



The Two Sides of Siponimod: Evidence for Brain and Immune Mechanisms in Multiple Sclerosis

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Abstract

Siponimod is a selective sphingosine 1-phosphate receptor subtype 1 (SIP₁) and 5 (SIP₅) modulator approved in the United States and the European Union as an oral treatment for adults with relapsing forms of multiple sclerosis (RMS), including active secondary progressive multiple sclerosis (SPMS). Preclinical and clinical studies provide support for a dual mechanism of action of siponimod, targeting peripherally mediated inflammation and exerting direct central effects. As an SIP₁ receptor modulator, siponimod reduces lymphocyte egress from lymph nodes, thus inhibiting their migration from the periphery to the central nervous system. As a result of its peripheral immunomodulatory effects, siponimod reduces both magnetic resonance imaging (MRI) lesion (gadolinium-enhancing and new/enlarging T2 hyperintense) and relapse activity compared with placebo. Independent of these effects, siponimod can penetrate the blood–brain barrier and, by binding to SIP₁ and SIP₅ receptors on a variety of brain cells, including astrocytes, oligodendrocytes, neurons, and microglia, exert effects to modulate neural inflammation and neurodegeneration. Clinical data in patients with SPMS have shown that, compared with placebo, siponimod treatment is associated with reductions in levels of neurofilament light chain (a marker of neuroaxonal damage) and thalamic and cortical gray matter atrophy, with smaller reductions in MRI magnetization transfer ratio and reduced confirmed disability progression. This review examines the preclinical and clinical data supporting the dual mechanism of action of siponimod in RMS.

1 Introduction

In patients with multiple sclerosis (MS), lymphocytes from peripheral lymphoid organs cross the blood–brain barrier (BBB) and enter the central nervous system (CNS) to attack oligodendrocytes and other brain cell types, leading

Key Points

Relapsing forms of multiple sclerosis (RMS) include relapsing–remitting multiple sclerosis, defined as disease relapses with full or partial recovery and mainly driven by peripherally mediated inflammation, and secondary progressive multiple sclerosis, defined as a gradual worsening of disability independent of relapses and driven by chronic central nervous system (CNS) inflammation.

Siponimod, an approved oral treatment for adults with RMS, exerts its effects by binding to sphingosine 1-phosphate subtype 1 and 5 receptors (SIP₁, SIP₅), located peripherally (outside) and/or within the CNS.

Studies have demonstrated that siponimod has a dual mechanism of action by inhibiting lymphocyte migration from the periphery to the CNS and subsequent inflammation, and by having direct effects on neural cells after crossing the blood–brain barrier, resulting in regeneration and neuroprotection, and potential beneficial effects on myelination.

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to localized inflammation, demyelination, astrogliosis, and, ultimately, axonal loss [1–4]. The mechanism of neuroaxonal injury in MS is believed to be largely inflammation driven, but neuroaxonal degeneration may also occur independently of inflammation [5]. Cortical demyelination and diffuse neurodegenerative damage of gray and normal-appearing white matter, which are typically found in patients with progressive forms of MS, may not solely occur as a direct result of interaction with inflammatory T and B cells, but in addition may be attributable to the effects of toxic cytokines released by inflammatory cells, acting either directly or indirectly through activation of microglia and macrophages [3]. Moreover, uptake of iron, probably released during the process of demyelination, by pro-inflammatory macrophages and microglia, may augment their pro-inflammatory status, and in the context of chronic active MS lesions, may contribute to tissue damage, disease severity or progression [6].

Disease progression in MS may be a process that reflects the mounting residual effects of clinical relapses and of clinically silent lesions that may contribute to disability as functional reserve declines [7]. It is hypothesized that, as MS progresses, CNS repair mechanisms are exhausted, resulting in progressive irreversible neurodegeneration [3, 4, 8], in addition to the contributory aging factors such as immunosenescence [9, 10]. Protection against neurodegenerative effects is a key aim of MS drug development [11]. The measure most commonly used to document physical disability progression in MS clinical trials is the Expanded Disability Status Scale (EDSS) score [12]. The score captures changes in one or more functional systems (pyramidal; cerebellar; brain stem; sensory; bowel and bladder; visual; mood and cognition). However, the EDSS emphasizes motor changes affecting physical disability and is heavily weighted by ambulation status, rather than recognizing equally important signs and symptoms associated with MS progression, such as increasing fatigue and cognitive decline, which are only cursorily addressed in one subcomponent of the EDSS [13–16].

1.1 Siponimod

Siponimod (Mayzent[®]) is a selective sphingosine 1-phosphate (S1P) receptor modulator. In the USA, siponimod is approved as an oral disease-modifying therapy (DMT) for the treatment of adults with relapsing forms of MS (RMS), including clinically isolated syndrome, relapsing–remitting MS (RRMS), and active (evidence of relapse in the previous 2 years) secondary progressive MS (SPMS) [17]. In the European Union (EU), siponimod is approved for the treatment of active SPMS evidenced by relapses or imaging features of inflammatory activity [18] and for SPMS in other countries, such as Australia and Japan

[19, 20]. S1P is a lysophospholipid with a broad range of extracellular signaling functions; it is found throughout the body, exerting major effects on the immune and cardiovascular systems, the BBB, and the CNS [21]. Most bioactivity of S1P is mediated via five G protein-coupled receptor subtypes (S1P_{1–5}) [22–24], which are members of the lysophospholipid receptor family and are expressed both broadly and differentially across many cell types [22, 24–26].

Fingolimod was the first S1P receptor modulator to be approved in MS, followed by siponimod, ozanimod, and, more recently, ponesimod [17, 25, 27–30]. Most of these molecules bind to S1P₁, but their high affinity and specificities for other S1P receptor subtypes vary (e.g., fingolimod, S1P_{1, 3–5}; siponimod and ozanimod, S1P_{1,5}; ponesimod, S1P₁) [25]. Siponimod is both an agonist of S1P₁ and, with continued exposure, is a functional antagonist, causing persistent ligand-induced internalization and deactivation of the receptor; it appears to be primarily an agonist of S1P₅ [25]. Outside the CNS, functional antagonism of S1P₁ renders lymphocytes unresponsive to S1P-induced egress from lymph nodes and secondary lymphoid organs [23, 31]. The resultant reductions in circulating central memory and naive B and T lymphocytes in the blood are thought to limit the peripherally mediated CNS inflammation that is characteristic of RMS [8].

In addition to peripheral effects mediated by S1P₁, siponimod can exert direct CNS effects mediated by both S1P₁ and S1P₅. Any drug in the S1P receptor modulator class has the potential to exert CNS effects, but there are differences in how readily their active forms can reach the CNS. This is influenced by factors such as lipophilicity, elimination half-life, and transport mechanisms. Once across the BBB, their metabolism within the CNS also becomes a factor [25]. For example, fingolimod must be phosphorylated for activity, but the polarity of the phosphorylated form likely impedes its ability to cross the BBB. The unphosphorylated pro-drug form can cross the BBB readily, but generally lower levels of kinase activity in the CNS than the periphery may limit the rate at which the active form is produced in the CNS compartment [25]; however, individual cell types such as astrocytes enable phosphorylation and receptor activation [32]. Evidence from preclinical and clinical studies that are summarized in this review supports a dual mechanism of action for siponimod that involves (1) suppression of peripherally mediated inflammatory autoimmune processes within the CNS that are characteristic of MS; and (2) neuroprotection through inhibition of astrogliosis, promotion of oligodendroglial precursor cell (OPC) maturation, oligodendroglial cell survival, and remyelination. Thus, siponimod is proposed to act systemically by inhibiting lymphocyte egress from lymph nodes and subsequent infiltration into the CNS

[23, 33] and centrally after crossing the BBB, to exert protective and regenerative effects, as well as targeting compartmentalized inflammation typical of progressive MS [2, 34, 35] (Fig. 1).

The aim of this review is to summarize the preclinical and clinical data supporting the dual mechanism of action of siponimod in RMS.

2 Immunomodulatory Effects of Siponimod in MS: Peripherally Mediated Effects on CNS Pathogenesis

2.1 The Role of S1P and its Receptors in Lymphocyte Trafficking and Function

Siponimod indirectly influences CNS pathogenesis in MS through differential effects on lymphocyte trafficking and function.

S1P₁ and S1P₄ are the most highly expressed S1P receptor subtypes on lymphocytes [36]. When S1P binds to these receptors, they are activated and internalized, then the receptors are recycled back to the cell surface [37, 38]. Lymphocyte egress from the thymus and peripheral lymphoid organs is dependent on the binding of S1P to S1P₁. S1P₁-deficient mice have extremely low levels of peripheral T cells and reduced numbers of B cells in both the lymph and peripheral blood compared with control mice [31], indicating that these cells remain trapped in the thymus or the peripheral lymph

nodes. Mouse strains that lack detectable circulating S1P have significantly more natural killer (NK) cells in peripheral lymph nodes and far fewer in the lymph compared with wild-type mice [32], indicating that these cells also remain trapped in the thymus or peripheral lymph nodes. Lymphocytes in wild-type thymus and lymph nodes express high levels of S1P₁ on their surface, indicating that extracellular S1P is normally very low in these tissues [37] and that S1P levels are key to the lymphocyte egress. Despite the low expression of S1P₅ in the periphery relative to the CNS, a significant accumulation of NK cells was observed in the lymph nodes of S1P₅-deficient mice, with reduced numbers in the lymph. This indicates that S1P₅ is important for NK-cell trafficking [32]. In addition to slowing T cell egress, interaction of S1P with S1P₁ influences T cell differentiation [39]. S1P increases T helper (T_h)17 polarization via the interleukin (IL)-6-driven, Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) pathway, which is common in central memory cells and blocks the differentiation of regulatory T cell precursors. An unrestrained autoimmune T_h17 response is a hallmark of MS; therefore, this implicates S1P in T_h17-related MS pathology.

Plasmacytoid dendritic cells (pDCs) modulate the severity of the CNS immune response, but their numbers are reduced in the peripheral blood of patients with MS [40]. Depletion or peripheral sequestration of pDCs increases the severity of murine experimental autoimmune encephalomyelitis (EAE) and is associated with increased levels of T_h1 and T_h17 CD4+ lymphocytes in the CNS [41, 42]. However,

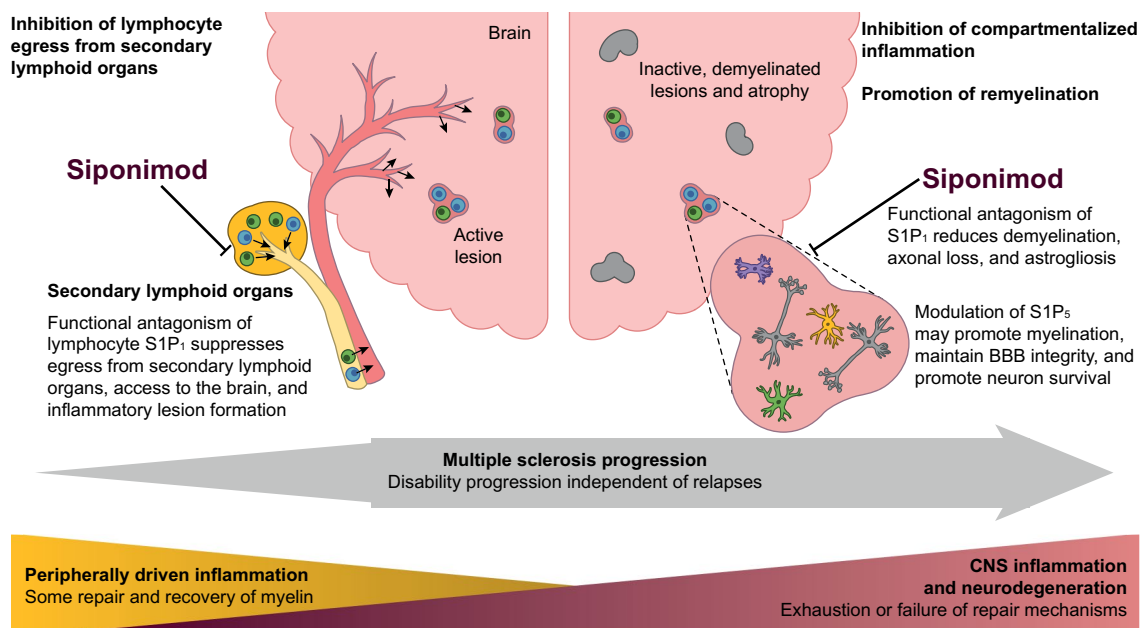


Fig. 1 The dual mechanism of action of siponimod in multiple sclerosis [2, 8, 23, 33–35]. *BBB* blood–brain barrier, *CNS* central nervous system, *S1P₁* sphingosine 1-phosphate receptor subtype 1, *S1P₅* sphingosine 1-phosphate receptor subtype 5

selective S1P₁ blockade in EAE mice reduced IL-17 and interferon- γ expression in the CNS, as long as pDCs were present [43]. Mobilization of pDCs from lymph nodes is S1P₄-dependent and, accordingly, pDCs were retained in lymph nodes in mice treated with fingolimod, which modulates S1P₄ [44]. Siponimod binds S1P₄ with about 0.1% of the affinity of fingolimod [21, 23], and therefore, would not be expected to interrupt significantly recruitment of pDCs into the CNS. These findings in the murine EAE model suggest that the failure of siponimod to inhibit pDC mobilization may be another pathway by which it could reduce CNS inflammation.

2.2 Effect of Siponimod on Lymphocyte Sequestration and Lymphocyte Composition in Humans

A key aspect of the mechanism of action of siponimod in MS is the inhibition of lymphocyte egress from lymph nodes, which is mediated by functional antagonism of S1P₁ [33]. When siponimod binds S1P₁, β -arrestins are recruited to the receptor–ligand complex, promoting its internalization [23, 25]. This state is perpetuated by continued drug exposure; thus, siponimod acts as a functional antagonist of S1P₁. In the lymphocyte population, stimulation of S1P₁ by S1P overrides retention signals induced by CCR7, leading to the release and egress of CCR7+ lymphocytes (naive and central memory cells) into the peripheral circulation [45]. Thus, functional antagonism of S1P₁ causes CCR7+ lymphocytes to remain sequestered in lymphoid tissues, while leaving CCR7– lymphocytes (effector memory cells) relatively unaffected [45]. Onset of the effects of S1P receptor modulators on lymphocyte trafficking is observed within 16 h of administration [46].

Siponimod administration causes a persistent, dose-dependent decline in absolute lymphocyte count (ALC). In healthy individuals ($n = 48$), siponimod resulted in a dose-dependent (0.3–10 mg per day) decline in ALC, with maximal reduction observed at 4–6 h after administration, which was maintained throughout 28 days of treatment [23]; in participants with SPMS ($n = 36$), siponimod treatment led to a 71% reduction in ALC at 6 months and 69% at 9–12 months compared with placebo [47]. Siponimod preferentially reduces the levels of some leukocyte subsets: in healthy volunteers, siponimod had the greatest effect on CD4+ cells (median-fold decrease from baseline [baseline cell count divided by post-baseline cell count], 22.8) compared with CD8+ T cells (median-fold decrease from baseline, 5.9) ($p = 0.0003$) [23]. Naive T cells were reduced the most, followed by central memory T cells, with peripheral effector memory T cells (T_{PEM}) largely unaffected. In participants with SPMS, siponimod had the greatest effect on CD4+ T cells (97% reduction from baseline at 6 months) and CD19+

B cells (93% reduction from baseline at 6 months), with CD8+ T cells reduced to a lesser degree (67% reduction from baseline) over the same period [47]. As a proportion of the overall CD4+ T cell population, naive T cells were significantly depleted (by approximately 50% at 6 months), but effector memory T cell levels were enriched by 240% after 6 months of treatment with siponimod compared with placebo. In addition, siponimod was associated with significant relative increases in T_{H2}, T regulatory, and B transitional regulatory cells. This pattern of lymphocyte composition, consistent with S1P₁-mediated peripheral lymphocyte trapping, was similar in healthy volunteers and participants with SPMS treated with siponimod, and suggests a shift in the immune system towards a more anti-inflammatory state [23, 47]. As with siponimod, absolute counts of central memory T cells decrease after fingolimod initiation, but two small studies in patients receiving fingolimod found an association between increased levels of central memory T cells before or during treatment and the likelihood of disease activity while on treatment. This suggests a role for this lymphocyte subset in MS relapse and thus possible value in predicting treatment response [48, 49].

3 Clinical Evidence for the Peripheral Immunomodulatory Effects of Siponimod

As noted earlier, siponimod acts as a functional antagonist of S1P₁ receptors causing lymphocytes to be sequestered in peripheral lymphoid tissues [23, 33]. By inhibiting the migration of lymphocytes from the periphery into the CNS, siponimod is believed to reduce peripherally mediated inflammation, manifested by evidence of reduced inflammatory lesion activity in the CNS seen on magnetic resonance imaging (MRI).

3.1 MRI Lesion Activity

The effect of siponimod on lesion activity was demonstrated in 297 participants with RRMS in the phase 2 BOLD study (NCT00879658) [50]. BOLD demonstrated a dose-dependent reduction in combined unique active lesions (CUALs), defined as new gadolinium-enhancing (Gd+) lesions on T1-weighted images and new or newly enlarged non-enhancing lesions on T2-weighted monthly MRI scans (without double counting), in participants receiving siponimod relative to participants receiving placebo (Fig. 2) [50]. Over 3 months of treatment, siponimod 10 mg per day reduced CUALs by 82% compared with placebo. A significant dose–response relationship was also identified across five doses of siponimod (0.25–10 mg; $p = 0.0001$) [50, 51]. When examining the effect of siponimod on Gd+ lesion activity exclusively, significant reductions in new lesions

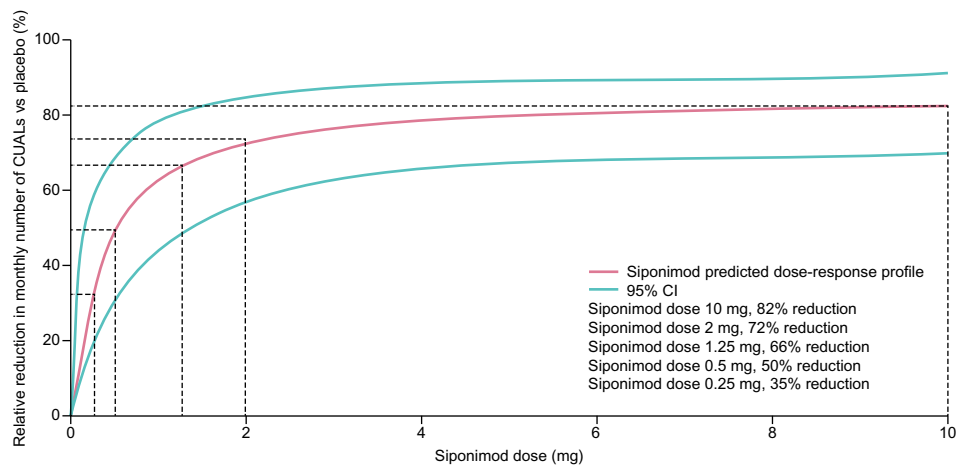


Fig. 2 Longitudinal dose–response curve at month 3 in BOLD and relative reductions versus placebo in number of CUALs (patients with RRMS) [50]. A negative binomial model was used to describe CUAL count over time and an E_{max} model was selected to fit the dose–response profile best over 3 months. A Bayesian model was also chosen to fit the CUAL count data observed at month 3. The resulting siponimod dose–response curve was summarized by a plot of the

were seen at all doses of siponimod, except 0.25 mg/day, compared with placebo. There were on average 0.38 new Gd+ lesions at 6 months in participants treated with siponimod 2 mg/day compared with 1.65 new lesions in participants receiving placebo ($p = 0.0051$). In participants with SPMS in the EXPAND study (NCT01665144), siponimod also significantly reduced the number of Gd+ lesions compared with placebo, despite the fact that the proportion of participants with Gd+ lesions at baseline was lower in the EXPAND population than in the RRMS population in BOLD (21% vs 44–57%) [50, 52]. Participants receiving siponimod ($n = 1099$) in EXPAND had on average 0.08 Gd+ lesions per scan from baseline to month 24, compared with 0.60 per scan among those receiving placebo ($n = 546$), a reduction of 86% (rate ratio, 0.14; $p < 0.0001$) [52]. Similarly, 89% of participants receiving siponimod who had MRI scans in EXPAND had no Gd+ lesions on study, compared with 67% of those receiving placebo [52].

Siponimod significantly reduced T2 lesion activity compared with placebo in participants with RRMS in the BOLD study at doses of 1.25 mg/day or higher. At the approved dosage of 2 mg/day, participants receiving siponimod had on average 0.41 new or newly enlarged T2 lesions at 6 months compared with 2.09 in participants receiving placebo ($p = 0.0012$) [50]. In EXPAND, participants receiving siponimod had on average 0.70 T2 lesions across all study visits, compared with 3.60 among those receiving placebo, for a reduction of 81% (rate ratio, 0.19; $p < 0.0001$); the increase in T2 lesion volume on study was also lower with siponimod treatment than with placebo ($p < 0.001$) [52]. Similarly, 57%

posterior median estimate and associated 95% CIs and by the dose achieving a 50% relative reduction in CUAL count versus placebo. Reproduced with permission from Selmaj et al. *Lancet Neurol.* 2013; 12: 756–67. *CI* confidence interval, *CUAL* combined unique active lesion, E_{max} maximum effect, *RRMS* relapsing–remitting multiple sclerosis

of participants receiving siponimod who had MRI scans in EXPAND had no new or enlarging T2 lesions, compared with 37% of those receiving placebo [52].

3.2 Relapse Activity

Although the BOLD study was not designed to assess treatment effects on relapse activity, annualized relapse rate (ARR) was significantly lower at 6 months with siponimod 2 mg/day than with placebo (0.20 vs 0.58, $p = 0.0408$) [50]. Patients with SPMS typically relapse less frequently than those with RRMS; however, siponimod still reduced ARR and increased the time to first relapse compared with placebo in EXPAND [52]. ARR was 0.07 with siponimod and 0.16 with placebo (rate ratio, 0.45; $p < 0.0001$), a 55% reduction; there was a 46% risk reduction on time to first relapse with siponimod compared with placebo ($p < 0.0001$).

4 The Role of S1P-Mediated Signaling in the CNS

In the CNS, S1P receptors are expressed on astrocytes, oligodendroglia, microglia, and neurons, and were shown to modulate processes relevant to MS neuropathology, including OPC migration and maturation, oligodendroglial cell survival, astrocyte activation, and microglial modulation [2, 21, 28, 35, 38, 53–58] (Fig. 3). S1P receptors are also found in embryonic brain areas undergoing active neurogenesis: in prenatal mice, S1P₁ expression colocalized with a marker

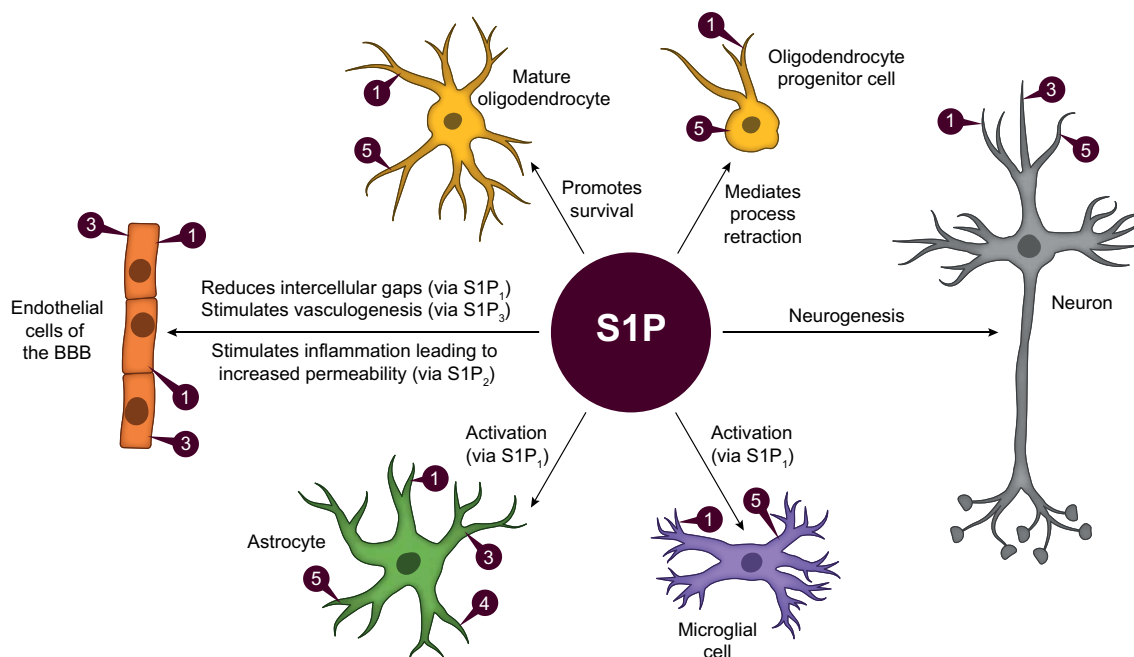


Fig. 3 Key roles of S1P-mediated signaling in the CNS [2, 21, 28, 35, 38, 53–58]. Owing to the ubiquitous nature of S1P receptors in the neural cells of the CNS, S1P has wide-reaching effects. Here, S1P receptor subtypes known to be expressed at high levels on CNS cells are represented as lollipops with the subtype number in white text. If

the effects of S1P are known to occur through a particular S1P receptor subtype, this is indicated in brackets. The figure is not intended to be comprehensive and illustrates the key roles and expression of S1P receptors. *BBB* blood–brain barrier, *CNS* central nervous system, *S1P* sphingosine 1-phosphate

of cell proliferation in the ventricular zone of the neocortex, the hippocampal primordia, and ganglionic eminence [59]. S1P also functions as a chemoattractant for neural progenitor cells, which migrate towards sites of neural injury [53]. In vitro studies suggest that S1P also modulates astrocyte proliferation and migration; all S1P receptor subtypes can be expressed on astrocytes, with particularly high levels of S1P₁ and S1P₃ [53]. Oligodendrocytes, the myelinating cells of the brain, express both S1P₁ and S1P₅ [21, 53, 54]; however, the effects of S1P on these cells appear to vary with the stage of cell development. S1P promotes the survival of mature oligodendrocytes but not OPCs, yet it mediates process retraction in OPCs but not in mature oligodendrocytes [55]; moreover, in mature oligodendrocytes deficient in S1P₁, the rate of process extension is lower than normal [60]. S1P₅ is highly expressed in the CNS and is most abundant in the white matter [21, 61].

4.1 S1P Receptors in the CNS

MS is associated with changes in the expression levels of S1P₁ and S1P₅ in the CNS, suggesting that alterations in expression of these receptors may play a role in disease pathology [28]. S1P₁ expression, as well as S1P₃ expression, is increased on astrocytes in MS lesions [35] and also on astrocytes isolated from donors without neurological

disease, after stimulation by tumor necrosis factor- α (TNF α), suggesting that S1P₁, along with S1P₃ activation, may have an early and persistent involvement in MS pathology [35]. Chronic activation of microglia and astrocytes drives progressive pathological responses in MS, even in the absence of infiltrating inflammatory cells from the periphery [2]. Pathological specimens from progressive MS lesions demonstrate primarily microglia, a small number of astrocytes, and absence of lymphocytes [62]. Studies using the EAE mouse model of MS have shown that genetic deletion or pharmacological functional antagonism of astrocyte-expressed S1P₁ reduces demyelination, axonal loss, and astrogliosis [38].

S1P₅ is expressed on oligodendrocytes and OPCs, and colocalizes with myelin [63]. In MS, S1P₅ expression is decreased in both active and chronic demyelinated lesions, compared with non-inflamed control tissue, which may be associated with myelin loss [63] or may reflect lower numbers of oligodendrocytes [1].

S1P_{1–3,5} are all expressed on the vascular endothelium of the BBB [57]. Perturbation of structures comprising the BBB, in particular post-capillary venules, plays a central role in the pathogenesis of MS, allowing extravasation of inflammatory lymphocytes into the brain [57]. S1P_{1–3} appear to increase while S1P₅ reduces the permeability of the BBB: S1P₁ modulates the endothelial cell (EC) cytoskeleton and

SIP₅ promotes the expression of intercellular binding molecules (cadherins) to reduce gaps between neighboring cells [56, 57]; SIP₃ stimulates the migration and proliferation of ECs, contributing to vasculogenesis [57]. In contrast, SIP₂ activation increases the permeability of the BBB, at least in part by preventing mobilization of cadherins to the extracellular surface of ECs [57].

5 Preclinical Evidence for Direct Effects of Siponimod on the CNS

As a small, lipophilic molecule, siponimod can penetrate the BBB, enabling direct effects on SIP receptors in the brain [64, 65].

5.1 Siponimod and Synaptic Neurodegeneration in Animal Models

Experimental animal models of MS have been used to demonstrate the direct effects of siponimod on disease pathology in the brain. In one study, intracerebroventricular infusion of siponimod (0.45 µg/day) significantly attenuated neural inflammation compared with vehicle control [66]. Glial fibrillary acidic protein levels (a marker of astrocyte activation) were reduced by 50% in the striatum of EAE mice treated with siponimod ($n = 5$) compared with control mice ($n = 5$) ($p < 0.05$), indicating reduced astrogliosis. Ionized calcium-binding adaptor protein-1 levels (a marker of microglia and macrophages) were reduced by more than 50% in the striatum of EAE mice treated with siponimod ($n = 5$) compared with control mice ($n = 4$) ($p < 0.05$), indicating reduced microgliosis [66]. CD3 levels, indicative of infiltrating lymphocytes, were also reduced to a similar degree in the striatum of siponimod-treated EAE mice compared with controls [66].

In a separate study using a modified EAE mouse model that targets induction of lesions in the cerebral cortex (focal EAE model), the effects of siponimod on the integrity of the auditory thalamocortical system were assessed using voltage-sensitive dyes on sagittal brain slices from mice with distinct focal cortical gray and white matter lesions. Changes in neuronal activity in the auditory cortex, evoked by electrical stimulation in the internal capsule, were detected as fluorescence changes. Stimulation evoked a response that subsided in controls but persisted in animals with lesions in the auditory cortical network. Incubation of brain slices with siponimod reduced this persistent state of activation and decreased the amplitude of the response relative to EAE controls, indicative of an improvement in the propagation of the stimulus. Flow cytometry revealed that the focal EAE model responded to oral siponimod, with peripheral lymphopenia and decreases in lymphocyte infiltration of lesioned

gray and white matter; treatment with oral siponimod was also associated with a less severe disease course compared with controls. Intracerebral administration of siponimod did not alter peripheral immunological responses, in contrast with the neuroprotective effects of siponimod in slice preparations. These findings are consistent with effects of siponimod in the brain occurring independently of effects on peripheral immune cell trafficking [67].

5.2 Effects of Siponimod on Key Neural Cells Involved in MS Pathology

The expression of SIP receptors and associated enzymes across the spectrum of cells within the human CNS [68–70] means that siponimod may affect these cells in multiple ways to modulate the inflammation and neurodegeneration characteristic of MS pathology (see data summary in Table 1).

5.2.1 Astrocytes

Astrocytes, which express SIP_{1–3,5}, play an important role in the development of MS lesions [53, 71, 72]. These cells contribute to both lesion development and repair, depending on lesion stage and topography [73]. Activated astrocytes in acute MS lesions secrete compounds that are potentially toxic to neurons and myelin [73]. However, following demyelination, astrocytes may express neurotrophic factors important for neuronal and axonal repair [74]. Siponimod activates protein kinase R-like endoplasmic reticulum kinase (pERK), phosphorylated protein kinase B/AKT (pAKT), and Ca²⁺ signaling through SIP₁ in cultured mouse and human astrocytes, suggesting an activation of pro-survival pathways in these cells [72], and inhibits nuclear factor kappa B translocation evoked by inflammatory cytokines via SIP₁-regulated downstream pathways in astrocytes generated from human fibroblasts that express SIP_{1,3} but not SIP₅ [75]. Siponimod-treated astrocytes also maintained high levels of the glial glutamate transporters GLAST and GLT1, which are typically downregulated by inflammatory cytokines, implicating an SIP₁-mediated mechanism that may help to avert accumulation of excitotoxic levels of glutamate under inflammatory conditions [75]. Astrocytes also provide metabolic and structural support to the BBB; SIP regulates the astrocyte cytoskeleton, modifying gap junctions between cells [57] and maintaining the low paracellular BBB permeability [76]. In MS, modulation of SIP₅ by siponimod may possibly maintain BBB integrity and impede the ingress of proinflammatory cells into the CNS.

5.2.2 Neurons

Neuronal degeneration is a major determinant of reduced function in patients with progressive MS. Axonal

Table 1 Preclinical evidence for the effects of siponimod on neural cells involved in MS pathology

Cell type	Role in MS	Effects of siponimod
Astrocytes	Contribute to lesion development and repair [73]	Activates pro-survival pathways in astrocytes through S1P ₁ [72] Inhibits NF-κB translocation evoked by inflammatory cytokines [75]
Neurons	Neuron degeneration is a major determinant of reduced function in patients with progressive MS [66]	Protects neurons from inflammation-induced damage by influencing astrocyte activity [75] Induces partial recovery of PV+ GABAergic interneurons in EAE mice [66]
Oligodendrocytes	Responsible for the remyelination of axons in the CNS [75]	Promotes myelin integrity through S1P ₁ internalization on oligodendrocytes [79, 81]
Microglia	Involved in neuronal damage and T cell recruitment in the CNS via the release of proinflammatory cytokines [66]	Attenuates microgliosis in preclinical models [66, 81, 84] This may be an indirect effect, mediated through other immune cells [72]
T cells crossing the BBB into the CNS	Lymphocyte infiltration of the CNS is a key stage in the neuropathology of MS	Reduces the lymphocyte proinflammatory activity in the CNS, but prevention of infiltration is unlikely to be a pivotal mechanism of action [81]
PMNLs	PMNLs (including neutrophils, eosinophils, and basophils) produce leukotrienes through the activity of 5-LO [98] Leukotrienes are proinflammatory lipid mediators found at high levels in the cerebrospinal fluid of patients with MS [97]	Inhibits 5-LO production by PMNLs by 50% via S1P ₄ modulation [98]

5-LO 5-lipoxygenase, BBB blood–brain barrier, CNS central nervous system, EAE experimental autoimmune encephalomyelitis, GABA γ -aminobutyric acid, MS multiple sclerosis, NF- κ B nuclear factor kappa B, PMNL polymorphonuclear leukocyte, PV+ parvalbumin-positive, S1P₁ sphingosine 1-phosphate receptor subtype 1, S1P₄ sphingosine 1-phosphate receptor subtype 4

transection and destruction occurs during the acute inflammatory process and is abundant in active and chronic active lesions [77]. Axonal degeneration is a chronic process that begins with sustained demyelination, which triggers a cascade of events (oxidative stress, mitochondrial injury, energy failure, ionic dysregulation) that lead to axonal and retrograde neuronal degeneration [78]. Since neurons may at times express S1P₁ and/or S1P₅, siponimod may have direct effects on their survival [66]. Human astrocyte cultures were treated with proinflammatory factors, and the resulting conditioned media added to pure cultures of spinal neurons, causing neuronal loss and neurite fragmentation. However, pre-treatment of the astrocytes with siponimod protected the spinal neurons from neurodegeneration, suggesting that siponimod may protect neurons from inflammation-induced damage by down-regulating astrocyte activity [75]. In EAE mouse models of MS, the loss of parvalbumin-positive (PV+) γ -aminobutyric acid (GABA)ergic interneurons in the striatum has been proposed as a major determinant of reduced function [66]. The striatum of EAE mice has been shown to have a reduced number of PV+ neurons (mean = 1885; $n = 5$), compared with control mice (mean = 2664; $n = 3$). Siponimod-treated EAE mice showed partial recovery to a mean level of 2293 neurons ($n = 5$; $p < 0.01$), indicating

that siponimod reduced GABAergic interneuron loss in the striatum of EAE mice [66].

5.2.3 Oligodendrocytes

The S1P₅-mediated action of siponimod on oligodendrocytes may promote myelin integrity. In both the *Xenopus* tadpole and mice, S1P₅ is highly expressed on myelin-forming oligodendrocytes, with markedly lower expression on other neural cells [55, 58, 79]. S1P₅ expressed on OPCs are preserved throughout developmental stages, including mature, myelinating oligodendroglia. S1P₅ stimulation/modulation promotes survival of mature oligodendrocytes [55]. Single-cell RNA sequencing identifies oligodendrocytes as the cell type with the highest expression of S1P₅ within the human brain [80]. In the *Xenopus* tadpole, siponimod demonstrated a promyelinating effect on oligodendrocytes [79]: tadpoles were treated with metronidazole for 10 days, inducing near complete demyelination, and then returned to normal water ($n = 3$) or water containing siponimod (3 nM) for 3 days ($n = 3$) or 8 days ($n = 3$). Exposure to siponimod for 3 days resulted in an increase in myelinated internodes compared with controls. Remyelination was also observed when a selective S1P₅ agonist was used, but not a selective S1P₁ agonist. Furthermore, the pro-remyelinating effect of siponimod was no longer apparent in knockout S1P₅

embryos ($S1P_5^{-/-}$), revealing the critical role of $S1P_5$ on oligodendrocytes in this process. In EAE mice, although the number of myelinating oligodendrocytes did not change in response to siponimod, demyelination was reduced [81]. The adoptive transfer of proteolipid protein (PLP)-primed T_h17 cells into naive mice resulted in aggregates of immune cells (called tertiary lymphoid tissues [TLTs]) in the brain, inducing acute EAE. Compared with controls, immunostaining for myelin identified a gradient of demyelination that was most pronounced in the layer proximal to meningeal TLTs, becoming less pronounced in deeper cortical layers. However, on histological detection of myelinating oligodendrocytes, there were no differences across the layers of the cortex in EAE mice and no differences in the EAE mice compared with controls, suggesting that myelin damage was not accompanied by loss of myelinating cells. Treatment with siponimod 5 days after adoptive transfer resulted in a sparing of myelin compared with untreated controls, but with no change in oligodendrocyte numbers [81]. A study utilizing three experimental models (cuprizone and autoimmune encephalomyelitis-optic neuritis mouse models and a conditional demyelination *Xenopus* tadpole model) observed a protective effect of siponimod on oligodendrocytes [82]. Finally, post hoc analyses of MRI data from the phase 3 EXPAND trial also suggest that siponimod might have a beneficial effect on myelination density in patients with SPMS (see Sect. 6.4) [83].

5.2.4 Microglia

Microglia are involved in neuronal damage and T cell recruitment in the brain via the release of proinflammatory cytokines such as IL-6 and regulated on activation normal T cell expressed and secreted (RANTES), both of which are implicated in EAE and MS pathogenesis [66]. Siponimod attenuated microgliosis in EAE mice [66] in a preclinical model of subpial cortical injury [81] as well as in a traumatic brain injury mouse model, in which it also preserved BBB tight junctions [84]. In mouse microglial cell cultures, siponimod reduced lipopolysaccharide- or $TNF\alpha$ /IL-17-induced levels of IL-6. However, this effect was more pronounced in organotypic cerebellar slice cultures, suggesting that reduction in microglial IL-6 was an indirect effect of siponimod, mediated through other immune cells [72]. Basal levels of receptor gene expression are highest for $S1P_1$, followed by $S1P_5$, and then the other $S1P$ receptors [58], although experimental or disease conditions can alter expression levels. Beneficial effects of siponimod in the CNS driven by shifting microglia to a regenerative state have been found [82].

5.2.5 Endothelial Cells

ECs are a key element of the BBB; astrocytes and pericytes cooperate with the ECs lining capillary walls to form the BBB [85]. In the BBB, ECs interact with the astrocytes and pericytes to form intercellular tight junctions and with operating efflux pumps to prevent migration of cells and molecules into the CNS [86–88]. $S1P_1$ is expressed on ECs [22], and under various conditions, astrocytes can express all $S1P$ receptor subtypes. Agonism of $S1P_1$ on ECs appears to be important in maintenance of the BBB [57, 89], and there is also some evidence for the involvement of $S1P_5$ [90]. The effects of siponimod on ECs, however, are yet to be fully elucidated.

5.2.6 T Cells Crossing the BBB into the CNS

Lymphocyte infiltration of the CNS is a key stage in the neuropathology of MS, so it is possible that siponimod could affect this process via $S1P$ receptors on the surface of lymphocytes. However, a recent study suggests that this is not a pivotal mechanism of action of siponimod. In acute EAE mice generated by adoptive transfer of PLP-primed T_h17 cells into naive mice, siponimod treatment 3 days after adoptive transfer completely prevented EAE symptoms, while treatment 8 days after adoptive transfer had no effect on clinical disease; treatment at 5 days resulted in a significant reduction, but not elimination, of symptoms assessed by composite clinical score ($p < 0.001$). Treatment at day 5 was associated with a selective reduction in the capacity of transferred T_h17 cells to produce proinflammatory cytokines, accompanied by a reduction in demyelination, microglia/macrophage accumulation, and oxidative injury [81]. This suggests that lymphocyte-induced, proinflammatory activity in the CNS may be downregulated by siponimod in the presence of lymphocyte infiltration [81]. Functional antagonism of $S1P_1$ by siponimod could reduce CNS inflammation by blocking $S1P_1$ -mediated activation pathways that lead to recruitment of the JAK/STAT3 pathway [91, 92]. In EAE mice, increased STAT3 expression in CD4+ T cells and monocytes in the CNS drives T_h17 polarization and inflammation initiated by stimulation of JAK/STAT3 by IL-6 [93–96].

5.2.7 Polymorphonuclear Leukocytes

Leukotrienes are proinflammatory lipid mediators found at increased levels in the cerebrospinal fluid of patients with MS [97]. Polymorphonuclear leukocytes (PMNLs) (including neutrophils, eosinophils, and basophils) produce leukotrienes through the activity of 5-lipoxygenase (5-LO) [98]. $S1P$ inactivated 5-LO in PMNLs isolated from human

blood, and this effect was shown to be mediated by $S1P_4$, identified as the predominant $S1P$ receptor on this cell type [98]. Siponimod, with low affinity for $S1P_4$, was able to inhibit 5-LO production by PMNLs by 50% compared with untreated PMNLs. Fingolimod, which has a higher affinity for $S1P_4$ than siponimod, achieved the same level of inhibition, but at a tenfold lower concentration [98].

5.3 Myelin Integrity in Preclinical Models

Chronic demyelination is a hallmark of MS, which disrupts signaling and eventually leads to axonal degeneration and neuronal death [8]. Toxin-induced models of demyelination can be useful for investigations of remyelination without the confounding impact of autoimmune processes. Siponimod significantly attenuated lysophosphatidylcholine (LPC)-mediated demyelination in organotypic slice cultures from the cerebellum of 10-day-old mice (53.8% vs 97.4% [presence vs absence of siponimod]; $p < 0.05$) [72]. In a study that used organotypic cerebellar slice cultures from rats, fingolimod and an $S1P_1$ -specific agonist also inhibited LPC-mediated demyelination [99]. Experiments to characterize the functional basis of remyelination of LPC-demyelinated cerebellar slice cultures with fingolimod showed that an $S1P_{3,5}$ antagonist co-administered with fingolimod countered the remyelination effect seen with fingolimod alone; co-administration of an $S1P_1$ antagonist with fingolimod only slightly decreased fingolimod-mediated remyelination [100]. In another LPC-mediated demyelination model, siponimod, but not a selective $S1P_1$ agonist, induced a significant increase in myelin levels in demyelinated CNS aggregates from fetal rats, compared with controls. These findings indicate that $S1P_1$ modulation alone is insufficient to elicit changes in remyelination, and suggest that the trophic actions of siponimod upon remyelination could be mediated in part by $S1P_5$ agonism [101].

6 Clinical Evidence for the Dual Benefit of Siponimod in the CNS

Much of the clinical evidence for the effects of siponimod in the CNS comes from patient data collected in the phase 3, randomized, parallel-group, double-blind, placebo-controlled EXPAND trial. As well as effects on inflammatory MRI lesion activity (see Sect. 3), siponimod reduced disability progression in the EXPAND population compared with placebo (see Sect. 6.3) [52]. Several MS DMTs that have been evaluated in progressive forms of MS have not slowed disability progression, possibly because they do not impact both inflammatory and neurogenerative mechanisms. For example, natalizumab, which has powerful anti-inflammatory activity, did not reduce disability progression (based on

changes in EDSS score and in timed 25-foot walk) compared with placebo in participants with SPMS in the ASCEND trial, even though exploratory analyses found improvements in relapse rates and focal inflammatory outcomes [102]. Ibudilast, which has been described as possibly neuroprotective [103], reduced gray matter atrophy and total brain volume loss compared with placebo in a mixed population of patients with PPMS or SPMS in the phase 2, placebo-controlled SPRINT-MS trial (NCT01982942) [104], but did not reduce physical disability progression (secondary endpoint) [105] or T2 lesion activity [104].

6.1 Inflammatory Disease and Neuronal Damage

Blood and cerebrospinal fluid neurofilament light chain (NfL) levels are markers of neuroaxonal damage and disease activity in patients with MS [106, 107]. Levels of NfL are increased in active MS, owing to ongoing neuroaxonal damage, and are associated with active inflammatory MRI lesion activity and relapses. One analysis found that high baseline levels of NfL, after age adjustment, were prognostic of increased risk of relapse and of new MRI lesion activity during the subsequent year [108]. There is evidence that changes in NfL levels are also associated with treatment response. Longitudinal reductions in NfL in patients with RMS have been associated with treatment with most of the established MS DMTs, and analysis of data from participants with SPMS in EXPAND showed a significant 5.7% reduction in NfL levels over 21 months among those treated with siponimod compared with a 9.2% increase among those on placebo ($p = 0.0004$). The greatest effects were observed

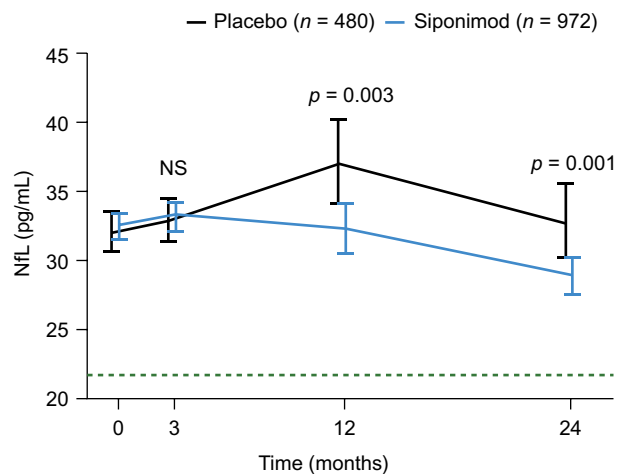


Fig 4. Effect of siponimod on blood NfL levels in patients with SPMS [107, 124]. Data are geometric means of NfL concentration in blood with 95% CIs. The green dashed line marks an estimate of the NfL level of healthy controls of similar age. *CI* confidence interval, *NfL* neurofilament light chain, *NS* not significant, *SPMS* secondary progressive multiple sclerosis

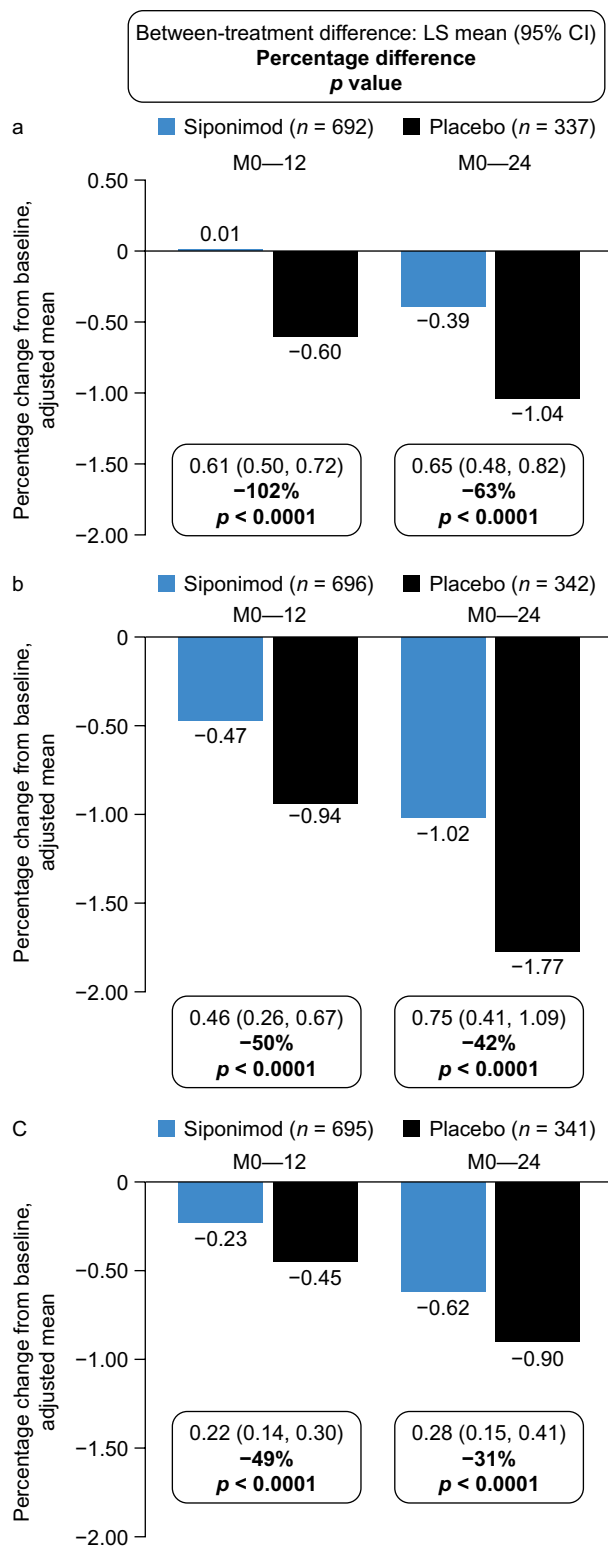
Fig. 5 Percentage change in brain volume in **a** cGM, **b** thalamus, and **c** total brain in the pooled MRI data set from participants with SPMS in EXPAND [83]. Neural atrophy, estimated by changes in brain volume from baseline, increases over time in patients with SPMS. In the EXPAND study, siponimod slowed brain atrophy over 1–2 years of treatment. Data are from the per-protocol set and include all participants who did not have any major protocol deviations that could confound interpretation. Reproduced with permission from Arnold et al. [83]. *cGM* cortical gray matter, *CI* confidence interval, *LS* Least squares, *M* month, *MRI* magnetic resonance imaging, *SPMS* secondary progressive multiple sclerosis

in the subgroup of participants with active SPMS at baseline, who experienced a 10.5% reduction in NfL levels with siponimod treatment (Fig. 4) [107]. By virtue of their specificity for neurons, increased levels of NfL in active disease support the association between active inflammatory lesions in MS and neuroaxonal damage [106].

6.2 Neurodegenerative Disease and Brain Atrophy

Diffuse and progressive axonal injury together with a loss of tissue volume in demyelinated plaques give rise to global brain atrophy [1]. Cortical atrophy is profound in MS, becoming very prominent at late stages of progressive disease; demyelinated plaques are seen in deep gray matter, associated with some neuroaxonal injury and loss [1]. In patients with progressive MS, gray matter atrophy steadily increases over time and is closely correlated with accumulation of long-term physical disability [109, 110] and cognitive impairment [8]. Data from the EXPAND study showed that siponimod significantly reduced brain volume loss [52] and both thalamic and cortical gray matter (cGM) atrophy compared with placebo in patients with SPMS, irrespective of whether they had recent active disease [83, 111]. Gray matter preservation was evident at both month 12 and month 24. The strongest effect was observed for cGM atrophy, for which siponimod was associated with a 0.01% increase in adjusted mean cGM volume at month 12, compared with a 0.6% decrease in volume in participants receiving placebo ($p < 0.0001$) (Fig. 5) [83].

Results of secondary analyses of data from the EXPAND study suggest that the beneficial effects of siponimod on brain atrophy are accompanied with improvements in cognitive processing speed (CPS). In EXPAND, CPS was assessed using the Symbol Digit Modalities Test (SDMT), which is thought to be the best measure of CPS for MS studies [112], with a 4-point change considered clinically meaningful [113]. Patients receiving siponimod had a 21% lower risk of having a sustained 4-point decrease on the SDMT ($p = 0.0157$), while the probability of having a 4-point sustained increase in SDMT score was 28% higher ($p = 0.0131$) than for participants in the placebo group [114].



Paced Auditory Serial Addition Test and Brief Visuospatial Memory Test–Revised scores were similar between the siponimod and placebo groups [114]. These results suggested that siponimod had a positive effect on CPS [114].

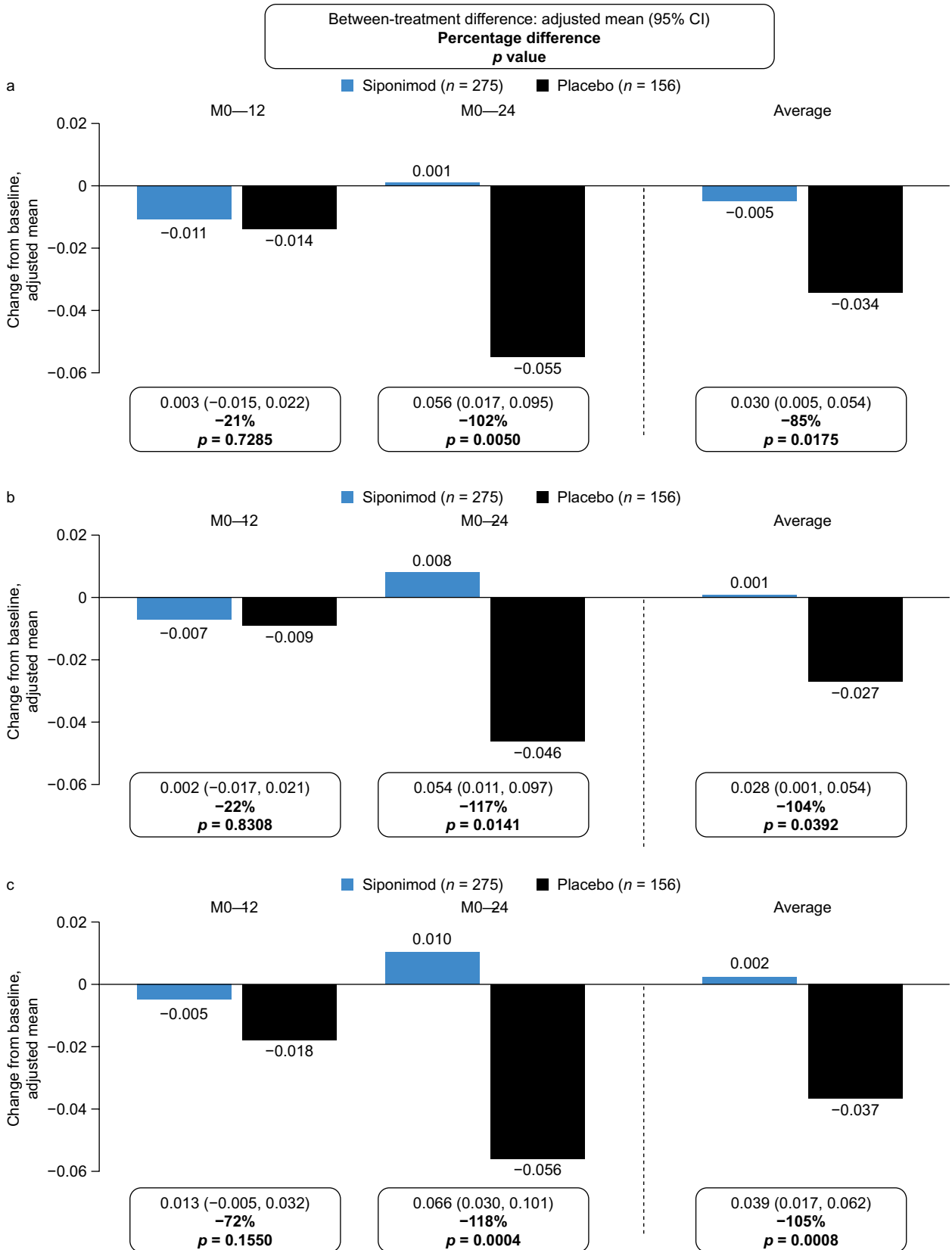


Fig. 6 Effect of siponimod on changes in median nMTR in **a** NABT, **b** cGM, and **c** NAWM in patients with SPMS [83]. MTR may reflect (at least in part) changes in myelin density, with decreases associated with acute demyelination and increases with remyelination. nMTR decreases in patients with SPMS. In the EXPAND study, siponimod versus placebo reduced the rate of nMTR decrease, and increased nMTR in some patients. Data are from the per-protocol set from EXPAND and include all participants who did not have any major protocol deviations that could confound interpretation. Reproduced with permission from Arnold et al. [83]. *cGM* cortical gray matter, *CI* confidence interval, *M* month, *MTR* magnetization transfer ratio, *NABT* normal-appearing brain tissue, *NAWM* normal-appearing white matter, *nMTR* normalized MRT, *SPMS* secondary progressive multiple sclerosis

6.3 Inflammatory and Neurodegenerative Components of Disability Progression

Long-term disability progression in MS can be attributable to the cumulative effects of unresolved relapses as well as underlying neurodegenerative disease progression that occurs independently of relapse activity. Indeed, disability progression in the absence of relapses is a defining characteristic of non-active SPMS [115]. The EXPAND study population included participants classified as relapsing or non-relapsing based on their relapse history at enrolment [52]. The primary analysis of EXPAND demonstrated reduced disability progression in the overall population [52], but analyses in the subgroup of participants classified as non-relapsing (i.e., with non-active disease) found the effect of siponimod on disability was not significant [116]. A complication to be overcome in such an analysis is the fact that even patients with no relapses for an extended period before enrollment cannot be assumed to have non-active disease. Moreover, if these patients are randomized to receive siponimod, subsequent absence of relapse activity may be attributable to the anti-inflammatory effects of treatment. Using methods to control for potential confounding treatment effects in a post hoc analysis, it was possible to estimate that siponimod reduced the risk of disability progression confirmed at 6 months by up to 33% among patients with non-active SPMS [117]. These findings support the notion that, after excluding anti-inflammatory effects, siponimod influences pathological processes in the CNS that contribute to disability progression in SPMS.

6.4 Remyelination

Changes in magnetization transfer ratio (MTR) are used as an MRI-based surrogate marker of changes in myelin density in the brain, with decreases indicative of acute demyelination and increases of remyelination [118–123]. However, it should be noted that MTR recovery represents resolution of edema, and therefore could be attributable to anti-inflammatory effects rather than being indicative of remyelination. In

a post hoc analysis of data from the EXPAND study [52], siponimod was associated with a smaller reduction in MTR over time compared with placebo in normal-appearing brain tissue and with improved MTR recovery in newly formed MTR lesions (Fig. 6) [83]. Importantly, consistent effects on MTR were seen with siponimod both in participants with active disease and in those without acute inflammation in EXPAND, lending weight to the possibility of treatment-mediated effects on myelination.

7 Conclusions

There is an increasing body of evidence that siponimod has a dual mode of action in MS: it suppresses characteristic inflammatory autoimmune processes through peripheral effects on migration and proinflammatory polarization of lymphocytes and may further afford neuroprotection through direct effects on the resident CNS cell population. Preclinical data indicate that such neuroprotective mechanisms could include activation of pro-survival pathways in astrocytes, augmentation of BBB function mediated by the activity of siponimod on astrocytes and ECs, attenuation of microgliosis, and attenuation of axonal demyelination and promotion of axonal remyelination by oligodendrocytes. Further, clinical data corroborate a beneficial effect of siponimod on prevention of disability progression in SPMS, attenuation of brain volume loss, and preservation of a clinically relevant cognitive performance outcome. The positive associations between regional brain volume at baseline and cognitive and physical outcomes at 2 years in EXPAND may provide a physiological basis for the improved volumetric MRI and disability outcomes seen among participants receiving siponimod compared with those receiving placebo. These findings suggest that, as well as modulation of the systemic immune system, the proposed interactions of siponimod with S1P receptors on multiple cell types in the CNS may lead to improvements in multiple MS clinical outcomes. Preclinical data also support the possibility that the therapeutic response to siponimod in progressive forms of MS could, at least in part, be driven by direct effects on the CNS.

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Authors' contributions All authors discussed and agreed on the concept for the article. APR and SG performed the literature research and data analysis and drafted the manuscript. All authors critically reviewed the data and each manuscript draft, have read and approved the final submitted manuscript, and agree to be accountable for the work.

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