

# Neurodegeneration in multiple sclerosis involves multiple pathogenic mechanisms

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**Abstract:** Multiple sclerosis (MS) is a complex autoimmune disease that impairs the central nervous system (CNS). The neurological disability and clinical course of the disease is highly variable and unpredictable from one patient to another. The cause of MS is still unknown, but it is thought to occur in genetically susceptible individuals who develop disease due to a nongenetic trigger, such as altered metabolism, a virus, or other environmental factors. MS patients develop progressive, irreversible, neurological disability associated with neuronal and axonal damage, collectively known as neurodegeneration. Neurodegeneration was traditionally considered as a secondary phenomenon to inflammation and demyelination. However, recent data indicate that neurodegeneration develops along with inflammation and demyelination. Thus, MS is increasingly recognized as a neurodegenerative disease triggered by an inflammatory attack of the CNS. While both inflammation and demyelination are well described and understood cellular processes, neurodegeneration might be defined by a diverse pool of any of the following: neuronal cell death, apoptosis, necrosis, and virtual hypoxia. In this review, we present multiple theories and supporting evidence that identify common biological processes that contribute to neurodegeneration in MS.

**Keywords:** lipid and one-carbon metabolism, hypoxia, oxidative stress, autoantibodies, nuclear receptors

## Introduction

Historically, neurodegeneration in multiple sclerosis (MS) was viewed as a secondary process resulting from inflammatory demyelination. While demyelination may play an important role in relapsing remitting stage, it doesn't correlate well with the progressive forms of the disease. Over the past several years, a major shift in thinking about the pathogenesis of progressive forms of MS has occurred.<sup>1-13</sup> Axonal loss, rather than demyelination, correlates better with clinical disability.<sup>5,14</sup> A new concept emerging in the MS literature theorizes that axonal loss may occur independently of or may even be the cause of the demyelination in MS.<sup>5,14</sup> Evidence indicates that neurodegeneration occurs in all stages of the disease.<sup>9,13,15,16</sup> In addition, the neurodegeneration seen in the progressive forms of MS does not correlate with white matter plaque location but instead, correlates with gray matter and cortical pathology.<sup>6,13,15,17-21</sup> A post-mortem analysis of spinal cords from MS patients showed that axonal loss in the white matter tracts did not associate with the demyelinated plaques in the region.<sup>4</sup> This indicates that there might be some pathological mechanisms independent of myelin loss that contribute to the axonal loss and neurodegeneration present in MS. Further evidence has shown that axonal injury can occur before myelin loss,<sup>4,5,9,22</sup> suggesting that axonal injury and neurodegeneration

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could be independent of demyelination and may occur prior to or in parallel with demyelination. Neurodegeneration is a very complicated mechanism that involves several factors. Perhaps the best way to understand the process of neurodegeneration is to dissect the protein targets and molecular pathways involved. In this review, we will discuss multiple theories of myelin loss and axonal degeneration as the basis of disease pathology, with the goal of shedding light on the common pathways of neuronal destruction.

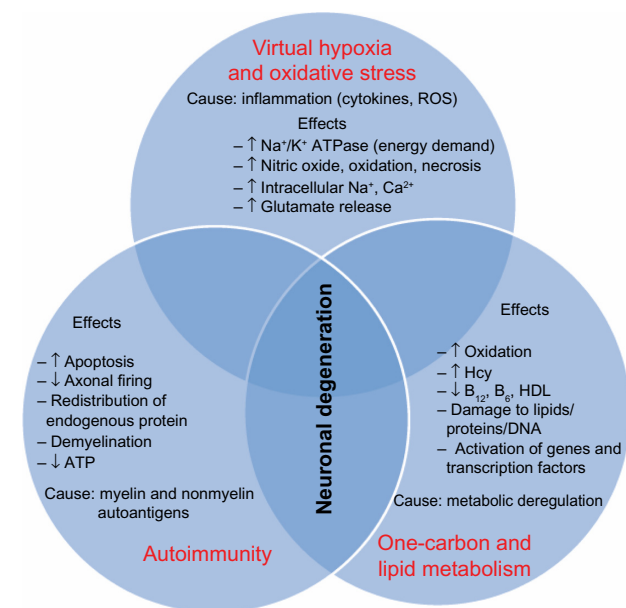
## Hypoxia

Over the years, multiple hypotheses have been proposed to explain the pathogenesis of MS, ranging from viral infection, cytokine-induced apoptosis, and oxidative stress (OS) to molecular mimicry and metabolic disorders.<sup>23–26</sup> However, none have successfully identified a single pathological mechanism, mainly because MS is a heterogeneous disease, with a multifaceted etiology.<sup>27,28</sup>

One school of thought suggests MS pathology is due to axonal damage and loss, which occurs when chronically demyelinated neurons reach a state of “virtual hypoxia” associated with reduced adenosine triphosphate (ATP) production, and ion channel and mitochondrial dysfunction. It is believed that the loss of myelin results in an increased energy demand and a relative cellular energy deficit, which eventually leads to neuronal death (Figure 1). In a viable neuron,  $\text{Na}^+/\text{K}^+$  ATPase is located at the nodes of Ranvier (regions between myelin

sheaths). Evidence suggests that after demyelination, the  $\text{Na}^+$  channels undergo redistribution, from localization predominantly on the nodes of Ranvier to a diffuse spread along the axon.<sup>29,30</sup> Thus,  $\text{Na}^+/\text{K}^+$  ATPase increases along a demyelinated axon in order to continue saltatory conduction. The increase in  $\text{Na}^+/\text{K}^+$  ATPase results in an increased energy demand for neuronal firing. In MS patients, this increased energy demand cannot be met because of impaired mitochondrial energy production in the central nervous system (CNS).<sup>4,22,31</sup> The impaired mitochondrial energy production leaves neurons in a depleted energy state, which has been shown to reduce the ability of  $\text{Na}^+/\text{K}^+$  ATPase function.<sup>32</sup> Depleted mitochondrial energy production and reduced firing ability in the overpopulated  $\text{Na}^+/\text{K}^+$  ATPase within demyelinated neurons in MS leads to several deleterious downstream effects, among which is impaired neurotransmission. With a lack of efficient  $\text{Na}^+/\text{K}^+$  ATPase, the cell, in theory, should enter a state of axonal depolarization. This state of axonal depolarization causes the overpopulated  $\text{Na}^+/\text{K}^+$  ATPase to become leaky, resulting in increased intracellular  $\text{Na}^+$  concentrations (Figure 1). It is believed that if axonal  $\text{Na}^+$  rises to a concentration greater than 20 mM, the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger will operate in reverse, thus acting as a system to dump  $\text{Ca}^{2+}$  into the axon.<sup>33</sup> The increase in  $\text{Ca}^{2+}$  within the axon is known as  $\text{Ca}^{2+}$  loading. Additional sources may contribute to axonal  $\text{Ca}^{2+}$  loading, including release from intracellular  $\text{Ca}^{2+}$  stores,<sup>34</sup> voltage-gated  $\text{Ca}^{2+}$  channels,<sup>35</sup> and  $\text{Ca}^{2+}$ -permeable cation channels, such as glutamate-gated receptors.<sup>36–38</sup> Large quantities of glutamate released by activated immune cells, in turn, activate glutamate receptors, which results in axonal  $\text{Ca}^{2+}$ -loading and subsequent neuronal death.<sup>39,40</sup> In hypoxic cells, the reversal of  $\text{Na}^+$ -dependent glutamate transporters results in glutamate release.<sup>41</sup> In addition, astrocytes can release glutamate, by exocytosis or hemichannels,<sup>42,43</sup> and unmyelinated callosal axons release glutamate in a vesicular manner, similar to the normal release at the synapse.<sup>44</sup> This increase in vesicular release of glutamate within the white matter has implications for the mechanisms of ischemia-induced myelin damage, which can possibly occur through the activation of glial cells.<sup>44</sup>

Hypoxia might also play a role in the formation of MS lesions. Decreased oxygen availability (hypoxia) is often seen in tissues at the sites of chronic inflammation. Inflamed tissue has increased metabolic activity, due to the presence of inflammatory cells and poor perfusion, which is related to blood vessel stenosis and microthrombosis. Therefore, chronically inflamed tissue has an increased demand for and a limited supply of oxygen. This imbalance results in hypoxia at inflammatory sites. Hypoxia also increases the permeability of the blood–brain barrier (BBB) and results in



**Figure 1** Schematic representation of the neurodegeneration theories.

**Note:** Arrows represent increase (upward) and decrease (downward) in cellular processes and metabolites.

**Abbreviations:** ATP, adenosine triphosphate;  $\text{B}_6$ , vitamin  $\text{B}_6$ ;  $\text{B}_{12}$ , vitamin  $\text{B}_{12}$ ; DNA, deoxyribonucleic acid; Hcy, homocysteine; HDL, high-density lipoprotein; ROS, reactive oxygen species.

the overexpression of proinflammatory genes.<sup>45–47</sup> Hypoxia is often accompanied by hypoperfusion. In about 50% of MS patients, the blood flow through normal-appearing white matter is reduced.<sup>48–52</sup> Taken together, both hypoxia and hypoperfusion might be a precipitating factor for MS lesion formation.

Hypoxia-inducible factor (HIF) is an important transcription factor that regulates cellular metabolism and survival under hypoxic stress. HIF is composed of an alpha and beta subunit (HIF- $\alpha$  and HIF- $\beta$ ). Active HIF requires a heterodimeric complex formation of the two subunits, which then translocates to the nucleus, binds to the hypoxia-response

**Table 1** Genes involved in oxidative stress, hypoxia, lipid, and one-carbon metabolism

Gene	Theory	Pathway	References
AMPA/kainate receptor	Oxidative stress	AMP kinase	8, 37, 82, 83
Apo A1	Oxidative stress	Inflammation, immune response,	23, 167, 169, 180
HDL	Inflammation	Lipid metabolism, cholesterol transfer	
CBP/p300	Hypoxia	Apoptosis NF-k $\beta$	47, 54
TNF $\alpha$	Inflammation	Apoptosis	57, 70
TNF $\beta$	Hypoxia oxidative stress	Cytokines	
INF $\gamma$	Inflammation	Inflammation	70, 167
INF $\beta$			
ICAM-1	Autoimmunity	Apoptosis,	40, 64
VCAM-1	Virtual hypoxia	Inflammation	
IL-1 $\alpha$	Inflammation	Cytokines	168–171
IL-2 $\alpha$	Virtual hypoxia	T cell survival	
IL-18	Oxidative Stress	Proliferation	
IL-6			
IL-1 $\beta$			
HIF	Hypoxia	Inflammation,	47, 53, 55–60, 71, 73, 74
HIF-1		Apoptosis	
HIF $\alpha$		PI3K/AKT	
HIF $\beta$			
NADPH oxidase	Oxidative stress	Apoptosis	62, 78, 149, 172, 180
NOS			
NMDA			
NF-k $\beta$	Hypoxia, oxidative stress	Apoptosis	40, 47,
	Autoantibodies	ERK	57–60, 107, 115, 149
	Metabolic disturbances		
HnRNP A1/B2	Autoantibodies	Autoimmune response	10, 11, 85,
NF-155,		Cell survival,	90–92, 94, 100, 106–108,
NF-186		Proliferation,	110–112, 117, 120, 122,
MOG		Apoptosis	123
MAG		Immune response	
PLP			
BHMT	Metabolic disturbances	One-carbon metabolism	23, 138, 144, 148, 151,
MAT			152, 157, 160, 173
GNMT			
CBS			
Methyl transferase			
Methionine synthase			
MTHFR			
PPAR $\alpha$	Oxidative stress,	Immune response	23, 180,
PPAR $\beta/\delta$	Hypoxia,	Lipid metabolism	182–201
PPAR $\gamma$	Lipid and amino acid metabolism	Cholesterol transfer	
		Apoptosis	
PLA2	Oxidative stress	Immune response	77, 78

**Abbreviations:** AKT, protein kinase B; AMP, adenosine monophosphate; AMPA, isoxazolepropionic acid; Apo A1, apolipoprotein A1; BHMT, betaine-homocysteine-S-methyl transferase; CBP, CREB-binding protein; CBS, cystathionine beta synthase; CREB, cyclic AMP response element binding protein; ERK, extracellular signal-regulated kinase; GNMT, glycine N-methyltransferase; HDL, high-density lipoprotein; HIF, hypoxia inducible factor; HnRNP, heterogeneous nuclear ribonuclear proteins; ICAM, intracellular adhesion molecule 1; IL, interleukin; INF, interferon; MAG, myelin-associated glycoprotein; MAT, methionine adenosyltransferase; MOG, myelin oligodendrocyte glycoprotein; MTHFR, methylenetetrahydrofolate reductase; NADPH, nicotinamide adenine dinucleotide phosphate; NF, neurofascin; NF-k $\beta$ , nuclear factor-kappa  $\beta$ ; NMDA, N-methyl-D-aspartate receptor; NOS, nitric oxide synthase; p300, E1A binding protein p300; PI3K, phosphoinositide 3'-kinase; PLA, phospholipase A; PLP, proteolipid protein; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; VCAM, vascular-cell adhesion molecule 1.

element, and associates with coactivators, such as CREB (Cyclic AMP response element binding protein)-binding protein (CBP)/E1A binding protein p300 (p300) (Table 1). The binding results in the activation or suppression of many genes involved in metabolism and cell survival. These changes include an increase in HIF-1 $\alpha$  protein levels, expression of HIF1-inducible genes in the MS brain,<sup>53</sup> blood vessel density, and endothelial cell proliferation.<sup>47,54</sup> HIF-1 $\alpha$  has been shown to play a proinflammatory role in cells of the myeloid lineage and anti-inflammatory role in intestinal epithelial cells.<sup>55,56</sup>

HIFs regulate cellular stress responses in tandem with nuclear factor-kappa  $\beta$  (NF- $\kappa$  $\beta$ ) to control hypoxic inflammation through activation of cytokine and hypoxia pathways.<sup>47</sup> In fact, there is a cross talk between these two transcription factors during hypoxic inflammation. HIF can be activated in response to multiple stimuli, such as bacterial lipopolysaccharide, microtubule disruption, interleukin (IL)-18 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), hepatocyte growth factor, and reactive oxygen species (ROS). The mechanism of HIF activation involves a NF- $\kappa$  $\beta$ -dependent upregulation of HIF-1 $\alpha$  messenger ribonucleic acid (mRNA) levels.<sup>57</sup> HIF-1 $\alpha$  contains an active binding site for NF- $\kappa$  $\beta$ , upstream of the transcription start position.<sup>58</sup> A recent study demonstrated that NF- $\kappa$  $\beta$  controls the HIF pathway in response to TNF- $\alpha$ .<sup>59</sup> While NF- $\kappa$  $\beta$  controls HIF-1 $\alpha$  expression levels, HIF-1 $\alpha$  can regulate NF- $\kappa$  $\beta$  signaling. Mice overexpressing HIF-1 $\alpha$  exhibited increase in pro-inflammatory NF- $\kappa$  $\beta$  targets.<sup>60</sup> As it appears from the evidence mentioned, hypoxia alters ATP potential and gene and protein expression, and may contribute to MS lesion formation.

## Oxidative stress

Oxidative stress (OS) resulting from the formation of ROS, secreted primarily by macrophages, is believed to play a role in the pathogenesis of MS. ROS are free radicals and related molecules that are defined as any chemical species that contain one or more unpaired electrons.<sup>24</sup> The most common ROS are hydroxyl radical (OH $\cdot$ ), superoxide radical (O $_2^{\cdot-}$ ), and nitric oxide (NO) as well as other molecules, such as hydrogen peroxide (H $_2$ O $_2$ ) and peroxynitrite (ONOO $^-$ ). Unpaired electrons cause ROS to act as electron acceptors, which results in the “stealing” of electrons by ROS (oxidation). ROS occur within a normal cell to a certain extent, and a number of mechanisms are in place to guard against ROS-induced damage; however, it appears that in patients with MS, the ROS exceed the capacity of the cellular defense mechanisms. ROS are known to cause damage to lipids, proteins, and deoxyribonucleic acid (DNA), leading to cellular death by necrosis and apoptosis

(Figure 1). Metals, such as iron, are normally stored within iron-binding proteins. However, injured cells release iron, which is then available to catalyze the free-radical reactions of ROS formation. Other sources of free radical production are the result of oxygen use in mitochondria and enzymatic pathways, such as xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lipoxygenases, and cyclooxygenase.<sup>24</sup> The “respiratory burst” system of activated microglia has also been shown to produce large quantities of ROS. In addition, reactive astrocytes have been shown to produce NO.<sup>24,61</sup>

The oxygen- and nitrogen-free radicals generated by macrophages have been shown to cause demyelination and axonal injury in experimental autoimmune encephalomyelitis (EAE) and MS.<sup>62,63</sup> Furthermore, free radicals activate transcription factors, resulting in the upregulation of the expression of many genes that are associated with MS, such as NF- $\kappa$  $\beta$ , TNF- $\alpha$ , intracellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1)<sup>40,64</sup> (Table 1). A study by Langemann et al revealed that MS plaques had increased free radical activity as well as decreased levels of important antioxidants, such as glutathione, alpha-tocopherol, and uric acid.<sup>65</sup> Further evidence has shown that oxidative damage to DNA in MS includes damage to mitochondrial DNA, implicating mitochondria not only in the formation of ROS but possibly as a pathway directly affected by OS.<sup>66</sup> Studies have also shown that nitric oxide synthase (NOS) is upregulated in inflammatory lesions<sup>62,67</sup> and that NO and its derivative peroxynitrite inhibit mitochondrial respiration.<sup>68</sup> NO is both essential for life and toxic. Its immunomodulatory effect helps sustain healthy homeostasis; however, large NO quantities damage axons.<sup>67</sup> Inflammation induces the production of NO. Excessive generation of NO is an indicator of aging and neurodegeneration. Increased NO concentration raises intracellular Ca $^{2+}$  and Na $^+$  levels and may be responsible for mitochondrial dysfunction.<sup>67,69</sup> The tissue damage in MS is caused, in part, by elevated levels of NO. In the CNS, NO is produced by macrophages and microglia following the induction of NOS by the proinflammatory cytokines TNF- $\alpha$  and interferon (INF)- $\gamma$ .<sup>70</sup> Notably, NO mediates the destabilization of HIF through increased ROS production.<sup>71,72</sup> Another oxidative agent, H $_2$ O $_2$ , has been shown to decrease the HIF-DNA binding capacity and the expression of its target genes.<sup>73,74</sup> Taken together, these studies suggest that the redox system plays an important role in HIF regulation.

NO can also react with the sulfhydryl groups of proteins, resulting in the S-nitrosylation of target proteins, which

initially can act as a protective mechanism in OS, to defend proteins from degradation. However, increased OS and the overaccumulation of NO results in irreversible cell damage caused by the oxidation of free thiols, nitration of tyrosine residues, and lipid peroxidation.<sup>75</sup> Increased S-nitrosylation has been detected in the normal-appearing white matter of MS patients' brain compared with that of normal controls, indicating that nitrosative damage is involved in the pathophysiology of MS.<sup>76</sup>

In addition to ROS, glutamate appears to be another major source of OS in the brain, through the activation of ionotropic glutamate receptors. It is possible that damage induced by free radicals can occur via the stimulation of phospholipase A2 and the release of amino acids, which in the presence of free radicals, results in an enhanced release of glutamate.<sup>77,78</sup> The cerebrospinal fluid (CSF) of MS patients has elevated levels of glutamate.<sup>79,80</sup> Increased glutamate, via an interaction with alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, has also shown to be deleterious to oligodendrocytes (which appear to be highly vulnerable to glutamate excitotoxicity).<sup>81,82</sup> Interestingly, AMPA/kainate receptors are known to have increased permeability to  $\text{Ca}^{2+}$ , resulting in  $\text{Ca}^{2+}$ -loading by cells.<sup>83</sup> As the OS theory stands, it appears that ROS in the presence of a weakened antioxidant cellular defense results in the damage of cellular components, such as lipids, proteins, nucleic acids, and mitochondrial DNA. These damaged components alter multiple pathways associated with ATP production, upregulation of the genes associated with MS pathology, and increase in glutamate levels (via AMPA/kainate receptors).

## Antibodies in neurodegeneration

The hypothesis of molecular mimicry explains the pathogenesis of MS as an autoimmune response to an environmental agent.<sup>84–89</sup> The antibodies resulting from molecular mimicry have profound effects on neurons and implicate molecular mimicry as a contributor to neurodegeneration and the pathogenesis of neurological disease<sup>90–96</sup> (Figure 1). The antibodies present in MS patients can be categorized into two major groups: myelin and nonmyelin antibodies. Both types of antibodies have sufficient evidence to support their involvement in the pathogenesis of MS<sup>11</sup> (Figure 1). The antimyelin antibody targets include myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and proteolipid protein (PLP).<sup>97–100</sup> These antibodies have been found in the sera and CSF of MS patients; however, the exact role of such myelin antigens in MS remains contradictory. One study

demonstrated that the development of clinically definite MS could be predicted based on the presence of anti-MOG and anti-MBP antibodies in patients' sera;<sup>101</sup> others found no association between anti-MOG and anti-MBP antibodies and MS progression.<sup>102,103</sup> The immunopathogenic effects of antimyelin antibodies might be epitope-specific<sup>98</sup> or depend on the antibody confirmation.<sup>104–106</sup> These earlier studies did not specify the cellular pathways affected by autoantibodies. In a recent study, Ho et al showed that MAG autoantibodies targeted the following natural brain lipids: 1-palmitoyl-2-glutarol-*sn*-glycero-3-phosphocholine (PGPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-L-serine), (POPS), 1-hexadecyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (azPC), and 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (azPC ester).<sup>107</sup> Moreover, the authors showed that POPS, PGPC, azPC, and azPC ester affected inflammatory, survival, and apoptotic signaling pathways – specifically, canonical NF- $\kappa$ B and extracellular signal-regulated kinase (ERK) pathways were activated, in stimulated T-cells isolated from EAE mice. Overall, their data suggest that myelin phospholipids are targeted by autoimmune responses in MS.

A growing number of studies point to the fact that antibody-mediated axonal injury could be initiated by antibodies to nonmyelin antigens. Nonmyelin antibodies present in MS patients have been found to target neuronal surface molecules (axolemma-enriched fractions, neurofascin, and gangliosides), cytoskeletal proteins (neurofilaments [NFs] and NF light chains [NF-Ls]), intracellular enzymes, signaling molecules and chaperones ( $\beta$ -arrestin, retinal arrestin, heat shock proteins, glutamate decarboxylase, and proteasomes), and nuclear antigens (nuclear ribonuclear proteins).<sup>108</sup> These antibodies present a different mechanism of immune-mediated axonal injury. For example, antineurofascin-186 and antineurofascin-155 antibodies were shown to cause an exacerbation of EAE without demyelination, in the spinal cord of rats. When antineurofascin antibodies were cotransfected with MOG-specific T-cells, they selectively targeted the nodes of Ranvier and inhibited neurotransmission in an MS animal model.<sup>109</sup> In other work, chronic progressive MS patients had significantly higher levels of NF-L-specific antibodies in their sera compared with that in patients with other neurological diseases.<sup>110,111</sup> How the anti-NF and anti-NF-L antibodies arise and their specific effects on the axon are unclear, but their correlation with disease progression appears applicable, in a biomarker-specific manner.<sup>109–111</sup>

Antibodies to heterogeneous nuclear ribonuclear proteins (hnRNP) A1 and B2 were present in the CSF of MS and



human T-lymphotropic virus 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) patients but not in normal controls.<sup>10,85,90–92,94,112</sup> These RNA-binding proteins play a major role in the adjustment of pre-mRNA splicing, through various factors.<sup>113</sup> They also participate in mRNA stability,<sup>114</sup> NF- $\kappa$ B-dependent transcription,<sup>115</sup> and telomerase activity.<sup>116</sup> HnRNP A1 plays several key roles in neuronal functioning, and its depletion, either due to debilitated cholinergic neurotransmission<sup>117</sup> or due to autoimmune reactions, causes drastic changes in RNA metabolism.<sup>10</sup> Recently, RNA-binding proteins have gained attention because a large number of these proteins were mutated in neurodegenerative diseases.<sup>118,119</sup> RNA-binding proteins use protein aggregation as part of a normal regulated, physiological mechanism that controls protein synthesis. The process of regulated protein aggregation is most evident in the formation of stress granules.<sup>120</sup> HnRNP A1 has been shown to relocate into cytoplasmic stress granules in the presence of stress stimuli, such as osmotic shock or OS.<sup>121</sup> Recent studies showed that the addition of antibodies to hnRNP A1 affected its distribution, from a primarily nuclear location to a mixed nuclear/cytoplasmic distribution.<sup>10,122,123</sup> It was known that anti-hnRNP A1 antibodies decreased neuronal firing in vitro, but it was not clear whether the antibodies to this intracellular protein could penetrate neurons and find its target.<sup>92</sup> Recent studies have revealed that anti-hnRNP A1 antibodies penetrate neuronal cells via clathrin-mediated endocytosis and cause deleterious effects.<sup>10,92,122,123</sup> Anti-hnRNP A1 antibodies were also shown to increase apoptosis, reduce ATP levels, and cause the redistribution of endogenous hnRNP A1 protein. Thus, anti-hnRNP A1 antibodies altered endogenous protein localization as well as inhibited normal cellular processes in vitro.

In MS patients, the presence of the two types of auto-antibodies may not be mutually exclusive. It is possible that antibodies to myelin antigens may have an impact on the early, relapsing stages of disease, while the nonmyelin antigens play a more dominant role in the progressive stages of MS. More importantly, both types of antibodies may cause neurodegeneration through the activation of apoptotic inflammatory cytokines and immune response pathways.

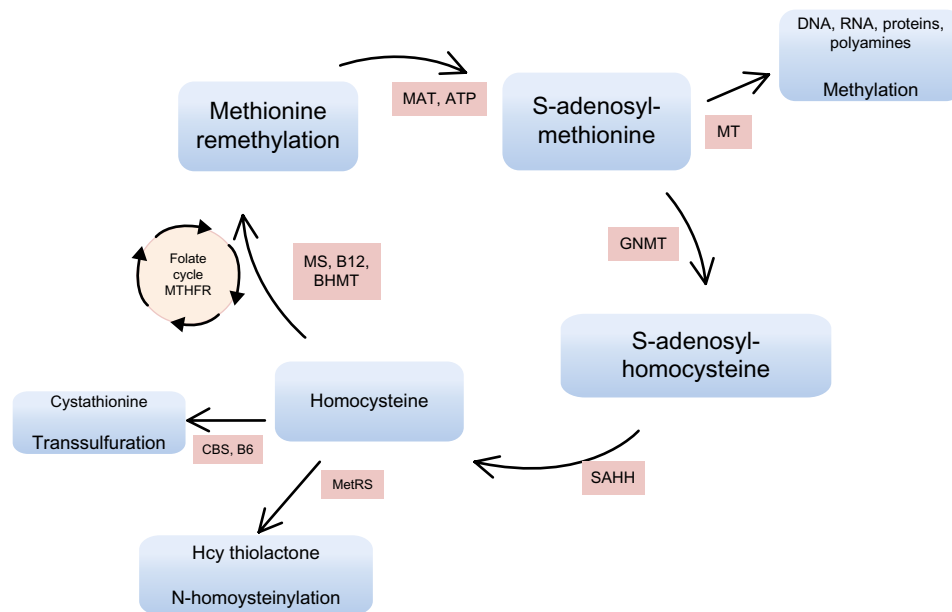
## Role of homocysteine in neurodegeneration

Axonal loss is a key contributor to disability in neurodegenerative diseases. In MS, many studies have suggested that axonal damage is a consequence of demyelination triggered by inflammation.<sup>124–127</sup> However, substantial axonal loss has also been

detected at the early stages of MS, and several studies suggest that axonal loss is independent of demyelination.<sup>128–130</sup> Exactly how CNS damage develops is unclear, but it is unlikely to be a direct result of viral infection.<sup>131</sup> Rather a complicated mechanism, involving innate immunity, genetic predisposition, and environmental agents, is at play. The penetration of blood-borne neurotoxins through the compromised blood–brain barrier might play a significant role in axonal degeneration in MS.<sup>132,133</sup> In addition, microglia cells can produce neurotoxins endogenously and seem to play an important role in neurodegeneration, by acting as an accelerator of neurotoxicity.<sup>134</sup> One of the neurotoxins is homocysteine (Hcy), a sulfur molecule produced from amino acid methionine. Hcy promotes the activation and proliferation of microglia.<sup>135</sup> Hcy is a major contributor to oxidative injury and DNA damage<sup>136</sup> (Figure 1). Elevated Hcy levels are toxic to neurons and might compromise the blood–brain barrier (a hallmark of MS pathology).<sup>137–142</sup> MS patients have been shown to have elevated Hcy levels, which were associated with cognitive decline.<sup>143–147</sup> Interestingly, a recent study from our laboratory revealed that patients with primary and secondary progressive MS had significantly higher Hcy levels in their plasma compared with relapsing remitting stage patients and controls.<sup>148</sup> Hcy can be elevated in biological fluids as a result of genetic or metabolic disturbances. Elevated Hcy levels induce adverse effects either directly, through lipid metabolism, or indirectly, via oxidative and endoplasmic reticulum stress (Figure 1). In addition, OS might stimulate the accumulation of Hcy because ROS impair the Hcy conversion to methionine. Hcy modulates substrate levels for various catalytic processes and regulates the expression of genes involved in complex diseases through the activation of NF- $\kappa$ B.<sup>149,150</sup> Sharma et al used a literature mining approach to identify the genes and related pathways affected by Hcy. They identified 112 genes modulated by Hcy levels and 23 genes that affected Hcy.<sup>149</sup> Not surprisingly, many of these genes were involved in hypoxia, apoptosis, ROS, inflammation, and lipid metabolism. According to their study, a common link between apoptosis and the inflammatory pathways was endoplasmic reticulum stress, which is closely related to OS. Hcy may induce OS and apoptosis through NADPH oxidase or through the activation of c-Jun N-terminal kinases (JNKs). Hcy is one of the metabolites in the one-carbon cycle (Figure 2), which plays an important role in disorders of the nervous system.

## Importance of the one-carbon metabolism in MS

The one-carbon metabolic pathway plays an important role in many biological processes and clinical symptoms, such



**Figure 2** Diagram of the one-carbon cycle.

**Notes:** The major metabolites are presented in the blue frames (methionine, S-adenosylmethionine, S-adenosylhomocysteine, homocysteine); enzymes and cofactors are highlighted in the pink rectangles.

**Abbreviations:** ATP, adenosine triphosphate; B6, vitamin B6; B12, vitamin B12; BHMT, betaine-homocysteine-S-methyl transferase; CBS, cystathionine beta synthase; DNA, deoxyribonucleic acid; GNMT, glycine N-methyltransferase; Hcy, homocysteine; MAT, methionine adenosyltransferase; MetRS, methionine-tRNA ligase; MS, methionine synthase; MT, methyl transferase; MTHFR, methylenetetrahydrofolate reductase; RNA, ribonucleic acid; SAHH, S-adenosylhomocysteine hydrolase.

as hypomethylation, homocysteinemia, liver dysfunction, and the accumulation of white-matter hyperintensities in the human brain.<sup>138,148</sup> In addition to Hcy, the one-carbon cycle contains other important molecules, such as S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and methionine (Figure 2). These metabolites are synthesized within the cycle, through a cascade of biochemical reactions involving vitamins, enzymes, and cofactors. For example, the essential amino acid methionine is converted to SAM in the presence of the enzyme methionine adenosyltransferase, ATP, and magnesium.<sup>151,152</sup> SAM is further metabolized into SAH, and in the presence of SAH hydrolase, SAH is converted to Hcy. Hcy can be remethylated or recycled back to methionine, in the presence of methionine synthase, vitamins B12, and folate; formed into cystathionine, in the presence of cystathionine beta synthase and vitamin B6; or transformed into Hcy thiolactone by methionyl-tRNA synthetase enzyme (Figure 2). The simultaneous measurements of major metabolites of the one-carbon cycle in MS patients uncovered aberrations in the Hcy conversion back to methionine, and the formation of SAM and SAH.<sup>148</sup>

MS patients often have low levels of vitamins B12 (cobalamin), B6 (pyridoxine), and B9 (folate).<sup>153–157</sup> A severe vitamin B12 deficiency can cause a breakdown of the myelin sheath.<sup>158</sup> B12 deficiency associated either with

poor nutrition, defects in absorption, or disease progression results in neuronal demyelination. The early studies on B12 status in MS patients produced conflicting results; however, as improved techniques became available, the consensus was reached that MS patients have lower levels of B12 in comparison with controls.<sup>138,153,156,157,159</sup> In addition, MS patients also have reduced B6 levels in their plasma.<sup>148</sup> Vitamin B6 plays a significant role in normal brain development and function, the formation of myelin, and the production of several neurotransmitters (such as serotonin and norepinephrine). A key interaction between vitamin B12 and folate in the one-carbon cycle occurs during the synthesis of methionine from Hcy by methionine synthase, in which both 5-methyltetrahydrofolate (enzyme of the folate cycle) and methyl-vitamin B12 are cofactors. The folate cycle is essential for many genomic and nongenomic methylation reactions via SAM and indirectly, for the synthesis of purines and thymidine, and therefore, of nucleotides, DNA, and RNA.<sup>160</sup> Methylation reactions of DNA and myelin, via SAM, are vitally important in the CNS. Folic acid (B9) deficiency also reduces the activity of methionine synthase.<sup>158</sup> The CNS lacks the alternate betaine pathway of homocysteine remethylation; therefore, if methionine synthase is inactivated, the CNS has greatly reduced methylation capacity.<sup>161</sup> Deprivation of folate and B12 increases neurodegeneration, through the activation of ROS and

apoptosis, and increase in cytosolic calcium and intracellular Hcy.<sup>162</sup> In summary, the one-carbon metabolism has profound effects on the CNS, where it plays a central role in DNA synthesis, methylation, gene regulation, synaptic function, and neurotransmission.

## Disturbances in lipid metabolism

The human brain has a high lipid content; therefore lipids and their turnover should be considered as good candidate contributors to diseases of the CNS. However, the importance of lipid metabolism in MS has been understudied, mainly due to the central focus on the immune system. Disturbances in lipid metabolism lead to myelin loss, neuronal degeneration, and metabolic distress (Figure 1). Myelin glycolipids received some attention because of their role in autoimmune-mediated demyelination. Apart from the myelin autoantibodies, there is now some evidence for a potential role of cholesterol and lipids in MS. Among the three major pathways of lipid metabolism (exogenous, endogenous, and reverse cholesterol), the cholesterol efflux from peripheral macrophages by microglia deserves special attention. Microglial cells regulate lipid homeostasis in the CNS by maintaining a careful balance between phagocytic and cytotoxic macrophages. A distorted lipid homeostasis results in an imbalance between the cytotoxic and phagocytic microglia.<sup>23</sup> In a comprehensive review of MS, Corthals described the disease as a dysfunction of the metabolism of lipids.<sup>23</sup> The author explained that the immune system relies on lipids for repair and for prevention of inflammation. Therefore, a disequilibrium in lipid metabolism causes deregulation of the immune system. Lipids, and especially oxidized lipoproteins, are the core agents of the immune response during acute inflammation. The oxidation of lipids could increase in hypoxic conditions and cause distortions in lipid metabolism. The importance of controlling dyslipidemia in MS patients has been recently emphasized in several studies.<sup>163–166</sup> Dyslipidemia was linked to an increased risk for disability progression in a study that analyzed 8,993 MS patients.<sup>163</sup> Lipid profiles were shown to be associated with magnetic resonance imaging (MRI) outcomes as well as lesion formation in IFN- $\beta$ -treated patients after the first demyelinating event.<sup>164–166</sup> Patients treated with intramuscular IFN- $\beta$  showed an association between higher serum low-density lipoprotein (LDL) cholesterol and total cholesterol, with an increased risk for developing new lesions on T2 weighted scans.<sup>165</sup> Interestingly, a different study showed that patients who had high levels of apolipoprotein A1 (ApoA1) adapted better to IFN- $\beta$  therapy.<sup>167</sup> ApoA1 is a

major component of high-density lipoprotein (HDL). ApoA1 inhibits contact-mediated activation of monocytes by binding to stimulated T-cells, thereby inhibiting TNF- $\alpha$  and IL-1 $\beta$  production.<sup>168,169</sup> Others also found that an increased total cholesterol was associated with increases in the number of contrast-enhancing lesions in clinically isolated syndrome following the first clinical event.<sup>164</sup> The results from these studies suggest the importance of controlling dyslipidemia in MS. Cholesterol-lowering drugs, such as statins, are used to lower cholesterol in humans; therefore it was logical to evaluate these therapies in MS. Animal studies showed that statins inhibited the production of NOS, TNF- $\alpha$ , and IL-6, and lowered disease scores.<sup>170,171</sup> However, such therapeutic approach in humans resulted in controversial findings.<sup>172–176</sup> A pilot study using 80 mg simvastatin reported a reduction in the number and volume of gadolinium-enhancing lesions.<sup>176</sup> The next double-blinded clinical trial with atorvastatin (40 or 80 mg) as an add-on to IFN- $\beta$  treatment showed that patients on statins had either new T2 lesions or more clinical relapses.<sup>177</sup> In 2012, a new randomized clinical trial showed a benefit of simvastatin use in secondary progressive MS patients.<sup>172</sup> Simvastatin reduced brain atrophy by 43% and improved clinical outcomes over the 2-year study period. Overall, statins are well-tolerated and widely used drugs that lower LDL, increase HDL, and reduce inflammation. However, these drugs have been shown to increase ROS generation and suppress the activation of the protein kinase B (PKB)/AKT and extracellular signal-regulated kinase (ERK) pathways, elevate lipid peroxidation, and induce oxidative DNA damage, in human peripheral blood lymphocytes.<sup>178,179</sup> Increased lipid peroxidation has been shown to be associated with disease exacerbation periods and lesion pathogenesis in MS patients.<sup>180</sup> Statins block the hepatic enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase responsible for the production of cholesterol in the body. The inhibition of this enzyme also affects the pathways responsible for leukocyte migration and activation, thus providing a beneficial outcome in autoimmune inflammation. Animal studies have shown that Hcy inhibits simvastatin-induced ApoA1 upregulation,<sup>181</sup> thus suggesting a link between statins and Hcy metabolism. However, human studies did not provide detailed ApoA1 and Hcy measurements in addition to the MS clinical outcomes in statin trials. At present, it is not entirely clear which statin drug, at what dose, and at which stage of the disease will provide the most benefit to MS patients. Therefore, future research is needed to uncover the protective and/or pathological effects of statins in MS.



## Peroxisome proliferator-activated receptors in MS

Perturbations in lipid metabolism negatively affect myelin.<sup>182,183</sup> Therefore, special consideration should be given to the factors that control lipid turnover in health and disease. Lipid metabolism is regulated by peroxisomes and the peroxisome proliferator-activated receptors (PPARs).<sup>23,184–186</sup> Remarkably, PPARs also control inflammation.<sup>184</sup> Peroxisomes are responsible for oxygen metabolism, and  $\alpha$ - and  $\beta$ -oxidation reactions.<sup>183</sup> PPARs regulate the function and the number of peroxisomes within the cell as well as numerous biological pathways associated with MS (Figure 3). PPARs form heterodimers with the retinoid X-receptor (RXR). These heterocomplexes regulate the inflammatory response and cytotoxic cell apoptosis, myelin synthesis, neuronal cell proliferation and differentiation, energy and lipid homeostasis, and reactive oxygen species.<sup>187–190</sup> There are three subtypes of PPARs: PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ . PPAR $\alpha$  and PPAR $\gamma$  are present in the lipid core of atherosclerotic lesions, macrophages, foam cells, and smooth muscle cells.<sup>188,190</sup> PPAR $\alpha$  is one of the several factors that regulate the expression of HDL and ApoA1.<sup>191</sup> PPAR $\alpha$  is involved in the acetylcholine metabolism, neurotransmission, and OS defense.<sup>192,193</sup> PPAR $\beta/\delta$  is involved in the control of brain lipid metabolism, epidermal cell proliferation, fatty acid adipogenesis, and preadipocyte proliferation.<sup>194</sup> All PPAR subtypes have been described in the adult and developing brain and in the spinal cord. Several publications have suggested that PPAR activation might directly affect the viability and differentiation of neuronal cells.<sup>195–199</sup> Remarkably, the expression of PPAR $\gamma$

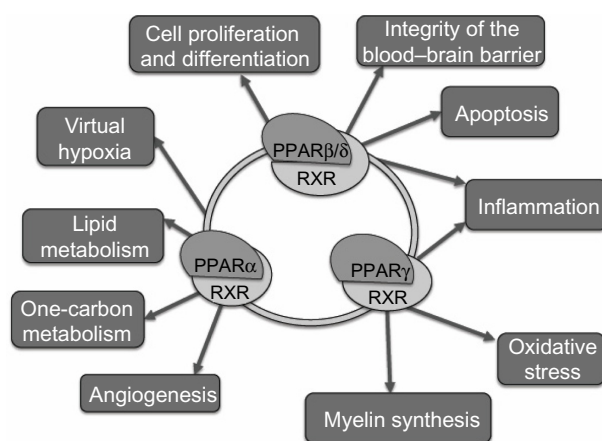
in the brain has been studied in relation to inflammation and neurodegeneration.<sup>195</sup>

For example, PPAR $\gamma$  was found to be increased in microglia and astrocytes during EAE.<sup>200</sup> This knowledge prompted an array of studies utilizing PPAR ligands to modulate the course of the disease in animals.<sup>198,200–202</sup> These studies have shown that PPAR activation reduced leukocyte infiltration into the brain parenchyma and decreased inflammation and axonal demyelination.

Use of the PPAR $\gamma$  antagonist GW347845 in peripheral blood mononuclear cells (PBMCs) from MS patients has been shown to result in suppressed T-cell proliferation and reduced secretion of TNF- $\alpha$  and INF- $\gamma$ .<sup>194</sup> However, these antiproliferative effects were accompanied by reduced cell viability and induced apoptosis in activated lymphocytes. Preincubation of PBMCs with pioglitazone was shown to increase the DNA-binding activity of PPAR $\gamma$  and decrease NF- $\kappa$ B DNA-binding activity, in the absence of an acute MS relapse.<sup>194</sup> Interestingly, Hcy is known to downregulate PPAR $\alpha$  expression by competing with its ligands (Figure 4).<sup>48,149,181,191</sup> These results underscore a cross talk between the two types of transcription factors NF- $\kappa$ B and PPARs in the regulation of the immune response. The Hcy downregulation of PPAR $\alpha$  suggests that PPAR activation could benefit patients with normal Hcy levels.

## Discussion: common pathways of destruction

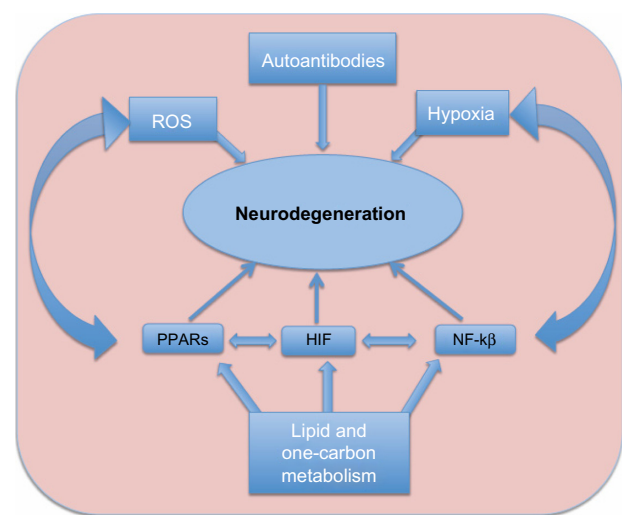
Hypoxia, OS, ROS, autoantibodies, and disturbances in lipid and one-carbon metabolism affect the health of neurons.



**Figure 3** PPARs regulation of cellular processes.

**Note:** PPARs form complexes with RXR to control multiple cellular processes.

**Abbreviations:** PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X-receptor.



**Figure 4** Common pathways of neurodegeneration.

**Note:** Metabolic disturbances affect major regulators of cellular processes.

**Abbreviations:** HIF, hypoxia inducible factor; NF- $\kappa$ B, nuclear factor kappa  $\beta$ ; PPARs, peroxisome proliferator-activated receptors; ROS, reactive oxygen species.

Hypoxia seems to be secondary to demyelination. Oxidative stress could be caused either by external factors (such as viruses, bacteria, and other environmental agents) or by internal toxins that have accumulated inside neurons due to impaired metabolic processes. Excess ROS generated by macrophages or microglia can lead to inflammation, demyelination, and neuronal degradation (Figure 4). Antimyelin and other autoantibodies could cause significant damage to neurons and activate other destructive pathways.

It is not clear which of the factors precipitate the first signs of neuronal demise. However, from the evidence at hand, it appears that each theory of neuronal degeneration and related pathway overlaps the next (Figures 1 and 4). Autoimmunity (caused by the presence of myelin and nonmyelin autoantibodies), metabolic deregulation in one-carbon and lipid metabolism, hypoxia and OS precipitated by inflammation, ROS, and cytokines can all result in neuronal degradation. Taken together, the studies suggest that all of these processes play important roles in neurodegeneration (Figure 4). Neurotoxins, such as Hcy, released in the vicinity of the CNS promote neuronal injury by inducing the cytokine or OS pathways. OS triggers lipid peroxidation, which in turn, negatively affects myelin. The reactive oxygen and nitrogen species released by invading inflammatory cells can cause demyelination and axonal destruction. Oxygen radicals cause damage by reacting with cellular lipids, proteins, carbohydrates, and DNA.

Impaired one-carbon metabolism adversely affects myelination, DNA methylation, and amino acid and protein conversion reactions, and can trigger inflammation through increased Hcy levels. Compromised Hcy conversion to methionine or cysteine might be a crucial factor responsible for the activation of transcription factors and stimulation of ROS formation (Figure 4). Very few studies have addressed the importance of lipid metabolism in MS; however, the current knowledge points to a possible missing link between the simple lowering of cholesterol and a reduced lesion load. Statins reduce inflammation and lower LDL cholesterol through the inhibition of HMG-CoA reductase. At the same time, statins induce the formation of NO through the induction of endothelial NOS. Therefore, a deeper understanding of the cross talk between inflammation, OS, and cholesterol transport could lead to novel therapeutic strategies.

Neurons have another mechanism of response to stress – through upregulation of transcription factors, such as PPARs. These transcription factors are involved in a plethora of vital cellular processes (Figure 3). PPARs seem to be a common link between ROS, hypoxia, and apoptosis. They are also

involved in the modulation of immune response and lipid metabolism. However, the activation of these transcription factors is deeply influenced by the cellular environment. For example, the presence of large quantities of Hcy and other toxins might result in PPAR inhibition. Hcy appears to play an important role in OS, lipid and one-carbon metabolism, and the regulation of NF- $\kappa$ B and PPARs (Figure 4). The cofactors affecting the one-carbon cycle metabolites, such as vitamins B6, B12, and folate, should be evaluated in MS patients. PPARs-activation agents are less likely to work in MS patients with high Hcy because of the ongoing production of Hcy in the one-carbon cycle. Hcy levels could be lowered with the increased consumption of vitamins B6 and B12. Therefore, future studies designed to combine PPAR-activation with homocysteine- and cholesterol-lowering strategies could lead to novel therapeutic approaches.

## Conclusion

MS is a complex disease, and most progressive MS patients develop a common final pathway of neurodegeneration. The molecules responsible for neurodegeneration remain an ongoing area of investigation. Neurons are very susceptible to OS, hypoxia, autoantibodies, and metabolic disturbances. This review highlighted several targets, mechanisms, and pathways that play important roles in neuronal degeneration. Because of the variability of MS, more than one pathway may contribute to neurodegeneration, and thus, targeted interventions designed to normalize these cellular processes could help delay neuronal degeneration and improve clinical outcomes in MS patients.

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## Disclosure

The authors report no conflicts of interest in this work.

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