed, histopathological evidence in MS lesions have identified traces of iron accumulation around blood vessels indicating the presence of hemorrhages. MS lesions usually have a central blood vein running through them and iron immunostaining studies have revealed hemosiderin depositions, which is a breakdown product of hemoglobin, inside as well as outside of white matter MS lesions. [3,96,97]

3.6 Importance of animal models in iron overload in MS

In order to study the histopathological changes associated with increased iron levels in the MS brain, a particular animal model has been developed. Mice with experimental auto-immune encephalitis (EAE), a neurological condition that strongly resembles the inflammation and neurodegeneration occurring in MS have been studied to compare similarities and differences between the MS mice brain and that of human patients suffering from MS. Susceptibility-weighted imaging studies and T2-weighted MRI scans in EAE mice have revealed hypo intense areas in the brain associated with increased iron deposits, some of which are located in microglia. In addition to that demyelination features were observed around areas with iron overload, in a similar way to those observed in phase imaging studies in the brain of MS patients. [98,99100]

Notwithstanding the above-mentioned similarities from imaging and histological studies that iron overload is present in both mice with EAE and in MS patients, a recent study highlighted the possibility that not every animal model of EAE demonstrate sings of iron accumulation in a similar fashion as in MS patients. The authors of this study observed that iron accumulation is not always present in animal models with EAE and that mice show limited to no global iron accumulation during ageing as opposed to humans. [101]

These findings indicate that rodent models with EAE have a lower iron overload overall and possibly lower levels of oxidative stress compared to that of patients with MS. If this is the case, these findings question the relevance of EAE animal models in order to study the role of iron accumulation and iron chelation treatments in MS patients.

4. The role of iron (re)myelination

Iron in the brain is of vital importance for the completion of processes ranging from normal cell function to repair mechanisms.

In the brain, iron is found in a number of different cell types including astrocytes, neurons, microglia and oligodendrocytes. [63]. The circulation and the uptake of iron is primarily mediated by the astrocytes, which also have the ability of redistributing iron to other cells within the central nervous system. [64] Another process regulated by iron is myelin preservation and that is the reason why iron is found in abundancy in oligodendrocytes, as a continuous iron supply is crucial for their proliferation and proper function. [65]

The critical role of iron in myelin formation is being supported by scientific evidence showcasing that uptake of iron in the brain corresponds with the commencement of myelination during the embryonic development. [103] Therefore, this might be the reason behind the accumulation of iron in oligodendrocytes and its significance in their physiological function.

Mitochondria, cells which exhibit high metabolic requirements and energy capacity, are in constant need of iron in order to complete vital cellular and enzymatic processes. [66]

Glucose-6-phospate 1-dehydrogonase is another important enzyme which requires iron in high quantities as it participates in the pentose phosphate metabolic pathway. The pentose phosphate pathway is vital to the synthesis of myelin by oligodendrocytes as it provides for NADPH that enables the myelin fatty acid synthesis. [102]

Furthermore, iron is crucial as a cofactor in enzymes involved in the synthesis of lipids and cholesterol like the 14-demythelase of lanosterol that participates in cholesterol biosynthesis. Cholesterol and lipids are prerequisites for myelin synthesis and growth. [104,105]

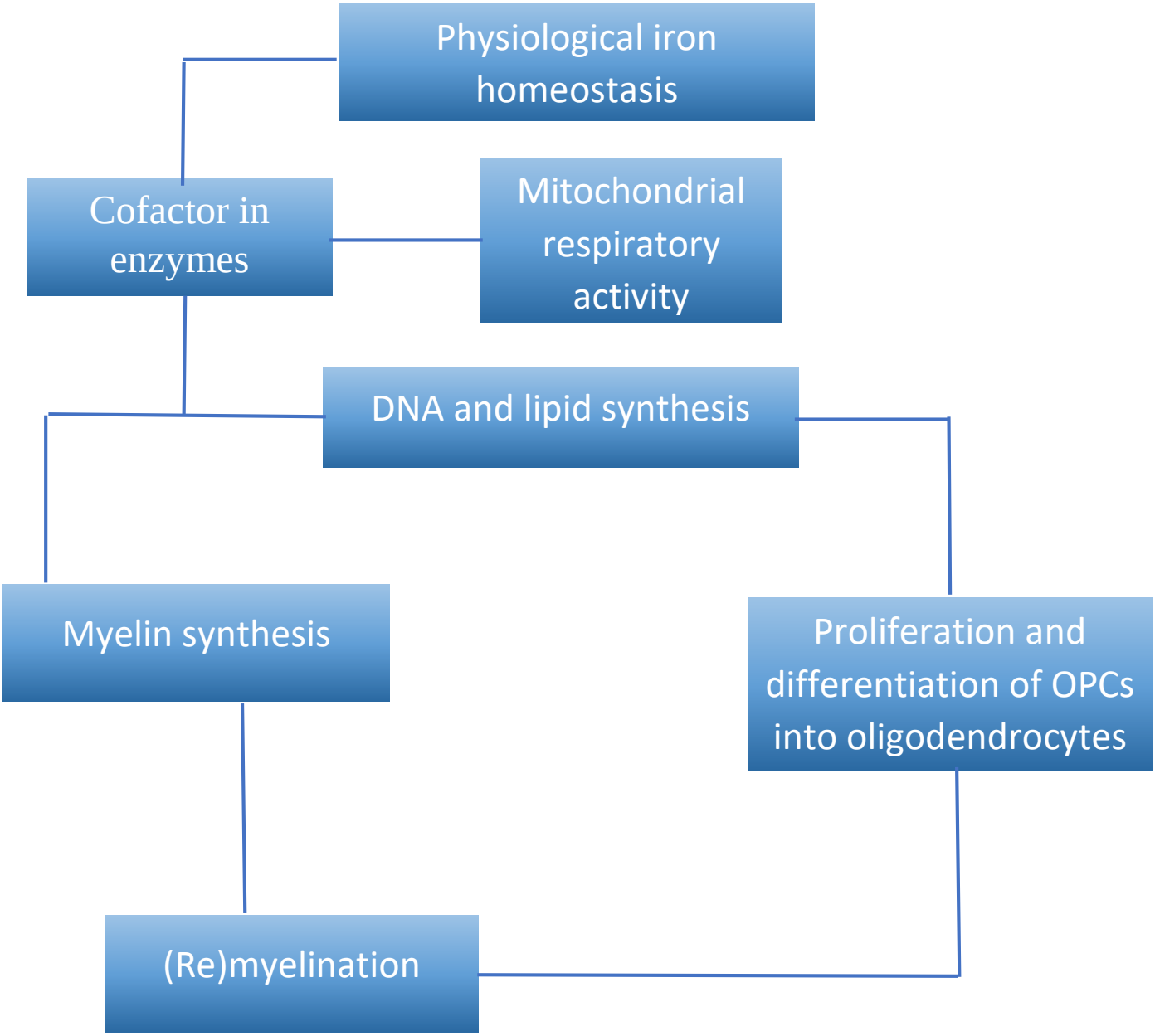
During remyelination, oligodendrocyte precursor cells are being generated, mature and differentiate to oligodendrocytes which help in the formation of the myelin sheaths. Taking into account the demand for iron-containing enzymes in order to complete these processes, iron accumulation in oligodendrocytes is crucial for repair mechanisms and for remyelination attempts overall in MS lesions. [106]

Physiological iron levels may be also indirectly involved in repair mechanisms by affecting other types of brain cells (apart from OPCs and oligodendrocytes) that participate in remyelination.M1 activated macrophages despite their excessive central nervous system infiltration induced by iron overload, can also express certain pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1β which promote remyelination.[107,108] The secretion of TNF has a direct effect on OPCs differentiation while IL-1β prompts astrocyte proliferation. [109,110] TNF and IL-1β further induce the release of fibroblast growth factor and insulin-like growth factor 1 (IGF-1) from astrocytes. [106] IGF-1 prevents oligodendrocyte apoptosis, initiate the generation and differentiation of OPCs and is an important prerequisite for oligodendrocyte build-up and myelination overall. [111,112]

Macrophages can also release ferritin which extends the survival of oligodendrocytes and promotes the production of new oligodendrocytes upon uptake by the already existing oligodendrocytes. [113,114]

An overview of the role of iron balance in remyelination and its trophic effects in the central nervous system are presented in Figure 3 below.

Figure 4: The role of iron homeostasis in (re)myelination and its trophic effects at the cellular level in the central nervous system



5. Discussion/Conclusions

MS is a multifactorial neurodegenerative disease but the main pathogenetic mechanism involved in MS neurodegeneration still remains elusive. Evidence arising from recent scientific studies suggest that iron has an important role in the onset and progression of the disease but whether iron is a key initializer of the MS pathogenesis or merely a by-product of the neurodegenerative processes is not yet understood.

Iron levels are pivotal for the sustainment of the physiological role of iron-containing enzymes involved in mitochondrial respiratory activity as well as in the generation, proliferation and differentiation of OPCs that will later differentiate into mature oligodendrocytes and participate in the process of myelination. Iron homeostasis is also important for the production of pro-inflammatory cytokines and growth factors (TNF, IL-1β and IGF-1) that promote remyelination after a demyelinating insult takes place. Consequently, dysregulation of iron metabolism in MS will not only initiate and contribute to enhanced cytotoxicity, but also impede the ensuing attempts for repair mechanisms.

The overall decreased iron levels in the normal appearing white matter of MS patients might have neuroprotective effects against demyelination and the production of oxidative stress [3] but this decrease in iron levels could also be a reason for reduced iron levels within oligodendrocytes. Taking into account the vital role of iron homeostasis for myelin synthesis by oligodendrocytes, further research needs to be done in order to assess whether and why oligodendrocytes in normal appearing white matter in MS patients demonstrate an iron deficiency. One hypothesis would be that iron-deficient oligodendrocytes are a consequence of the overall reduction in oligodendrocyte number, or that iron-deficient oligodendrocytes is a normal phenomenon to balance iron dysregulation since other neuronal cells exhibit higher and higher cellular iron levels.

Regarding the role of lipocalin 2, evidence arising from research studies highlight its importance as a biomarker for identifying MS onset and progression. Although the presence of LCN 2 is well established in MS, the role of LCN 2 is still under debate. As mentioned before, some studies have demonstrated the toxic effects that LCN 2 has on disease severity and in demyelinating lesions in MS patients, [129,130] at least one study argues that it may also exhibit neuroprotective effects. [131] Specifically, LCN 2 deficient mice exhibited increased disease severity which is accompanied by increased lesion burden and expression of proinflammatory cytokines TNF-α and IFN-γ. This observation suggests that rather than the deleterious effects of LCN 2 described in previous studies, LCN 2 has a neuroprotective role against the development of EAE. In addition to that, the IL-4 mRNAs of the LCN 2 deficient mice showed no alterations and consequently IL-4 is not upregulated by LCN 2 but LCN 2 controls the T1 helper cells immune response instead of promoting it. The majority of the neuroprotective effects of LCN 2 were shown to occur in the macrophages, microglia and reactive astrocytes, but not on neurons, given the fact

that the former ones express the 24p3R receptor on their cell surface. [131] Nevertheless, the scientific evidence currently supports the notion that LCN 2 has detrimental properties in the CNS and the production of LCN 2 by reactive astrocytes is crucial for the onset of neurodegeneration in EAE models and also in MS patients.

Understandably, the results arising from scientific studies regarding the role of LCN 2 are controversial and this can be attributed to the use of the LCN 2 knockout mouse model which has important limitations. Further research needs to be done in order to determine the role of LCN 2 in a context-depend manner and exactly which factors are mediating to up and down regulate LCN 2 expression and subsequently LCN 2 toxic or protective properties.

All in all, the well-documented presence of the 24p3R cell surface receptor of LCN2, and its ability to iron-binding and transportation supports the node that LCN2 is a key player regarding intracellular iron trafficking and maintaining iron homeostasis.

Ferroptosis a newly discover non-apoptotic and iron-dependent form of cell death is characterized by iron-mediated production of reactive oxygen species via the Fenton reaction and subsequent iron-dependent lipid peroxidation. Despite the fact that the mechanisms of iron in the molecular pathway of ferroptosis are not yet fully clear, the involvement of iron imbalance in the ferroptotic cell death is undoubted. Iron is not only directly involved in the generation of oxidative stress, but also participates in the synthesis of LOXs that oxidize PUFAs an event known as the lethal lipid signal. One of the reasons why iron mechanisms have not been elucidated yet is the difficulties arising from the measurement of its concentration. The design and synthesis of a novel fluorescence resonance energy transfer (FRET) probe, FRET Iron Probe 1 (FIP-1), is currently under investigation for measuring changes in the levels of labile iron pool (LIP) during ferroptosis in order to provide further insight in the constant dynamic balance between iron overload and iron deficiency. Moreover, studies have shown that GSX 4 a unique enzyme capable of converting toxic lipid hydroperoxides to non-toxic lipid alcohols have been found to be reduced in MS leading to insufficient levels of GSH and enhanced lipid peroxidation. Therefore, some of the most important ferroptosis markers are found present in MS.The fact that certain iron chelators are effective against ferroptosis has important implications in MS that is a disorder characterized by iron dysregulation. Studies from EAE mouse models have indicated that treatment with iron chelators protects against further neurodegeneration and improves the clinical manifestations of the disease. Lipophilic anti-oxidants are also an efficient treatment against lipid peroxidation which is present in EAE by maintaining high levels of r vitamin E (α-tocopherol), an antioxidant against lipid peroxyl radicals. Lastly, although GPX 4 like agents that could mimic the action of GPX 4 haven’t been developed yet treatment with GPX 4-mimetic diphenyl diselenide inhibits at a certain degree the development of EAE in mouse models. [131-136]

While these studies support the fact that ferroptotic cell death occurs in EAE , it is still not known whether the effects of the afore-mentioned treatments can only be contributed to the inhibition of ferroptosis or whether their effects can be a result of the modulation of inflammation responses as well.

Data from a recent scientific study linking ferroptosis and MS/EAE reveal that expression of GPx4 is reduced in EAE, accompanied by abnormalities in mitochondrial morphology, decreased GSH levels and increased lipid peroxidation, all hallmarks of ferroptosis occurring in demyelinating diseased like MS and EAE. GPX4 was found in considerable deficient levels specifically in neurons and this may be the cause for neuronal damage in these disorders. This means that ferroptosis may also affect neurons while oligodendrocytes are damaged by immune responses promoting proinflammatory mediators. Consequently, therapies with ferroptosis inhibitors and GPX 4 like activators might be a promising new treatment for MS patients. [137]

The complicated interplay between iron overload and iron deficiency in MS makes it harder for medical interventions to be effective and efficient. For instance, iron chelators, which are used as a therapy for iron accumulation, have neuroprotective effects against iron-mediated neurodegeneration but can also diminish global iron levels which are important for remyelinating processes.

Determining the role of iron in MS will bring the scientific community one step closer to understanding the neurodegeneration causes in MS and provide for new insights for the development of efficient treatments against MS.

Lastly, revealing the causes of increased iron deposition in MS will further help scientists understand and re-evaluate the role of iron in other neurological conditions such as Alzheimer’s Disease, Parkinson’s disease, Friedrich’s ataxia and Huntington disease, since iron overload is also an aspect of the above-mentioned conditions.

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