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Sterol metabolism in multiple sclerosis

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"In all things of nature there is something of the marvelous."

Aristotle (384 BC – 322 BC)

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List of abbreviations

24S25-EC	24(S),25-epoxycholesterol
24SOHC	24(S)-hydroxycholesterol
DHCR24	24-dehydroxycholesterol
27OHC	27-hydroxycholesterol
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
Aβ	Amyloid beta
ANOVA	Analysis of variances
APC	Antigen presenting cell
ApoA	Apolipoprotein A
ApoE	Apolipoprotein E
ABC	ATP-binding cassette transporter
ActB	Beta actin
BDNF	Brain-derived neurotrophic factor
CRD	Carbohydrate recognition domain
CNS	Central nervous system
CSF	Cerebrospinal fluid
CCR	Chemokine receptor
CETP	Cholesteryl ester transfer protein
cpMΦ	Choroid plexus
CIS	Clinically isolated syndrome
CD	Cluster of differentiation
CL-P1	Collectin placenta 1
CCR	Corpus callosum
CycA	Cyclophilin A
DC-SIGN	Dendritic cell-specific ICAM3-grabbing non-
DMSO	Dymethylsulfoxide
ER	Endoplasmatic reticulum
EC	Esterified cholesterol
EAE	Experimental autoimmune encephalomyelitis
FH	Familial hypercholesterolemi
GC/MS	Gas chromatograph mass spectrometer
GM	Grey matter
HSC	Hematopoietic stem cell
HDL	High-density lipoprotein
HLA	Human leukocyte antigen
Hmbs	Hydroxymethyl-bilane synthase

Ig	Immunoglobulin
IHC	Immunohistochemistry
IGF-1	Insulin-like growth factor 1
IFN-γ	Interferon gamma
IL	Interleukin
IDL	Intermediate-density lipoprotein
IPP	isopentenyl pyrophosphate
LCAT	Lecithin-cholesterol-acyltransferase
LOX1	lectin-like oxLDL receptor
LIF	Leukemia inhibitory factor
LPC	lysophosphatidylcholine
LPS	Lipopolysaccharide
LRP	Lipoprotein related protein
LXR	Liver X receptor
LXRRE	Liver X receptor response element
LDL	Low-density lipoprotein
LRP1	Low-density lipoprotein receptor-related protein 1
LPCAT3	Lysophospholipid acyl-transferase 5
MRI	Magnetic resonance imaging
MHC	Major histocompatibility complex
MDA	Malondialdehyde
Vmax	Maximum velocity
mMΦ	Meningeal macrophages
Mrna	Messenger RNA
MCP-1	Monocyte chemoattractant protein 1
MS	Multiple sclerosis
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
MAG	Myelin-associated glycoprotein
NG2	Neural/glial antigen 2
NOGO-A	Neurite outgrowth inhibitor-A
NPC1	Niemann Pick C1
NO	Nitric oxide
NDC	Non-demented controls
NAWM	Normal appearing white matter
ORO	Oil red O
OLN	Oligodendrocyte
OPC	Oligodendrocyte precursor cell

oxLDL	Oxidized LDL
PBMC	Peripheral blood mononuclear cell
pvMΦ	Perivascular macrophages
PPAR	Peroxisome proliferator-activated receptor
PBS	Phosphate buffered saline
Pgk1	Phosphoglycerate kinase 1
PDGFRα	Platelet-derived growth factor receptor alpha
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
PPMS	Primary progressive MS
PGE2	Prostaglandin E2
PLP	Proteolipid protein
QPCR	Quantitative polymerase chain reaction
RRMS	Relapse-remitting MS
RXR	Retinoid X receptor
RCT	Reverse cholesterol transport
RNA	Ribonucleic acid
Rpl13a	Ribosomal protein 13a
SR	Scavenger receptor
SPMS	Secondary progressive MS
shRNA	Short hairpin RNA
STAT1	Signal transducer and activator of transcription 1
SREBP	Sterol regulatory element-binding protein
TCR	T cell receptor
Tbp	TATA box binding protein
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation zeta
MERTK	Tyrosine protein kinase MER
VLA-4	Very late antigen 4
VLDL	Very low-density lipoprotein
WM	White matter

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Introduction and aims

1 Introduction and aims

1.1 Basic concepts of multiple sclerosis

1.1.1 Clinical aspects and diagnosis

The first description of multiple sclerosis (MS) dates back to the 14th century, but it was Jean-Martin Charcot who made the first correlations between the clinical features of MS and the pathological changes noted post-mortem (1). In 1868 he described MS as an accumulation of inflammatory cells in a perivascular distribution in the brain and spinal cord of patients with episodes of neurologic dysfunction (2). Since then, advances in life sciences have resulted in a thorough analysis of the etiology and pathogenesis of MS.

Clinical aspects of MS

Currently, MS is regarded as a chronic inflammatory disease of the central nervous system (CNS), characterized by multiple sites of inflammation and the destruction of the insulating myelin sheath surrounding axons. The loss of myelin impairs signaling between neurons and gives rise to a plethora of symptoms including impairment of motor, sensory, visual and autonomic systems (3). Today, there is substantial evidence that both genetics (e.g. Human Leukocyte Antigen D Related (HLA-DR), Interleukin-7 Receptor A (IL-7RA), IL-1R and IL-1β) and environmental factors such as pathogens, viruses, smoking and vitamin D intake can determine a person's susceptibility to MS and play a role in modulating the disease (4-8).

Clinically, MS starts as a first and single neurologic episode of inflammation or demyelination of the CNS, termed clinically isolated syndrome (CIS). When patients exhibit new symptoms or lesions at different locations and times (dissemination in space and time), the disease can be diagnosed as MS. The prevalence of MS is approximately 2 million people worldwide with an incidence of 1:1000 in Europe and North America (9, 10). It is the most common non-traumatic neurological disability among young adults between the age of 20 and 40, and affects women twice as often as men (11). Although MS is a heterogeneous disease concerning clinical symptoms, about half of the patients require help with walking within 15 years after disease onset (12). MS is typically categorized as being either relapsing-remitting (RRMS) or primary-progressive

(PPMS). Patients with RRMS tend to experience a series of attacks that result in varying degrees of disability followed by complete or partial remission. The majority of RRMS patients will ultimately develop secondary progressive (SPMS). This subtype is characterized by a steady progressive neurological deterioration with or without relapses and minor remissions that eventually leads to irreversible loss of axons and neurons (13). In a small subset of patients the relapsing phase is lacking and the disease is progressive from the onset (PP-MS) (14).

Diagnosis

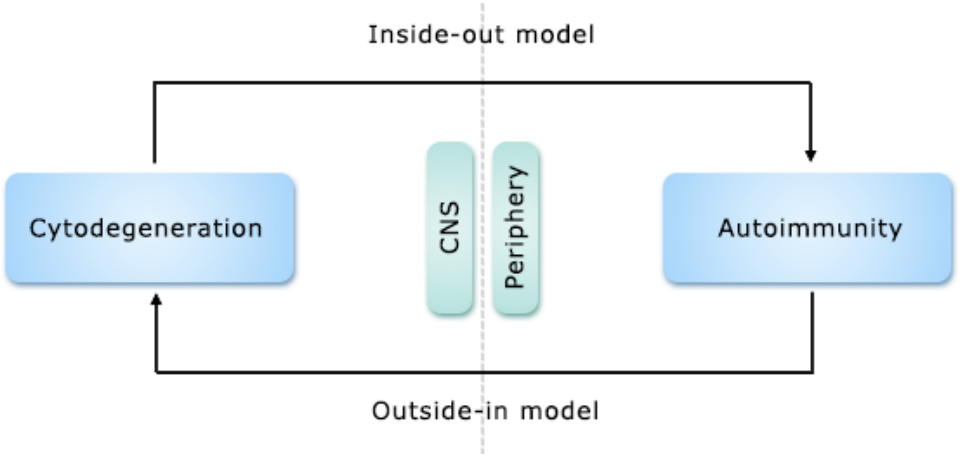
Although no straightforward single test for MS exists, in most cases the person's medical history and neurologic exam by means of magnetic resonance imaging (MRI) provide enough evidence to establish a diagnosis. Originally, MS was diagnosed using the McDonald criteria, which required repeated attacks (disease dissemination in time, DIT) and evidence of inflammation in at least two different sites (disease dissemination in space, DIS) in the CNS. In 2010, these criteria were reevaluated to allow diagnosis after a first clinical episode of the disease (15). Since 2010, MRI technology has improved, which has led to the discovery of better acquisition sequences and new insights into MS disease progression as evidenced by high-field and ultra-high-field scanners. In 2016, the European collaborative research network that studies MRI in MS (MAGNIMS) has proposed another revision of the McDonald criteria (16). MRI has proven to be pivotal in identifying the number, size, inflammatory activity and the evolution over time of brain lesions, which are present in 95% of patients at the time of diagnosis (17).

1.1.2 MS pathogenesis

Although the etiology of MS is unknown, it is traditionally regarded to be an autoimmune inflammatory disorder mediated by an aberrant T cell attack against CNS elements, particularly myelin (18). The leading cause of the permanent neurologic disability is axonal loss caused by inflammatory responses, disruptions of axonal ion homeostasis, energy failure or cytotoxic Ca^{2+} accumulation (19, 20). The most accepted "outside-in" hypothesis stipulates that early pathogenesis of MS is characterized by activation of autoreactive T cells in the periphery (fig. 1.1). In the CNS, T cells become reactivated and release proinflammatory cytokines. These cytokines attract macrophages and B cells into the CNS. Macrophages are

the most important effector cells in MS. They phagocytose myelin and produce several proinflammatory cytokines. B cells contribute to MS pathogenesis by producing autoantibodies and proinflammatory cytokines, and functioning as antigen presenting cells (APCs) for the activation of T cells (21-23). Combined, these immune interactions will eventually result in the vicious circle of local CNS inflammation seen in MS patients (10, 24).

Figure 1.1 | the inside-out and outside-in models for MS. MS has been classically categorized as an autoimmune disease in which peripheral activation of autoreactive T cells is the initial trigger (outside-in model). Recently, a new model has emerged postulating local CNS cytodegeneration as the initial event (inside-out model). Reprinted with permission from Nature Publishing Group (25).



Peripheral T cell activation is believed to result from functional recognition of antigenic peptides by T cell receptors (TCRs) present on the surface of cluster of differentiation 4 ($CD4^+$) T cells. Myelin-reactive T cells are found in the blood, cerebrospinal fluid (CSF), and CNS tissue of MS patients (26). Currently, there is no consensus about the nature of these, likely patient-specific, antigenic peptides or autoantigens. They may be derived from myelin-associated proteins like myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin-associated glycoprotein (MAG). Experimental autoimmune encephalomyelitis (EAE) an animal mode of MS, can be induced in a variety of animals (e.g. rats, mice, rabbits, guinea pigs and rhesus monkeys) by adoptive transfer of myelin reactive T cells or by generating a T cell mediated

immune response against CNS antigens like MOG, MBP or PLP (27). Although myelin antigens are major targets, there are possible other candidates, such as alpha B-crystallin (28).

Recently, this "outside-in" model has been challenged by a competing view that states that the initial trigger for MS is a cytodegenerative event in the CNS that promotes inflammation and autoimmunity by releasing antigenic constituents (18, 29-31). Irrespective of the initial trigger, the immune system is indisputably crucial in orchestrating the immunopathological cascade involved in demyelination and neurodegeneration.

1.1.3 Lesion pathology

A cardinal hallmark of MS is focal demyelination, which is present during all stages of the disease (32). Different classifications of MS lesions exist, depending on the stage of MS, lesion location, extent of inflammation and extent of remyelination (33-35). It should be noted that determining lesion pathology is difficult and primarily based on post-mortem material from patients with long-lasting MS. Our knowledge about lesion pathology thus might not reflect lesions in the initial stages of MS.

White matter lesions

Most commonly described are active white matter (WM) lesions that are predominantly found in RRMS patients (36, 37). They are defined by the accumulation of activated myelin-phagocytosing macrophages and microglia present throughout (active lesion) or at the rim of the lesion (chronic active lesion). Additionally, blood-brain barrier (BBB) leakage, axonal damage and infiltrating T and B cells are present in active plaques (34, 38). These active demyelinating plaques eventually convert into chronic lesions with loss of oligodendrocytes, myelin and glial scarring (39). Chronic lesions can be subdivided into chronic active and inactive lesions. Chronic active lesions have a hypercellular rim with ongoing demyelination and a hypocellular center containing naked demyelinated axons and fibrous astrocytes (39, 40). The most frequent lesion type in progressive MS is the chronic inactive lesion. They are devoid of myelin and show extensive axonal damage and astrocytic scarring (39). Immune cell

infiltration is rare. Microglia density is even lower in inactive lesions than in surrounding normal-appearing WM (41).

Gray matter lesions

Cortical gray matter (GM) lesions in MS have been neglected for long time due to predominant interest in WM demyelination and the difficulty in visualizing GM lesions with conventional histochemical staining and MRI methods (42). The appearance of cortical lesions is most prominent in progressive MS, but can already occur in early MS (37, 43-45). Despite the extensive pathological characterization and MRI imaging of GM lesions in MS, it remains enigmatic whether cortical tissue damage is secondary to WM pathology or is a primary pathological process (46). From a neuropathological point of view, GM lesions can be classified into three subtypes. Type 1 lesions extend through the GM into the WM and do not usually reach the surface of the brain. Type 2 lesions are located within the GM, surrounded by normal appearing gray matter (NAGM) and are often located around blood vessels. Type 3 lesions are subpial lesions that sometimes stretch over several gyri and are the most predominant GM lesion (47). GM lesions are generally characterized by demyelination and, to a lesser extent, microglial reaction whereas lymphocyte infiltration and BBB disruption are not usually found (48, 49). In post mortem samples taken from individuals in advanced stages of MS, high numbers of immune cell infiltrates were detected in type 1 GM lesions. Type 2 and 3 GM lesions were categorized as relatively non-inflammatory (43, 48, 50). The most common lesion type consists of purely cortical lesions extending inward from the surface of the brain and is grossly underestimated by standard histochemical myelin staining methods (51). Some MS patients even have subpial demyelination in all cortical areas of the brain (52). Recently, large multicenter studies have demonstrated that GM loss may be the most important contributor of cognitive impairment in MS patients. Specifically, volumes of the hippocampus and deep GM nuclei correlate strongly with cognitive function of MS patients (53, 54), making GM volume a suitable biomarker for predicting cognitive deterioration over time.

1.1.4 Therapeutic strategies

In the past decades, our understanding of MS pathology has significantly increased. This has led to the development of several new disease modifying therapies (DMTs) (55). Available drugs focus largely on suppressing or altering the activity of the immune system and can prevent exacerbations and the formations of new lesions in RRMS patients. However, only ocrelizumab (Ocrevus™), has been demonstrated to halt the neurodegenerative changes that are associated with the progressive phase of the disease (56).

First-line therapeutics

Commonly used first-line DMTs are interferon-β 1b (IFN-β1b; available under the trade names Avonex™, Rebif™, Betaferon™, and Extavia™) and glatiramer acetate (GA) (Copaxone™). IFN-β1b exerts its effect by decreasing MHC class II expression, upregulating IL-10 production, reducing Th1 and Th17 differentiation and suppressing the trafficking of immune cells into the CNS (57). It has been shown to reduce annualized relapse rates, delay progression of disability and lower the number of MRI lesions (58, 59). However, the body can produce neutralizing antibodies against IFN-β1b, which reduces therapeutic efficacy (60). GA is a synthetic copolymer that structurally resembles myelin basic protein (MBP). It stimulates the production of Th2 cells, regulatory T cells (Tregs) and regulatory $CD8^+$ T cells, inhibits antigen-specific T cell activation, promotes regulatory B cell properties and skews macrophages towards an anti-inflammatory phenotype (61-63). Treatment with GA reduces annualized relapse rates, prevents the development of new lesions in RRMS patients, and has no adverse effects (59, 64). Recently approved first-line therapeutics include teriflunomide (Aubagio™), dimethyl fumarate (Tecfidera™), alemtuzumab (Lemtrada™), and daclizumab (Zinbryta™), respectively FDA approved in 2012, 2013, 2014, and 2015. Teriflunomide inhibits pyrimidine *de novo* synthesis, which blocks rapidly dividing cells such as activated T cells (65). Dimethyl fumarate modulates oxidative pathways by activating nuclear factor erythroid-derived-like2 (Nrf2) (66) and alemtuzumab reduces circulating immune cells (67). Finally, daclizumab blocks IL-2 receptor and dramatically expands and activates CNS-resident NK cells that can kill autologous activated T cells (68). Importantly, long-term effects of these new therapeutics have yet to be evaluated.

Second-line therapeutics

When first-line DMTs fail, fingolimod (Gilenya™), natalizumab (Tysabri™), rituximab (Rituxan™), ocrelizumab, and mitoxantrone (Novantrone™) are used for second-line or third-line treatment, albeit with more safety risks (69). Fingolimod is an oral sphingosine-1 phosphate receptor (S1Pr) modulator. It blocks lymphocytes from exiting lymph nodes by downregulating the expression of S1Pr. Moreover, *in vitro* research has demonstrated a direct cytoprotectively effect in oligodendrocyte precursor cells (70-72). Treatment reduces annualized relapse rates, the risk of disability progression and the formation of new lesions (73). However, adverse effects such as bradycardia, hypertension, macular edema and skin cancer have been observed in patients (74). Natalizumab is a humanized monoclonal antibody that binds the alpha-4 integrins (VLA-4), which blocks the entrance of inflammatory leukocytes into the CNS. It reduces annualized relapse rates, the risk of disability progression and the formation of new lesions (75). The most notable side effect is the occurrence of progressive multifocal leukoencephalopathy (PML), caused by the John Cunningham virus (76). Rituximab and ocreluzimab are monoclonal antibodies that bind and deplete $CD20^+$ B cells. Compared to the mouse chimeric monoclonal rituximab, ocreluzimab is a humanized monoclonal antibody that has a higher capacity for direct, antibody-dependent cell toxicity and a lower capacity for indirect, complement-mediated cell killing thereby limiting damaging effects on the immune system. As with natalizumab, their most notable side effect is the occurrence of PML. Mitoxantrone works by intercalating with DNA to suppress repair processes. This induces apoptosis in proliferating immune cells. Important side effects include cardiomyopathy and acute leukemia, thereby limiting widespread use (77, 78).

1.2 Phagocytes in multiple sclerosis

1.2.1 Monocyte-derived macrophages

Circulating monocytes are generally considered to give rise to tissue-resident macrophages after extravasation into the tissue with their phenotype defined by the presence of environmental cues (79). Proinflammatory stimuli (e.g. LPS and IFN-γ) induce "classically activated" M1 macrophages. In contrast, anti-inflammatory stimuli (e.g. IL-4, IL-5 and IL-13) induce "alternatively activated" M2 macrophages. The M1 (proinflammatory) and M2 (anti-inflammatory) paradigm has been useful to characterize the functional phenotypes of phagocytes in divergent disorders. However, recent studies show that phagocytes show remarkable plasticity and likely adopt a spectrum of polarizations depending on environmental cues (80-82). Human monocytes express large quantities of the CD14 antigen, which is part of the receptor for lipopolysaccharide (LPS), and CD16, a low affinity Fc receptor (FcγRIII). When monocytes exit the bone marrow, they adopt two distinct fates. In humans, $CCR2^{lo}CD14^{hi}CD16^{hi}$ monocytes, analogous to mice $LyC6^{lo}CX3CR1^{hi}$, have a "patrolling" function as they migrate along the vascular endothelium in search for tissue damage or infections (83). They lack C-C chemokine receptor type 2 (CCR2) expression and cannot react to monocyte chemoattractant protein-1 (MCP-1) (84), resulting in slow recruitment to damaged or infected tissues (85). In contrast, human $CCR2^{hi}CD14^{hi}CD16^{lo}$ resembling $LyC6^{hi}CCR2^{hi}$ monocytes, express high levels of CCR2 and are rapidly mobilized during inflammation via the inflammatory chemokine C-C motif ligand 2 (CCL2) (85-87). The first population is the major monocyte population, while the latter only accounts for 10% of blood monocytes (86, 88).

Phagocytes play a crucial role in MS (fig. 1.2). Monocyte-derived macrophages are abundantly present in active demyelinating MS lesions, and are often found near terminal ovoids of transected axons (89, 90). They are characterized by an increased secretion of inflammatory and toxic mediators, and an elevated expression of costimulatory molecules (91, 92). A substantial number of foamy phagocytes, containing degenerated myelin, is present in active MS lesions (93, 94). These cells are likely derived from resident microglia and infiltrated monocyte-derived macrophages as both cell types have been shown to internalize

myelin *in vitro* (94, 95). Scavenger receptors (SRs) such as CD36, lectin-like oxLDL receptor (LOX1) and scavenger receptors A (SRA) (96), complement, and Fc are the principal receptors for recognition and internalization of myelin components (97-101). This is evidenced by the fact that anti-myelin antibodies are present in MS that are shown to cause demyelination in animal models of MS (102-104), the massive activation of complement in MS patients (105, 106), and the reduced demyelination in $SRA^{-/-}$ EAE mice (107). Recently, the low-density receptor-related protein (LRP1) has been added to the list of receptors involved in myelin phagocytosis (108).

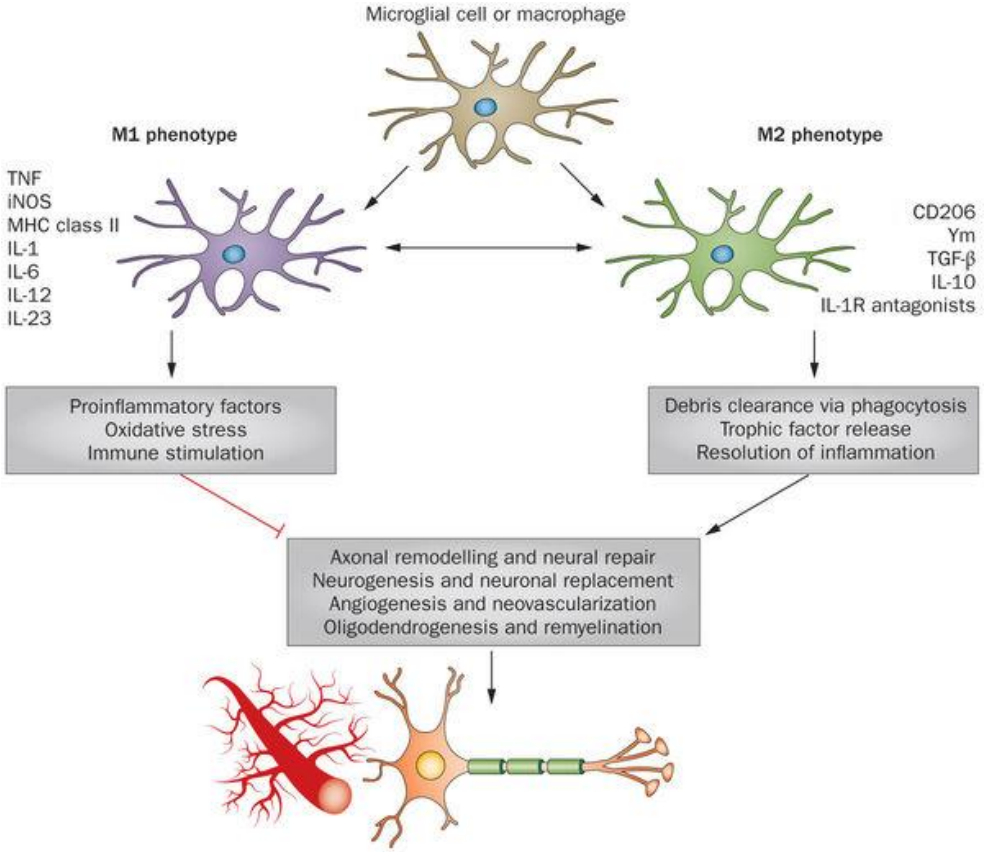


Figure 1.2 | Polarized phagocytes in MS. Phagocytes polarize toward M1 or M2 phenotype based on environmental cues. M1 phagocytes, characterized by the expression of TNFα, iNOS, IL-6, etc. release proinflammatory factors and free radicals that impair CNS regeneration. In contrast, M2 phagocytes, characterized by the expression of TGFβ, CD206, IL-10, etc. improve CNS regeneration by enhancing the clearance of myelin debris, releasing neurotrophic factors, and resolving CNS inflammation. Reprinted with permission from Nature Publishing Group (109).

Although monocyte heterogeneity was unexplored, early studies already established a causal relation between monocytes and the severity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (110). Recent studies elaborated on this by showing that LyC6^{hi} monocytes are highly mobilized into the circulation before EAE symptoms, and that they are pivotal in the effector phase (111-113). Although LyC6^{hi} monocytes appear to promote inflammation and cytodegeneration in the CNS, other studies provide evidence of repair-promoting activities. For example, infiltration of LyC6^{hi} monocytes coincides with CNS remyelination after lysolecithin-induced demyelination in mice (114). Moreover, monocyte-derived macrophages may promote CNS repair by clearing damaged myelin, a strong inhibitor of remyelination and axonal sprouting (115, 116). It is currently not clear whether human analogues of patrolling LyC6^{lo} monocytes play a role in MS pathology or if they promote repair processes. Thus far, only one paper addressed the role of LyC6^{lo} monocytes in EAE. Researchers found a small number of LyC6^{lo} monocytes in the inflamed CNS, suggesting a minor or redundant role of this monocyte subtype in the pathogenesis of EAE (113). Altogether, more research is needed to determine if there is a role for patrolling macrophages in MS.

1.2.2 Microglia

Microglia reside in the parenchyma of the CNS and fulfill a pivotal role in the conservation of CNS homeostasis (117). They differ from monocyte-derived macrophages in their ontogenetic trajectories. While monocyte-derived macrophages stem from hematopoietic progenitor cells, microglia originate from lineage-committed precursor cells in the yolk sack (118). Although microglia vigilantly guard the CNS, they are involved in the development and progression of MS. Microglia activation is widespread in early and late MS and EAE, and corresponds with axon and OLN pathology (89, 119). In concordance, activated microglia display a high expression of MHC class II and costimulatory molecules, and secrete a range of inflammatory and neurotoxic mediators in MS and EAE lesions (91, 94, 120-122). Moreover, it is known that microglia activation precedes EAE onset, and that the suppression of their activation inhibits the development and preservation of inflammatory lesions in the CNS (123-125).

In MS, clusters of activated microglia termed preactive lesions are present throughout the normal appearing white matter (NAWM). They occur in the absence of demyelination and leukocyte infiltration (126). Given the high frequency of preactive lesions, it is unlikely that most of them will progress into full-blown demyelinating lesions. However, they may represent an initial stage in the development of demyelinating lesions (127). Although preactive lesions correlate with the number of active lesions, they may support the inside-out hypothesis that postulates that local CNS cytodegeneration is the initial event (18, 126).

Microglia can also exert a key role in neuroprotection and CNS repair. Similar to monocyte-derived macrophages, microglia acquire a more anti-inflammatory phenotype after myelin phagocytosis both *in vivo* and *in vitro* (94, 95, 128). Importantly, microglia can promote oligodendrocyte precursor cell differentiation in animal models by clearing myelin debris and producing growth factors such as insulin-like growth factor 1 (IGF-1) and activin A (115, 129, 130). Collectively, these studies indicate the complex nature of microglia behavior and seemingly opposing roles in MS. The eventual outcome of CNS inflammation and healing may thus depend on a subtle balance of microglia immunomodulatory cues.

1.2.3 Other CNS macrophages

In addition to microglia, other macrophage populations are present in the CNS. Perivascular macrophages (pvMΦ), together with choroid plexus (cpMΦ) and meningeal macrophages (mMΦ) represent the bulk of resident CNS macrophages (131), and are distinct from monocyte-derived macrophages (132).

PvMΦ reside in the perivascular space demarcated by the glia limitans, formed by astrocytic endfeet processes along the vascular basement membrane (Virchow-Robin space). PvMΦ originate from hematopoietic precursors and are juxtaposed to the outer vessel wall (131). Substantial evidence indicates that pvMΦ are involved in the initiation and maintenance of neuroinflammation in MS and EAE (133-135). Moreover, they are competent APCs and are known to reactivate primed myelin-reactive T cells infiltrating the CNS. This likely explains their disease-promoting role in the EAE model (136). In contrast, pvMΦ may also perform neuroprotective functions. In early MS, pvMΦ produce neuroprotective

factors such as leukemia inhibitory factor (LIF) and brain-derived neurotrophic factor (BDNF) (137, 138). Moreover, myelin-laden pvMΦ exhibit characteristics of anti-inflammatory macrophages in MS lesions (93, 94). These studies suggest pvMΦ may fulfill neuroprotective roles in MS. However, more research is needed to determine whether a change in their functional properties alters the effect they have on neurons, glial cells and leukocyte extravasation.

CpMΦ are a distinct population of CNS macrophages. In contrast with other CNS macrophages, they originate from circulating bone-marrow-derived monocytes and embryonic progenitors. They are relatively short-lived and can be replenished by blood monocytes (131). The choroid plexus is a secretory tissue responsible for producing CSF in the vertebrate brain. Together with the BBB, the choroid plexus forms the interface between periphery and CNS (139). The presence of immune cells in the choroid plexus strongly indicates that it plays a role in neuroinflammatory disorders. In concordance, choroid plexus inflammation is widespread in MS patients as well as in other neurologic disorders (140, 141). Moreover, it precedes lesion formation and infiltration of leukocytes into the CNS in the EAE model (142), indicating the choroid plexus likely acts as a primary gateway for entry of T cells into the CNS (143, 144). Although the expression of adhesion molecules and secretion of chemokines by the choroid plexus has been extensively studied, the role of cpMΦ remains largely unknown (141, 145, 146).

Ontologically similar to pvMΦ and cpMΦ, mMΦ reside in the meninges of which the primary function is to protect the CNS from injury. However, the meninges also modulates brain development, reabsorbs CSF and stimulates glial scarring (147). Several lines of evidence indicate that meningeal inflammation is present in and promotes the progression of MS and EAE (147). Similar to cpMΦ, mMΦ express MHC class II indicating APC function and can maintain parenchymal pathology by releasing inflammatory and toxic mediates into the CNS (148, 149). Moreover, *in vitro* and *in vivo* activation of mMφ and conditioned media from mMφ activates microglia and astrocytes (150-153). These studies support the relation between meningeal inflammation and cortical demyelination in MS (154). Interestingly, activated mMφ produce also neurotrophic prostaglandin E2 (PGE2), which regulates the production of transforming growth factor β (TGFβ) by neurons and glial cells (155). The neuroprotective and immunosuppressive nature of TGFβ

indicate that mMφ may have disease-resolving functions in MS. However, PGE2 has also been known to exacerbate EAE pathology. Collectively, more research is needed to determine the phenotype functions and complex interactions of mMφ in a healthy and diseased CNS.

1.3 Sterol metabolism

Cholesterol was first discovered by François Poulletier de la Salle in 1769 in gallstones. In 1815, was rediscovered by the chemist Michel Eugène Chevreul who called it cholesterol after the two Greek words chole- (bile) and stereos (solid), and added the chemical suffix –ol for the alcohol group on position 3 (fig. 1.3) (156). Cholesterol is an important component of myelin and serves as a precursor for the biosynthesis of steroid hormones, bile acids, oxysterols, and vitamin D (157). In this chapter, I address how cholesterol is synthesized and transported throughout the body. I focus on low-density lipoprotein (LDL), and high-density lipoprotein (HDL), key components necessary for transporting cholesterol in the body's aqueous environment. Next, I will focus on how cholesterol homeostasis is maintained in the body. As one of the most intensely regulated processes in the human body, cholesterol uptake and removal are tightly controlled by the LDL receptor (LDLr) and the liver X receptors (LXRs). Lastly, I will focus on apoE, which is a surface constituent of lipoproteins that plays a key role in maintaining plasma cholesterol homeostasis.

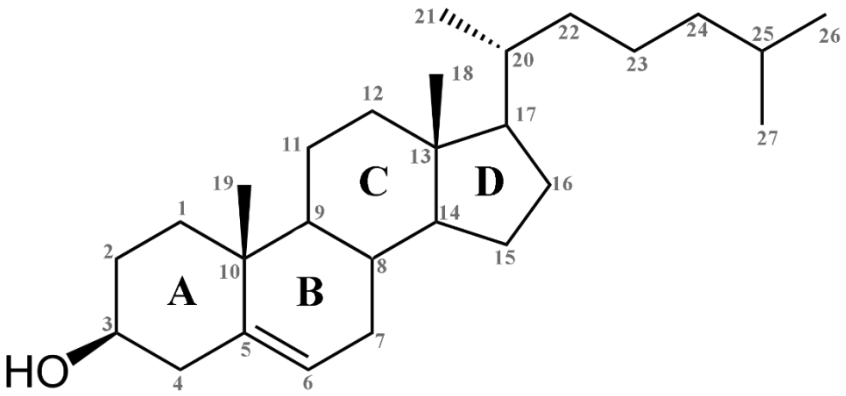


Figure 1.3 | The molecular structure of cholesterol.

1.3.1 Cholesterol synthesis

Cholesterol biosynthesis is a complex process involving more than 20 reaction steps and intermediates. In short, cholesterol biosynthesis begins with acetyl coenzyme A (acetyl-CoA) that is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Next, the rate limiting and irreversible step is the reduction of HMG-CoA into mevalonate. Mevalonate is converted to the isoprene-based molecule,

isopentenyl pyrophosphate (IPP), with the concomitant loss of CO_2. IPP is then converted to squalene, which in turn is converted to 5-cholesten-3β-ol, or cholesterol. This conversion occurs either via the Bloch pathway involving desmosterol or via the Kandutsch-Russel pathway involving lathosterol as the most important intermediate (158). 24-dehydroxycholesterol reductase (DHCR24) or Seladin-1 is an oxidoreductase, which catalyzes the reduction of the delta-24 double bond of sterol intermediates of the Bloch pathway. For example, it converts lanosterol and desmosterol to 24,25-dihydro-lanosterol and cholesterol, respectively (159).

1.3.2 Cholesterol transport

Cholesterol is transported in the body's aqueous extracellular environment by five major types of lipoproteins: chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL, and HDL, with a decreasing size and lipid content and an increasing density and protein content, respectively. Cholesterol can be synthesized *de novo* (endogenous pathway, ~2/3rd) or be absorbed in the intestines from dietary and biliary sources (exogenous pathway, ~1/3rd). VLDL, IDL and LDL transport cholesterol from the liver to the tissues. Because high LDL levels can lead to plaque formation in arterial walls it is often called 'bad cholesterol'. In contrast, HDL is termed 'good cholesterol' because it removes cholesterol from the tissues and transports it to the liver for excretion. Cholesterol plasma levels are coordinated by the liver, which serves as the key organ for cholesterol metabolism and homeostasis. Cholesterol, either from gut absorption or *de novo* synthesis, is repackaged by the liver with proteins, triglycerides, and phospholipids into VLDL. VLDL then enters the blood circulation and is converted by lipoprotein lipase (LPL) and, in humans, cholesteryl ester transfer protein (CETP) into more cholesterol-enriched lipoproteins, IDL, followed by LDL. In contrast, HDL is generated through the secretion of apolipoprotein A1 (APOA1), APOA2 and phospholipids by both the liver and intestine. HDL receives cholesterol from peripheral tissues via reverse cholesterol transport (RCT), mediated by ATP-binding cassette transporter (ABCA1) (160, 161). The unesterified (free) cholesterol in HDL is then esterified by lecithin-cholesterol-acyltransferase (LCAT), forming mature HDL. Mature HDL also promotes RCT via ABCG1 (162, 163).

Comparable to the lipoproteins in the periphery, the brain contains HDL-like transport vehicles for lipids (164). The principal apolipoprotein of the CNS is apolipoprotein E (apoE), which is predominantly secreted by astrocytes and microglia (165). In the CNS, astrocytes are the most important lipid suppliers as full myelination membrane synthesis requires astrocyte lipid supply in addition to endogenous oligodendrocyte lipid synthesis (166). When sterols are synthesized, they are released by lipidation of discoidal apoE or lipid-poor lipoproteins mediated by ABC transporters of which ABCA1 and ABCG1 are the most important ones in the CNS (167). Neurons and oligodendrocytes subsequently express LDL and VLDL receptors to take up sterols from these lipoprotein particles (168).

High-density lipoprotein

HDL comprises a heterogeneous class of lipoproteins with a density of 1.06-1.21 g/mL and a size ranging from 6-20 nm (169). Depending on its maturation stage, HDL contains about 55% proteins, 26-46% phospholipids, 15-30% esterified cholesterol, 3-15% triglycerides, and 2-10% free cholesterol (170). The normal range for healthy individuals is 40-60 mg/dL. The primary protein components of HDL are dual APOA1 peptides, which form the initial structure of the nascent, discoidal HDL (171). APOA1 is recognized by some of the most important proteins involved in RCT: LCAT, ABCA1 and scavenger receptor BI (SR-BI) (172). ApoA1-deficient mice do not form normal HDL particles and exhibit 70-80% reductions in both plasma cholesterol and HDL (173). Therefore, APOA1 is not only the principal structural component, but it also functions as the recognition molecule for most of the proteins that interact with HDL during RCT. In cholesterol-loaded macrophages, the efflux process starts as discoidal HDL binds to ABCA1 on the cell surface via APOA1. Next, HDL is internalized and transported into endocytic vesicles inside the cell (174). There, ABCA1 and Niemann Pick C1 (NPC1) proteins mediate the transfer of free cholesterol into intracellular HDL (175). The complex then returns to the cell surface and dissociates, releasing the cholesterol-enriched particles (174). Inside the discoidal HDL, free cholesterol is converted to esterified cholesterol (CE) by LCAT (176). CE are more hydrophobic than free cholesterol and accumulate in the center of HDL, causing a change in the geometry from discoidal to spherical or mature HDL. This process is pivotal to cholesterol efflux,

because it helps to maintain a concentration gradient favoring the addition of free cholesterol to lipoproteins (177, 178).

Low-density lipoprotein

LDL is the principal transporter of cholesterol and fat in human blood. The normal range for healthy individuals is below 100 mg/dL. LDL functions by mediating cellular uptake via receptor-mediated endocytosis followed by lysosomal degradation (179, 180). Apolipoprotein B100 (APOB100) is the sole protein component of LDL being mainly located on its surface. Apart from their well-established role as a lipid transporter, LDL is strongly involved in the progression of cardiovascular diseases such as atherosclerosis or stroke, which are among the most prevalent causes of death in developed countries (181). LDL is highly heterogeneous in nature, varying in its size, densities, and chemical composition (182, 183). These characteristics also determine its biological nature. For example, in RRMS patients, we found smaller LDL particles compared to healthy controls (184). LDL size is described to be important for LDL function and may play a role in the pathophysiology of MS and other diseases as smaller particles are more atherogenic, susceptible to oxidation, and have a decreased LDL receptor (LDLr) affinity (185-187).

1.3.3 Low-density lipoprotein receptor

Cellular cholesterol homeostasis (fig. 1.4) is one of the most intensely regulated processes in biology (188). Cholesterol uptake and removal are tightly controlled by feedback mechanisms that operate at both transcriptional and post-transcriptional levels (189, 190). When intracellular levels are low, sterol regulatory element-binding proteins (SREBPs) coordinate the transcriptional activation of HMG-CoA and almost all down-stream enzymes of the mevalonate pathway (191), and activate the transcription of LDLr (190).

The LDLr is a 160kDa cell membrane glycoprotein that mediates clearance of cholesterol and cholesteryl ester-containing LDL particles from blood (179). It binds two physiologically important ligands, APOB100 and apoE. The only known ligand for the LDLr in the CNS is apoE since APOB cannot pass the BBB and, in contrast to apoE, is not synthesized in the CNS (192). LDLr is ubiquitously expressed and genetic mutations result in a condition with extremely elevated

serum LDL levels and early onset atherosclerosis known as familial hypercholesterolemia (FH) (193). FH is characterized by defective catabolism of LDL, which results in elevated plasma cholesterol and lipid accumulation in immune cells. This stimulates proinflammatory responses, including enhanced NF-κB signaling, inflammasome activation, and increased production of monocytes and neutrophils in the bone marrow and spleen (194-198).

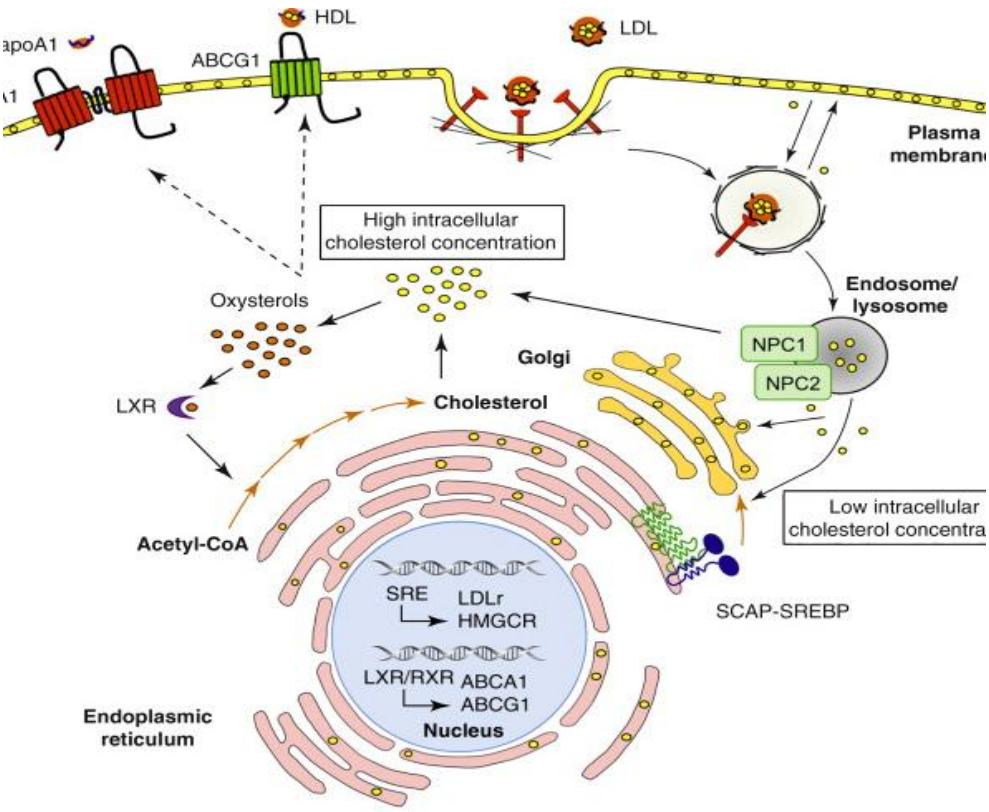


Figure 1.4 | Regulation of cellular cholesterol metabolism. When intracellular cholesterol levels are too low, the SCAP-SREBP complex moves to the Golgi apparatus where it is cleaved into its active form (SREBP). SREBP1 then migrates to the nucleus and enables cholesterol biosynthesis (HMGCR) and cholesterol uptake (LDLr). Conversely, when intracellular cholesterol levels are too high, the generated oxysterols bind and activate liver X receptors, which heterodimerize with retinoid X receptors and activate the expression of ATP transporters (ABCA1 and ABCG1). ABCA1 and ABCG1 promote cholesterol efflux via apoA1 and HDL respectively. Reprinted with permission from Elsevier (199).

Receptor-mediated endocytosis of LDL-cholesterol initiates upon binding of LDL to its membrane receptor via APOB100 or apoE. The LDL-LDLr complex is taken up via clathrin-mediated endocytosis. In endosomes, the receptor dissociates from its ligand due to low pH and recycles back to the cell surface. LDL is targeted to lysosomes where esterified cholesterol is hydrolyzed, freeing cholesterol for incorporation into cell membranes (200). To prevent intracellular overload, excess free cholesterol is re-esterified in the cytoplasm where it accumulates as cytoplasmic cholesteryl ester droplets (201). Moreover, rising intracellular cholesterol levels inhibit the biosynthesis of cholesterol and the expression of LDLr, preventing further LDLr-mediated cholesterol uptake. Unfortunately, oxidized LDL (oxLDL) is readily taken up by macrophages via scavenger receptors. In atherosclerosis, the uptake of oxLDL results in cholesterol-overloaded foam cells that display a proinflammatory and proatherogenic macrophage phenotype (202). In contrast with foam cells in atherosclerosis, foamy macrophages present in active demyelinating MS lesions express various markers that are involved in anti-inflammatory processes including IL-1RA, IL-10, CCL18 and TGFβ (94). Interestingly, we recently demonstrated that myelin-derived lipids can activate the anti-inflammatory nuclear liver X receptors in rat macrophages and skew these macrophages towards a less inflammatory phenotype *in vitro* (203).

1.3.4 Liver X receptors

Because cells cannot degrade cholesterol, excess cholesterol must be removed via RCT, which is controlled by liver X receptors (LXRs). The LXRs, LXRα (*NR1H3*) and LXRβ (*NR1H2*), are nuclear transcription factors that directly control the expression of genes involved in cholesterol metabolism. The tissue distribution of the two isoforms differs considerably, suggesting that both isoforms have different roles in regulating cholesterol homeostasis. Whereas LXRβ is ubiquitously expressed, LXRα is highly expressed in the liver, lung, spleen, intestine, kidney, fat cells, and macrophages. LXRs form heterodimers with retinoid X receptors (RXRs) and are activated by cholesterol biosynthetic intermediates, such as desmosterol and oxysterols (204). Oxysterols are oxygenated derivatives of cholesterol derived from diet or generated upon oxidation of cholesterol metabolites or lipoproteins (205-207). For example, oxidized LDL is rich in oxysterols (208, 209). Aside from natural agonists, about 50 potent synthetic

agonists have been developed, including T0901317 and GW3965 (210-212). Although these agonists do not discriminate between LXR isoforms, they have been widely used in the last decades to elucidate the biological actions of LXRs. The binding of an agonist to LXRs triggers a conformational change in the receptor that enhances interaction with coactivator proteins facilitating transcription of genes involved in cholesterol efflux, including ABCA1, ABCG1, ABCG5, and ABCG8 (213). ABCA1 and ABCG1 promote cellular cholesterol efflux to HDL and APOA1, while ABCG5 and ABCG8 promote cholesterol secretion into the bile (214, 215).

In addition, LXRs exert anti-inflammatory effects through multiple pathways. First, activation of LXRs causes SUMOylation of specific residues in their ligand-binding domain, leading to the transrepression of genes involved in inflammation (216). For example, several research groups have demonstrated that SUMOylated LXRs are required for the suppression of NF-κB-dependent and (LPS)-induced TLR4 inflammatory responses by LXRs (216-218). Second, LXRs suppress TLR-mediated inflammatory processes by stimulating cholesterol efflux from macrophages, as induction of ABCA1 and ABCG1 transporters suppresses these inflammatory responses (219, 220). Moreover, hematopoietic stem cells (HSCs) highly express the LXR-target apoE on its cell-surface proteoglycans, which promotes cholesterol efflux via ABCA1 and ABCG1, suppressing the proliferative responses of these cells (221). Third, LXRs induce the expression of several genes involved in the elongation and unsaturation of fatty acids, which ultimately leads to the formation of long-chain polyunsaturated fatty acids (PUFAs) (222). The increase of long-chain PUFAs alters histone acetylation in enhancer and/or promotor regions of NF-κB target genes, thereby suppressing inflammatory responses (222). Moreover, LXRα induces lysophospholipid acyl-transferase 5 (LPCAT3) in the liver, resulting in both decreased stress levels in the endoplasmic reticulum (ER), and reduced inflammatory responses (223). Finally, in phagocytes LXRs stimulate the expression of tyrosine protein kinase MER (MERTK), which decreases TLR4-dependent upregulation of ABCA1 and ABCG1 by promoting the uptake of apoptotic cells (224, 225). Based on these studies, activation of LXRs represents an interesting therapeutic option for neuroinflammatory disorders such as MS. As 25% of the lipid content in myelin consists of cholesterol, it is likely that myelin-loaded phagocytes in MS display a phenotype that is in part dictated by a myelin-mediated activation of LXRs (226). However, it remains to be elucidated if

activation of LXRs is similarly beneficial in myelin-phagocytosing macrophages present in demyelinating MS lesions.

1.3.5 Apolipoprotein E

ApoE is a 34kDa surface constituent of lipoproteins that plays a key role in maintaining plasma cholesterol homeostasis (227). It directs movement of lipids from the periphery to the liver, where high affinity binding of apoE to the LDLr and many other membranes of the LDLr family facilitate the uptake of lipoprotein particles (228). In addition, to its role as a lipid transporter, apoE has also been shown to have anti-inflammatory, anti-atherogenic and immune modulatory properties. It suppresses the activation and proliferation of T cells (229), promotes conversion of M1 to M2 macrophages (230), modulates the clearance of apoptotic bodies (231), and suppresses the systemic type I inflammatory response (232). ApoE has two major domains, a 22kDa N-terminal domain that binds to members of the LDLr family, and a 10kDa C-terminal domain that contains the primary lipoprotein-binding elements (233). In humans, the polymorphism of the apoE gene results in three alleles (ε2, ε3, and ε4). The ε3 allele accounts for about 70-80% of the gene pool. The ε2 and ε4 alleles account for only 5-10% and 10-15%, respectively (234). ApoE4 has distinct structural and biophysical properties that differentiate it from other isoforms by domain interaction and lack of cysteine, which are possibly associated with astrocyte dysfunction and impaired maintenance of neurons (233-235). Individuals with ApoE4 are at increased risk of Alzheimer's disease (AD) compared with those carrying ε3, whereas the ε2 allele decreases risk (236).

ApoE is primarily synthesized in the liver. Although the liver generates about 70% of the total body apoE, it is also secreted by other cell types including macrophages and adipocytes (228, 237, 238). In macrophages, apoE is synthesized in the ER and transported to the Golgi apparatus where it is post-translationally modified and incorporated into vesicles before being transported to the plasma membrane and secreted (228). ApoE represents up to 8% to 10% of protein constitutively secreted from macrophages (239). ApoE transcription in macrophages is directly regulated by LXRs and to a lesser extent by peroxisome proliferator-activated receptors (PPARs) and activator protein-1 (240-243). LXRs regulate apoE levels by interacting with a conserved LXR response element

(LXRRE) present in the promoter region of the apoE gene in macrophages (242). Although the LXR-induced increase of apoE expression is reduced in LXRα$^{-/-}$ or LXRβ$^{-/-}$ mice and abolished in LXRαβ$^{-/-}$, the basal apoE level is not compromised, indicating that LXRs mediate the lipid-induced expression of apoE (244). This is especially relevant in the context of MS where the breakdown and phagocytosis of the lipid-rich myelin by phagocytes leads to elevated intracellular free cholesterol and oxysterol-induced toxicity (245). LXR activation and subsequent upregulation of apoE may help protect these cells by inducing RCT.

In the CNS, apoE plays a key role in the lipid transport between neurons and glial cells (246). Because the CNS is shielded from lipids in the circulation by the BBB, most neutral lipids are synthesized *in situ* (226). Astrocytes are one of the most important cellular sources of lipid synthesis and secretion in the CNS (159, 247-249). Coincidentally, apoE is predominantly synthesized and secreted by astrocytes to generate apoE-containing lipoprotein-like particles that supply neurons and oligodendrocytes with cholesterol (166, 250). Since virtually all cholesterol is synthesized locally, astrocytes are the main hubs of cholesterol distribution throughout the CNS (251, 252) and activation of LXRs directly orchestrates cholesterol turnover in astrocytes (251, 253). In MS, chronic neurodegeneration leads to lowered levels of cholesterol precursors, apoE, and oxysterols in the circulation (254-256). Therefore, the metabolism of myelin lipids, such as cholesterol, could be a therapeutic target (257). For example, synthetic LXR agonists ameliorate the functional deficits associated with AD, possibly via their ability to modulate the cholesterol metabolism (251, 258). However, these LXR agonists induce severe hypertriglyceridemia and liver steatosis, rendering them unsuitable for use as a therapeutic application (259). Therefore, LXR agonists that favorably affect cholesterol metabolism without unfavorably affecting on hepatic triglyceride metabolism are of particular interest.

1.3.6 Plant sterols

Partially based on:

Plant sterols: Friend or foe in CNS disorders?

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Plant sterols and plant stanols, collectively known as phytosterols, are naturally occurring compounds that structurally and functionally resemble cholesterol in mammals. Over the last decades, more than 260 different phytosterols (derivatives) have been described (260). By using cell-free and cell-based assays, multiple studies defined that plant sterols, such as sitosterol, fucosterol, stigmasterol, schottenol, 24(S)-saringosterol, and spinasterol, bind and activate LXRα and/or LXRβ (261-266). Interestingly, in contrast to synthetic LXR agonists such as T0901317 and GW3965 (204, 210, 267), phytosterols do not induce hypertriglyceridemia and hepatic steatosis (204, 210, 267-271).

The chemical structure of phytosterols consists of a sterol-derived core that is decorated with divergent ring and/or C17 side-chain modifications (fig. 1.2). Plant stanols are saturated plant sterols, without double bonds in the sterol ring moiety. Mammals are unable to synthesize phytosterols and therefore can only obtain them from their diet. Food rich in phytosterols include vegetables, fruits, nuts, cereals, vegetable oils, and phytosterol-enriched diary spreads. Typically, the daily intake of plant sterols and stanols in humans is estimated on 300 mg and 20 mg, respectively (272, 273). Interestingly, whilst the daily intake of cholesterol approaches that of phytosterols, plasma levels of plant sterols (7-24 µmol/L) and stanols (0.05-0.3 µmol/L) are markedly lower than those of cholesterol (~5 mmol/L) (274, 275). In the jejunum, both phytosterols and cholesterol are incorporated into mixed micelles and are subsequently absorbed at the apical site of enterocytes via the Niemann Pick C1 Like 1 (NPC1L1) transporter (276). However, in contrast to cholesterol, phytosterols are poor substrates for the

esterifying enzyme acetyl-sterol O-acyltransferase 2 (SOAT2/ACAT2) in enterocytes (277). Due to incomplete or inefficient esterification, the bulk of phytosterols is transported back into the intestinal lumen. Moreover, phytosterols that enter the circulation are quickly excreted into the bile by hepatocytes (277). The obligatory heterodimeric complex ABCG5/G8 exerts a crucial role in the selective excretion of phytosterols by enterocytes and hepatocytes, and thereby contributes to the relatively low plasma and tissue levels of phytosterols (278-280).

Plant sterols in the CNS

In parallel to cholesterol, transport of plant sterols towards the CNS is also limited under physiological conditions. However, a dysfunctional BBB may disturb the balanced exchange of sterols between the CNS and the circulation. For instance, *pdgfb*$^{ret/ret}$ mice, which are characterized by an increased permeability of the BBB, show a significant flux of phytosterols into the brain (281). Additionally, it has been shown in an BBB model and in a dietary mouse study that sterol accumulation in CNS cells depends on the molecular complexity of the sterol side chain (282). Sterols with a lower molecular side-chain complexity such as cholesterol and campesterol cross an endothelial barrier more easily as compared to phytosterols that contain a more complex hydrophobic side chain such as sitosterol and stigmasterol (281-283). Interestingly, sitosterol and campesterol crossed a brain endothelial monolayer less efficient than to cholesterol. Whether cholesterol and plant sterol use similar transport mechanisms to cross the BBB remains to be elucidated. Compared to wild type mice, Abcg5$^{-/-}$ and ApoE$^{-/-}$ mice display up to 12-fold increased levels of phytosterols in their circulation. Remarkably, only Abcg5$^{-/-}$, but not ApoE$^{-/-}$ mice, show increased phytosterol levels in the CNS (284). Importantly, the Abcg5/g8 transporters are not detectable within the brain (284). Therefore, it is unlikely that the Abcg5/g8 transporter complex modulates phytosterol transport across the BBB. In addition, whereas in wild type and Abcg5$^{-/-}$ mice phytosterols are predominantly located in high density lipoproteins (HDL), phytosterols are mainly incorporated in very low density lipoproteins (VLDL) in ApoE$^{-/-}$ mice (284). Although it remains speculative, the latter supports a role for HDL-mediated transport of sterols across the BBB. Interestingly, the scavenger receptor class B member 1 (SR-BI) is the major

receptor for HDL and is expressed at the apical membrane of BBB endothelial cells (285). Collectively, these studies make us speculate that, although limited in quantity, sterols may be transported across the BBB into the CNS via HDL/SR-BI-dependent mechanism.

The consumption of phytosterol-enriched functional foods over five years roughly doubles circulating plant sterol and stanol levels (286). Moreover, phytosterols accumulate in peripheral tissues, such as aortic valves, liver, and the CNS (287-289). Quantitative data on temporal and spatial plant sterol accumulation in the human brain parenchyma is rather scarce. One study measured plant sterols in non-demented and demented brain samples (289). Herein, Saeed and colleagues found that sitosterol and campesterol are present in the "5 to 10 ng/mg wet tissue"-range in the temporal and parietal cortex. These concentrations were comparable to the oxysterol concentrations in these regions (24SOHC: 15-25ng/mg; 27OHC: 1-3ng/mg). In contrast, Vanmierlo *et al.* found that the concentration of the most prominent plant sterol in the CSF, sitosterol (2.48 µg/dl), was 10-fold higher than the major CNS cholesterol metabolite in the CSF, 24SOHC (0.264 µg/dl) (287, 290). Moreover, in an animal study, it was shown that a 2% plant sterol-enriched diet over six weeks resulted in a stable doubling of plant sterols in the CNS of mice (282). Once dietary phytosterols have entered the CNS, in particular upon increased dietary intake, they tend to accumulate within lipid rafts of CNS parenchymal cells (282, 284). The incorporation of sterols in biological membranes can result in structural and functional changes in membrane properties. The majority of lipid membrane bilayers occur as a homogeneous liquid-disordered (ld) phase. However, transient lateral heterogeneities coexist in a liquid-ordered (lo) phase, so called lipid rafts (291). Raft domains are enriched in free sterols, mostly cholesterol, and saturated lipids, including sphingomyelin, glycosphingolipids, cerebrosides, and gangliosides. The main membrane phospholipids – phosphatidylcholine, and phosphatidyl-ethanolamine - are mostly excluded from the lipid rafts (292). Cholesterol increases the lipid order and rigidity of the lipid rafts at the cytofacial leaflet of the membrane (293). Moreover, cholesterol is essential to promote the separation between the ld and lo phase, allowing lipid raft-specific cell signaling (294, 295). Lipid rafts are integral membrane scaffolds, acting as a movable platform for processes involved in membrane trafficking and signaling, and regulation of the

activity of membrane proteins (292, 296, 297). Incorporation of less compact lipids reduces the rigidity of lipids rafts and alters their function. Indeed, phytosterols reduce the molecular order of membranes and therefore alter the membrane fluidity and functionality (298, 299). It was demonstrated that the magnitude of the lipid order in membranes depends on the geometry of the side chain (cholesterol >> campesterol > sitosterol > stigmasterol) (298). Interestingly, Vanmierlo *et al.* found that feeding mice a 2% w/w plant sterol enriched diet for six weeks, resulted in a two-fold increase in lipid raft-associated plant sterol concentration (~3.5 ng/mg protein to ~7 ng/mg protein), whereas cholesterol concentration in the lipid raft remained stable (~2,300 ng/mg protein) (282). It remains to be defined whether this 1:300 plant sterol-to-cholesterol ratio is sufficient to functionally modulate membrane and/or raft properties. However, most of the sterols in the CNS are trapped in the sturdy oligodendrocyte myelin pool. The actual, biological active sterol pool in the CNS is limited and consequently more vulnerable for changes in their micro-constitution. Although speculative, the "plant sterol-to-cholesterol balance" is expected to shift more towards the plant sterol side of the balance in those metabolic more active cells. Furthermore, incorporation of 15μM sitosterol in HT22 hippocampal cells improved mitochondrial function by lowering the liquid order in mitochondrial membranes (300). Collectively, although it remains topic of debate whether physiologic relevant plant sterol concentrations are reached within the lipid rafts, a role for plant sterols in modulating lipid raft functioning is gaining support.

Plant sterols in MS

The impact of cholesterol metabolism on normal brain functioning and the pathophysiology of MS has been extensively scrutinized (301-306). Even more, cholesterol-lowering statins reduce lesion relapse rate in early MS patients and have recently been found to curtail the annualized rate of whole-brain atrophy in SPMS patients (307-309). Remarkably, despite their immunomodulatory properties, ability to cross the BBB, and capacity to lower blood cholesterol levels, the effect that phytosterols have on the MS disease progression remains largely elusive.

Numerous studies have reported immunomodulatory properties of phytosterols (310-316). For instance, both non-modified and modified phytosterols suppress

an inflammatory transcriptional profile in macrophages (312, 315-321). Furthermore, plant sterols and plant stanols skew T cells towards a Th1 phenotype independently of their effect of APCs and leaving the activity of Th2 cells unaffected (311, 322, 323). With respect to MS, sitosterol has been reported to decrease the secretion of the inflammatory mediators TNFα and IL-12 by PBMCs from MS patients at physiological relevant concentrations (313). In line with this finding, daily administration of a mixture of sitosterol (60%), campesterol (25%), and stigmasterol (15%) inhibits inflammatory CNS demyelination in the EAE model (324). Even more, this phytosterol mixture delays the onset and decreases disease severity in EAE. The protective effect of phytosterols on EAE severity was paralleled by a reduced infiltration of lymphocytes and macrophages into the CNS, and a dampened inflammatory activity of these immune cells. These findings indicate that phytosterols modulate the inflammatory and migratory activity of leukocytes in EAE-affected animals, thereby affecting neuroinflammation and neurodegeneration. Of note, the reduced infiltration of leukocytes into the CNS of EAE animals is in line with the fact that phytosterols decrease the chemotactic and docking properties of endothelial cells. However, EAE only mimics the inflammatory aspects of MS pathology, not considering modifiable and genetic risk factors. In conclusion, the role of plant sterols in the prevention and treatment of MS holds promise, but caution should be taken extrapolating findings in animal models to the clinic.

Apart from modulating the autoimmune response in MS, phytosterols may affect the pathophysiology of MS by affecting the viability and activity of parenchymal cells. It has been defined that phytosterols stably accumulate in the CNS, especially in glial cells, such as oligodendrocytes and astrocytes, and to a lesser extent in neurons (282, 284, 325). This finding suggests that phytosterols can directly affect the integrity and functioning of these cells, and thereby MS disease progression. Notably, incorporation of *β*-sitosterol in the cell membrane of hippocampal neurons prevents glucose oxidase (GOX)-induced oxidative stress and lipid peroxidation (326). Thus, phytosterols may protect neurons, and likely glial cells (282, 284, 325), from oxidative stress in MS lesions. On the other hand, oxides of β-sitosterol and campesterol are cytotoxic *in vitro* (327). Similar, phytosterol β-D-glucosides are neurotoxic when administrated to astrocytes, neurons, cortical slices, and mice through glutamate excitotoxicity and their

stimulatory effect on the generation of ROS (328-330). In addition, microglia and astrocyte activation is apparent in *β*-sitosterol *β*-D-glucoside treated mice, supporting a role for glial cells in *β*-sitosterol *β*-D-glucoside-induced neurotoxicity (329). To date, it is unclear if the neurotoxic properties of phytosterol glucosides at some point overwhelm the neuroprotective effects of free and esterified phytosterols in the healthy or diseased CNS. Moreover, considering that phytosterol glucosides also lower cholesterol levels in humans (331, 332), it is unknown whether the neurotoxic impact of glycosylated phytosterol outweighs their protective cholesterol-lowering effect in MS. The above studies indicate that the impact of phytosterols on CNS resident cells is dual and largely depends on structural modifications.

Nuclear receptors play a key role in CNS repair processes and neuroinflammation. Activation of LXRs ameliorates EAE and signaling through retinoid X receptor gamma (RXRγ), a heterodimeric partner of LXRs, accelerates CNS remyelination (333-335). Hence, a phytosterol-mediated activation of LXRs may suppress neuroinflammation and promote CNS repair processes in MS patients. Similar, sitosterol binds and activates the estrogen receptor (ER), particularly the ERβ subtype (326, 336, 337). Activation of both ER subtypes provides disease protection in the EAE model and estrogens are currently being evaluated in clinical trials of MS (338, 339). ERβ specific ligands have been reported to enhance endogenous remyelination by increasing the number of myelinating oligodendrocytes (340, 341). It should be noted that, similar to LXR activation, phytosterols only mildly activate ERs as compared to synthetic compounds such as 17-β-Estradiol. Future studies should determine if phytosterols affect CNS repair processes and whether a phytosterol-mediated activation of ERs and LXR represents the biological foundation for their impact on CNS repair.

1.4 Oligodendrocytes in multiple sclerosis

Oligodendrocytes (OLNs) are the myelin-forming cells in the CNS. They originate from oligodendrocyte precursor cells (OPCs), which are the last cells to be generated after neurons and astrocytes during development (fig. 1.5) (342). In the developing brain, several waves of OPCs migrate and generate the entire OLN population. In mice, at E12.5, a first wave of OPCs originates in the ganglionic eminence (343). As development continues, a second and third wave of OPCs derived from the lateral and caudal ganglionic eminences at E15.5 and from the cortex at birth, respectively, give rise to the majority of adult OLNs in mice (344).

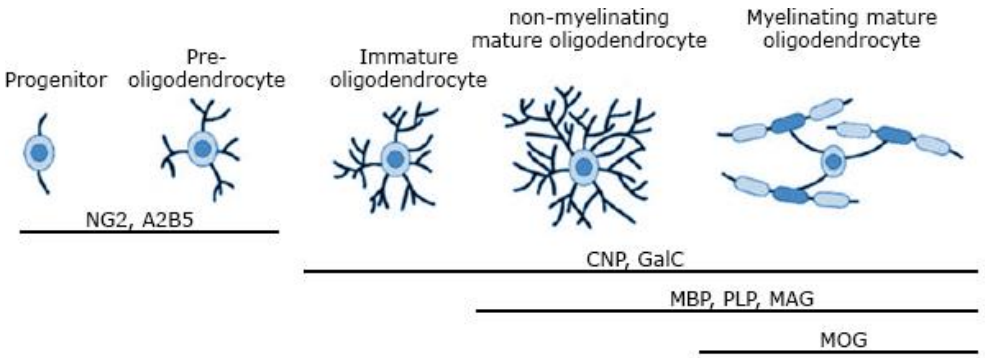

Figure 1.5 | Differentiation of oligodendrocyte cells. Oligodendrocyte precursor cells are positive for NG2 and A2B5 expression. When differentiating into mature oligodendrocytes expression of MBP, PLP and MAG increases significantly (up to 500-fold), confirming maturation stages. Reprinted with permission from John Wiley and Sons (304).

A small number of OPCs generated during development are maintained in an immature and slowly proliferating state in the adult CNS (345). These adult OPCs are present in all brain areas and represent about 2-9% of the CNS cell population (345). Various markers are used to identify adult OPCs, including the proteoglycan NG2, PDGFRα, Olig1 & 2, Nkx2.2 and O4 antigen (346). Because some of these markers are also expressed by other CNS cells, a combination of markers is often suitably to precisely identify OPCs in the adult CNS. Interestingly, lesions containing both cortex and adjacent WM, the cortex showed greater remyelination, more actively remyelinating OLNs and fewer reactive astrocytes (347). Compared to WM, astrocytes in GM do not significantly upregulate CD44, hyaluronic acid and versican, which inhibit remyelination. Moreover, in GM more

neighboring cells are present that provide support for OLNs by supplying lipids necessary for efficient remyelination (301).

Once at their final station, OPCs differentiate to mature myelin-forming cells expressing proteins typical of myelin (MBP, PLP, MOG and MAG, among others) and start the myelination process (348). Myelination is a complex sequence of events that is best understood when conceptualized as separate steps, including (1) membrane outgrowth and axonal wrapping, (2) trafficking of membrane components such as MBP and PLP, (3) myelin compaction, and (4) node formation (342, 349). Most OLNs generate between 20 and 60 myelinating processes with internodal lengths of ~20-200 µm and up to 100 membrane turns (350). The insulated myelin sheath significantly reduces axonal energy consumption, while increasing saltatory conduction 10- to 100-fold (351-353).

1.4.1 Remyelination in MS

A cardinal hallmark of MS is demyelination, which is present during all stages of the disease (32). Demyelinated lesions in the brain can be repaired by remyelination. OLNs start repairing myelin sheaths during early stages of lesion development, even when demyelination is still ongoing (354-356). Interestingly, remyelination not only occurs in RRMS patients, but also in a subset of PPMS patients (357). In MS, remyelination eventually fails, resulting in the loss of neurons (358). As few as 10 to 20% of chronic MS lesions are completely remyelinated (the so-called shadow plaques) (359, 360). Understanding why endogenous remyelination in MS fails is essential to the development of effective remyelination and repair strategies. One hypothesis for remyelination failure is that the number of adult OPCs available for remyelination is exhausted over time (361-363). However, post-mortem analysis of patients with long-lasting MS, revealed the presence of OPCs throughout the CNS, including within MS lesions (364, 365). Even though many OPCs are present, they fail to mature into myelin-forming OLNs. This implies that the lesion microenvironment is prohibitive to OPC differentiation and subsequent remyelination. Many changes occur during demyelination that could hinder remyelination: aberrant deposition of extracellular matrix (ECM) components, including fibronectin, hyaluronic acid, versican, and chondroitin sulfate proteoglycans (347, 366, 367), and exposing OPCs to inhibitory cues including components of damaged myelin such as MAG,

MOG, and neurite outgrowth inhibitor-A (NOGO-A) (115, 368-370). In addition, activation of both the innate CNS and peripheral immune cells leads to the release of proinflammatory mediators that can also negatively impact remyelination (371-373).

There are several promising strategies to stimulate the remyelination capacity of endogenous OPCs, by manipulating signaling pathways within OLNs to override the inhibition of remyelination, or altering the microenvironment to be more tolerant to OPC differentiation and remyelination (374). Likely, to be successful, one would need to dampen lesion inflammation, support the survival and differentiation of endogenous OPCs to allow for remyelination, and increase survival of naked axons so that they may be effectively remyelinated.

1.4.2 The role of sterols in remyelination

An important biochemical characteristic of myelin is its high lipid-to-protein ratio. Lipids, such as cholesterol, account for at least 70% of the dry weight of myelin (375), which is twice that of regular plasma membranes (376). The availability of lipids is a rate-limiting step in myelination, which is illustrated by the perturbed myelin formation in mice with defective lipid synthesis in OLNs (301, 377, 378). Similar, MS patients have a disturbed CNS lipid metabolism as HDL and apoE activity, and concentrations of 24SOHC, oxysterols, and cholesterol precursors have been shown to be altered in MS patients (184, 254, 256, 379, 380). Interestingly, uptake of extracellular cholesterol by OLNs partially rescues myelination over time (378). In concordance, mice carrying an OLN-specific deletion of squalene synthase (SQS), an enzyme pivotal in cholesterol synthesis, initially display signs of hypomyelination in the CNS. However, mutant OLNs survive by using cholesterol from neighboring wild type cells, such as astrocytes. This was evidenced by increased steady-state levels of apoE and low-density lipoprotein receptor-related protein 1 (LRP1), suggesting an enhanced lipoprotein particle-mediated cholesterol transfer in mutant mice (301). Results from these studies indicate a role for lipid-supplying glial cells during myelination.

Indeed, a recent study from the group of Verheijen *et al.* showed that abrogated astroglial synthesis resulted in CNS hypomyelination. More importantly, a lipid-enriched diet rescued full myelin membrane synthesis as OLNs incorporated

circulating lipids into the myelin membrane (166). The importance of dietary sterols in stimulating remyelination is further demonstrated by a recent study from Saher *et al.* in which the authors found that a high-cholesterol diet in mice improved remyelination after cuprizone-induced demyelination by increasing the rate of OPC differentiation (381). Collectively, these studies point to a role for dietary sterol supplementation in facilitating CNS repair mechanisms, leading to improved remyelination and neurological outcome.

1.5 Aims of the study

The pathological hallmarks of MS are focal demyelination and neurodegeneration. Infiltrated macrophages and activated microglia are present in active lesions, contributing to disease progression. However, recent studies have shown that phagocytes also reduce demyelination and promote remyelination by clearing the inhibitory myelin debris and the secretion of neurotrophic factors, indicating a key role in dampening MS disease progression and enhancing CNS repair. During the inflammatory response, lipid homeostasis of MS patients is disturbed and is correlated to neurodegeneration (184, 254, 256, 379, 380). Moreover, a disrupted cholesterol homeostasis can change the inflammatory phagocyte phenotype, affecting disease progression. Cholesterol is the most abundant lipid within myelin and plays a key role in (re)myelination. As ingestion of its biosynthetic intermediates by phagocytes activates LXRs, it is possible that these phagocytes undergo an anti-inflammatory phenotypic switch, suppressing MS lesion inflammation. Moreover, the induction of apoE expression by LXRs may stimulate the lipid-supplying function of glial cells and provide the necessary sterols for remyelination to oligodendrocytes. Currently, it is unclear how changes in the sterol metabolism contribute to repair mechanisms in the CNS. We hypothesize that modulating the sterol metabolism can induce repair mechanisms in MS. To test this hypothesis, the following aims (fig. 1.6) will be addressed:

AIM I: To determine the role of scavenger receptor collectin placenta 1 in the uptake of myelin by phagocytes

Using genome wide gene expression analysis, we previously found that internalization of myelin alters the expression of 676 genes in rat peritoneal macrophages (139). Collectin placenta 1 (CL-P1) was one of the most potently induced genes in macrophages upon uptake of myelin. Recent studies have associated CL-P1 with amyloid-β clearance and the innate immune system and complement activation (382-384).

In **chapter 2**, we unravel the dynamics of CL-P1 expression on myelin-containing phagocytes and define the myelin-clearing role that it plays in MS lesion development.

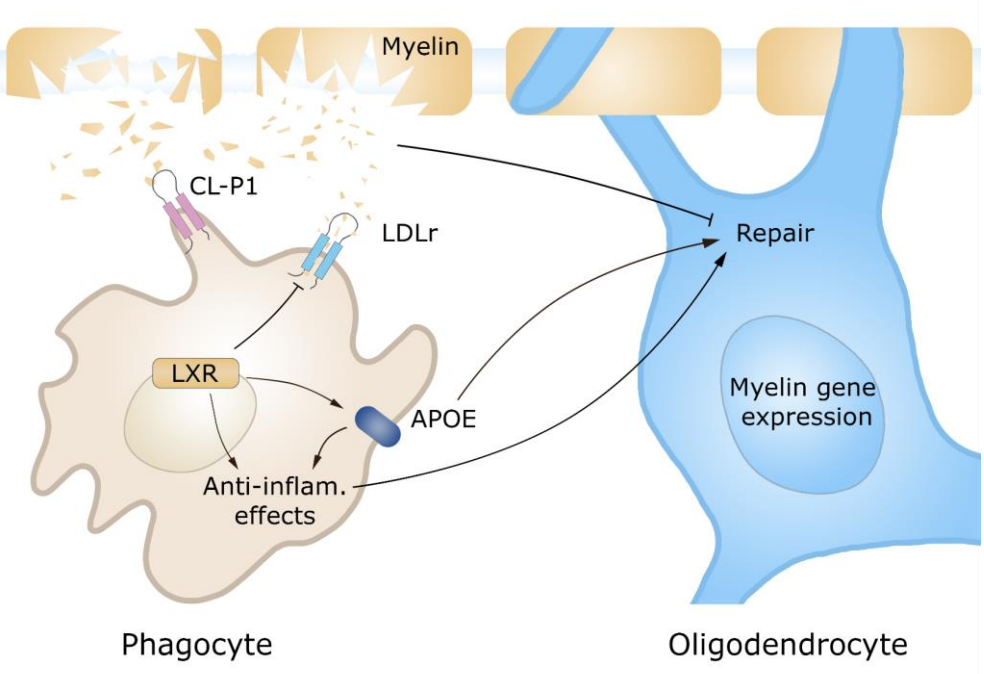

Figure 1.6 | Current state of the art & overview of aims. AIM I: To explore the role of scavenger receptor collectin placenta 1 (CL-P1) in the uptake of myelin by phagocytes. Aim II: Identification of pathways underlying the myelin-directed phenotype change in phagocytes in MS lesions. AIM III: The role of low-density lipoprotein receptor in neuroinflammation. Aim IV: Determine if dietary plant sterols boost repair in the central nervous system by activating liver X receptor signaling.

Aim II: Identification of pathways underlying the myelin-directed phenotype change in phagocytes in MS lesions.

Infiltrating macrophages and resident microglia phagocytose myelin leading to an increased uptake of cholesterol. This may lead to the formation of intracellular oxysterols. Oxysterols and other intermediates of the cholesterol biosynthetic pathway, such as desmosterol, are natural LXR ligands, fueling cellular sterol efflux (385). Recently, we demonstrated that myelin-derived lipids are able to activate LXRs in rat macrophages and skew these macrophages towards a less inflammatory phenotype *in vitro* (203). However, it is currently unknown if LXRs

are activated in phagocytes in active MS lesions. As 25% of the lipid content in myelin consists of cholesterol, it is likely that myelin-loaded phagocytes in MS display a phenotype that is in part dictated by a myelin-mediated activation of LXRs (226). (226).

In **chapter 3**, we elucidate the LXR ligands present in macrophages upon myelin phagocytosis and whether LXRs are activated in MS lesions. We use real-time quantitative PCR and immunohistochemistry to determine the expression of LXRs and their response genes in human phagocytes upon myelin phagocytosis and in active MS lesions. In addition, we use gas chromatographic/mass spectrometric analysis to determine LXR-activating oxysterols and cholesterol precursors present and formed in myelin and myelin-incubated cells, respectively.

AIM III: Identify the involvement of the low-density lipoprotein receptor in neuroinflammation

The LDLr mediates endocytosis of cholesterol-rich LDL and thus maintains plasma LDL levels. In the CNS, LDLr functions as an important regulator of cholesterol homeostasis by regulating apoE levels (386). The ability of lipoproteins to modulate neuroinflammation has been demonstrated in recent studies. Statins, which lower LDL plasma levels, have been known to improve the clinical disease score in EAE mice (387), whereas elevated LDL plasma levels increase neurotoxicity in a mouse model for Alzheimer's (388). However, the contribution of LDL and LDLr to neuroinflammatory responses remains unclear.

In **chapter 4**, we determine the role of the LDLr in neuroinflammation by inducing EAE in *ldlr*-knock out (*ldlr*$^{-/-}$) mice and elucidate the underlying mechanism.

Aim IV: Determine if dietary plant sterols boost repair in the central nervous system by activating liver X receptor signaling

Plant sterols are naturally occurring compounds that structurally and functionally resemble cholesterol in mammals. Several studies have recently shown that plant sterols bind and activate LXRα and/or LXRβ (261-266). LXRs form heterodimers with RXRs and agonists for both receptors activate the LXR/RXR heterodimer (389). Interestingly, activation of specific RXR subtypes was recently shown to be critically involved in CNS (re)myelination (304, 390).

For decades, plant sterol enriched dairy products have been marketed as functional food to lower plasma cholesterol concentrations. Interestingly, in contrast to dietary cholesterol, plant sterols can cross the BBB, enabling them to affect CNS-specific cell types (282). In EAE, they have been shown to reduce clinical manifestations and CNS inflammation (324). Furthermore, in monocytes derived from MS patients, treatment with the plant sterol β-sitosterol decreased the release of proinflammatory cytokines such as TNFα and IL-12 (313). The ability of plant sterols to cross the BBB, their anti-inflammatory potential, their LXR agonist activity and their potential to induce the expression of myelin-associated genes, make them an excellent tool to stimulate remyelination in MS.

In **chapter 5**, we determine if dietary plant sterols boost repair in the CNS by activating LXR signaling. We use primary OPCs to determine the impact of plant sterols on myelin-associated gene expression. In addition, we investigate the effects of increased dietary plant sterols on remyelination using the cuprizone model. In this model, behavioral parameters are assessed using a complex wheel assay.

2

Scavenger receptor CL-P1 is a novel receptor involved in the uptake of myelin by phagocytosis

Based on:

Scavenger receptor collectin placenta 1 is a novel receptor involved in the uptake of myelin by phagocytes.

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2 Scavenger receptor collectin placenta 1 is a novel receptor involved in the uptake of myelin by phagocytosis

2.1 Abstract

Myelin-containing macrophages and microglia are the most abundant immune cells in active multiple sclerosis (MS) lesions. Our recent transcriptomic analysis demonstrated that collectin placenta 1 (CL-P1) is one of the most potently induced genes in macrophages after uptake of myelin. CL-P1 is a type II transmembrane protein with both a collagen-like and carbohydrate recognition domain, which plays a key role in host defense. In this study, we sought to determine the dynamics of CL-P1 expression on myelin-containing phagocytes and define the role that it plays in MS lesion development. We show that myelin uptake increases the cell surface expression of CL-P1 by mouse and human macrophages, but not by primary mouse microglia *in vitro*. In active demyelinating MS lesions, CL-P1 immunoreactivity was localized to perivascular and parenchymal myelin-laden phagocytes. Finally, we demonstrate that CL-P1 is involved in myelin internalization as knockdown of CL-P1 markedly reduced myelin uptake. Collectively, our data indicate that CL-P1 is a novel receptor involved in myelin uptake by phagocytes and likely plays a role in MS lesion development.

2.2 Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, neurodegenerative disease of the central nervous system (CNS). Macrophage- and microglia-mediated myelin destruction is considered to be the primary effector mechanism in MS lesion development (139). Previous studies defined that complement-receptor 3, scavenger receptors I/II, and Fcγ receptors, facilitate the clearance of myelin by macrophages and microglia (100, 391). However, considering the complexity of myelin, it is unlikely that solely these receptors are involved in the uptake of myelin by activated microglia and macrophages in MS lesions.

Using genome wide gene expression analysis, we previously found that internalization of myelin alters the expression of 676 genes in rat peritoneal macrophages (203). Collectin placenta 1 (CL-P1) was one of the most potently induced genes in macrophages upon uptake of myelin. CL-P1 is structurally related to scavenger receptor class A (SRA) due to its collagen-like domain (392). However, CL-P1 also contains a C-type lectin/carbohydrate recognition domain (C-type CRD) (393, 394), typically found in C-type lectin receptors, such as dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (395). Functionally, CL-P1 is associated with binding and internalization of bacteria, yeast, and oxidized low-density lipoproteins (392-394, 396). Furthermore, CL-P1 recognizes carcinoma-associated antigens, possibly via interaction with Lewis^x trisaccharide on tumor cells (397, 398), hereby mediating tumor cell-endothelium interactions (399, 400). Finally, a recent study showed that the collagen-like domain of CL-P1 facilitates amyloid beta (Aβ) clearance by microglia and that uptake of Aβ increases the expression of CL-P1 (384). These findings indicate that CL-P1 plays a role host defense and cellular uptake in different diseases.

In this study, we sought to determine if myelin internalization increases surface expression of CL-P1 on peripheral and CNS-resident phagocytes, its involvement in internalization of myelin, and its cellular distribution in MS lesions. We show that myelin uptake increases the cell surface expression of CL-P1 by mouse and human macrophages, but not by primary mouse microglia *in vitro*. In active MS lesions CL-P1 immunoreactivity was localized to parenchymal and pervivascular myelin-containing phagocytes. Finally, we show that silencing of CL-P1 strongly

reduces myelin uptake. Collectively, our data indicate that CL-P1 mediates the uptake of myelin and likely plays a role in MS lesion development.

2.3 Methods

2.3.1 Cell isolation and culture

Bone marrow-derived macrophages were obtained as described previously (401). Briefly, femoral and tibial bone marrow suspensions from 12 week-old C57Bl/6J mice (Harlan, Horst, Netherlands) were cultured in 10 cm plates at a concentration of 10×10^6 cells/plate and differentiated in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS, Gibco, Merelbeke, Belgium), 50 U/ml *penicillin* (Invitrogen), 50 U/ml streptomycin (Invitrogen), and 15% L929-conditioned medium. Microglia cultures were prepared from postnatal P3 C57BL/6J mouse pups. Isolated forebrains of mice pups were placed in L15 Leibovitz medium (Gibco) containing 1:10 Trypsin (Sigma-Aldrich, Diegem, Belgium) (37°C, 15 min). Next, high glucose DMEM medium (Invitrogen) supplemented with 10% FCS, 50 U/ml *penicillin*, 50 U/ml streptomycin, (DMEM 10:1 medium), and 100 µl/ml DNase I (Sigma-Aldrich) was added to the forebrain tissue. Nervous tissue was dissociated by trituration with serum-coated Pasteur pipettes (Sigma-Aldrich). The dissociated mix was passed through a 70 µm cell strainer, rinsed with 5 ml of DMEM 10:1 medium, and centrifuged (170g, 10 min, RT). After a second centrifugation step, cell suspension was seeded at 2 forebrains/75 cm^2 flask. After 2 days, DMEM 10:1 medium was changed and after reaching confluence (± 6 days later), 2/3 DMEM 10:1 medium containing 1/3 L929-conditioned medium was added. Six days later, microglia isolation was performed using the shake-off method (200 rpm, 2h, RT). Microglia were centrifuged (170g, 10min, RT), suspended in DMEM 10:1 medium containing B27 supplement (Invitrogen), and cultured at 250.000 cells/well in poly-L-lysine (Sigma-Aldrich)-coated 24-well plates. Animals were housed in the animal facility of the Biomedical Research Institute of Hasselt University. All experimental protocols and methods involving animals within this study were conducted in accordance with institutional guidelines and approved by the Ethical Committee for Animal Experiments Hasselt University.

Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation on lympholyte-H cell separation media (Cedarlane, Ontario, Canada). Blood samples were collected from healthy controls after

obtaining informed written consent. Subjects with signs of infection were excluded. All experimental protocols and methods were conducted in accordance with institutional guidelines and approved by the Medical Ethical Committee Hasselt University. $CD14^+$ monocytes were collected using the EasySep human CD14 positive selection kit (Stemcell Technologies, Grenoble, France) according to manufacturer's instructions. After isolation, cells were cultured ($1x10^6$ cells/ml) in RPMI 1640 supplemented with 10% human serum (Sigma-Aldrich, Saint Louis, USA), 50 U/ml *penicillin* and 50 U/ml streptomycin.

The immortalized mouse macrophage (RAW 264.7), mouse microglia (BV-2), and human embryonic kidney (HEK293.1) cell lines were cultured in DMEM (Invitrogen) with 50 U/ml *penicillin*, 50 U/ml streptomycin), and 10% FCS. To determine the effect of myelin and LXR and PPARβ/δ agonists for LXR and PPARβ/δ on the expression of CL-P1, cells were treated for 24 hours with 100 µg/ml of isolated myelin, 10 µM T0901317 (T09; LXR agonist; Cayman Chemical, Huissen, The Netherlands), or 10 µM GSK0660 (PPARβ/δ agonist; Sigma-Aldrich). To determine the impact of inflammation on CL-P1 expression, cells exposed to 100 ng/ml LPS (Sigma-Aldrich) and/or IFNγ (Peprotech, Hamburg, Germany).

2.3.2 Myelin isolation, labelling, and phagocytosis

Myelin was purified from postmortem mouse and human brain tissue by means of density gradient centrifugation, as described previously (375). Experimental protocols and methods were conducted in accordance with institutional guidelines and approved by the Medical Ethical Committee Hasselt University and the Ethical Committee for Animal Experiments Hasselt University. Written informed consent was obtained from all donors. Myelin protein concentration was determined by using the BCA protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium), according to manufacturer's instructions. Endotoxin content was determined using the Chromogenic Limulus Amebocyte Lysate assay kit (Genscript Incorperation, Aachen, Germany). Isolated myelin contained a negligible amount of endotoxin ($\leq 1.8x10^{-3}$ pg/µg myelin). To obtain oxidized myelin, myelin was exposed to 10 µM CuSO4 at 37°C for 20 hours. Myelin was fluorescently labelled, according to the method of Van der Laan *et al*. (98). In short, 10 mg/ml myelin was incubated with 12.5 µg/ml 1,1"-diotadecyl-3,3,3',3',-tetramethylindocarbocyanide perchlorate (DiI; Sigma-Aldrich) for 30 min at 37°C.

To determine the capacity of cells to phagocytose myelin, cells were exposed to 100 µg/ml DiI-labeled myelin. The amount of myelin phagocytosed was determined using a FACSCalibur (BD Biosciences, Erembodegem, Belgium). HEK293.1 were used to define the impact of CL-P1 on myelin phagocytosis as BV-2 and RAW264.7 cells are not easily transfectable. Of note, HEK293.1 are often used as a model system to study phagocytic receptors (402, 403).

2.3.3 Western blot

CL-P1 protein expression was determined via SDS-PAGE and western blot analysis. Briefly, samples were denaturated and separated on an 8% polyacrylamide gel containing Tris-glycine and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshore, UK). Non-specific binding was blocked by incubating the membranes in 5% (w/v) nonfat powdered milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) for 1 hour. Subsequently, membranes were incubated with primary antibodies goat anti-human CL-P1 (R&D Systems, Abingdon, UK 1:1000), goat-anti-human CL-P1 (Novus Biologicals, Abingdon, UK, 1:1000), and rabbit anti-human B-actin (1:10000, Santa Cruz Biotechnology, Heidelberg, Germany) in TBS-T overnight at 4°C. Membranes were incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated rabbit-anti goat and goat anti-rabbit antibodies (Dako, 1:2000) in 5% milk in TBS-T. For stripping and reprobing, a mild stripping buffer was used (0.2M glycine, 0.1% SDS, 1% Tween-20, pH 2.2). An ECL Plus detection kit (Thermo Fisher Scientific) was used and the generated chemiluminescent signal was detected by a luminescent image analyzer (ImageQuant LAS 4000 mini; GE Healthcare).

2.3.4 shRNA and transfection

The X-tremegene HP transfection kit (Roche Diagnostics, Mannheim, Germany) was used to transfect HEK293.1 cells according to the manufacturer's instructions. In short, $0.25x10^6$ HEK293.1 cells were transfected with 1.5 µg of shRNA in 50 µl Opti-MEM® I Reduced Serum Media (Thermo Fisher Scientific). Cells were then resuspended in complete culture medium and incubated for 48 hours at 37°C. CL-P1 (shRNA-1); AACATCTCGCCAAACCTATGA, CL-P1 (shRNA-2); CAGGCTATCCAGCGAATCAAGAA, CL-P1 (shRNA-3); AAGAAATGAAGCTAGTAGACT,

CL-P1 (shRNA-4); AACGATTTCCAATGTGA AGAC, scrambled; CCTAAGGTTAAGTCGCCCTCG.

2.3.5 Flow cytometry

Flow cytometry was used to assess the expression of CL-P1 on all cell types. Cells were stained with goat-anti-mouse CL-P1 (R&D Systems), goat-anti-human CL-P1 (R&D Systems), or normal goat IgG (R&D Systems). Alexa fluor 488 $F(ab')_2$ fragment of rabbit-anti goat (Invitrogen) was used as a secondary antibody. The FACSCalibur was used to quantify cellular fluorescence.

2.3.6 Immunohistochemistry

Frozen brain material from active MS lesions was obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands). Human monocytes were cultured on glass cover slides (Thermo Fisher Scientific) and fixed in 4% PFA for 30 minutes. Cryosections were fixed in acetone for 10 minutes. Cryosections and human monocytes were blocked for 20 minutes with 10% normal serum from the same species as the secondary antibody (Dako, Heverlee, Belgium). For 3, 3' diaminobenzidine (DAB) staining, slides were incubated with goat-anti-human CL-P1 (R&D Systems). After washing, HRP-conjugated rabbit-anti-goat (Dako) was added. Subsequently, DAB substrate (Dako) was used to stain slides. Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). For fluorescence staining, cryosections were incubated with goat-anti-human CL-P1 (R&D Systems), goat-anti-human CL-P1 (Novus Biologicals), mouse-anti-human CD68 (Ebioscience, Vienna, Austria), mouse-anti-human Human Leucocyte Antigen DR/DP/DQ (HLA-DR/DP/DQ; Dako), or rabbit-anti glial fibrillary acidic protein (GFAP; Dako). Cryosections were stained with Alexa flour secondary antibodies (Invitrogen). Nuclei were visualized using 4,6'-diamidino-2-phenylindole (DAPI; Invitrogen). Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 3.10 software (Nikon, Tokyo, Japan). Intracellular myelin degradation products were defined with oil-red O (ORO), which stains neutral lipids, as described previously (404).

2.3.7 Quantitative PCR

Total RNA from cultures was prepared using the RNeasy mini kit (Qiagen, Venlo, The Netherlands), according to manufacturer's instructions. The RNA quality was determined with a NanoDrop spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). RNA was converted to cDNA using the reverse transcription system (Quanta Biosciences, Gaithersburg, USA) and quantitative PCR was performed on a StepOnePlus detection system (Applied Biosystems, Gaasbeek, Belgium), as previously described (203, 405). Relative quantification of gene expression was accomplished using the comparative C_t method. Data were normalized to the most stable reference genes (406, 407). Primers: *CL-P1* (fw); TGGTAGGGAGAGAGAGCCAC, *CL-P1* (rv); CCCATCCAGCCACTTCCATT, cyclophilin A (*Cyca)* (fv); AGACTGAGTGGTTGGATGGC, *Cyca* (rv); TCGAGTTGTCCACAGTCAGC, ribosomal protein L13A (*Rpl13a)* (fv); AAGTTGAAGTACCTGGCTTTCC, *Rpl13a* (rv); GCCGTCAAACACCTTGAGAC.

2.3.8 Statistical analysis

Data were statistically analyzed using GraphPad Prism for windows (version 4.03) and are reported as mean ± SEM. D'Agostino and Pearson omnibus normality test was used to test normal distribution. An analysis of variances (ANOVA) or two-tailed unpaired student T-test (with Welch's correction if necessary) was used for normally distributed data sets. The Kruskal-Wallis or Mann-Whitney analysis was used for data sets which did not pass normality. *P≤0.05, **P≤0.01 and ***P≤0.001.

2.4 Results

2.4.1 Myelin increases the surface expression of CL-P1 on phagocytes

By using a transcriptomic approach, we previously demonstrated that myelin induces gene expression of CL-P1 in peritoneal rat macrophages (203). Here, we validated this increase in CL-P1 mRNA expression on protein level in mouse and human primary phagocytes and phagocyte cell lines. By using western blot (fig. 2.1a-b), immunohistochemistry (fig. 2.1c), and flow cytometry (fig. 2.1d), we show that human primary monocytes express CL-P1 and that myelin internalization increases the expression of CL-P1. For western blot analysis, two separate antibodies were used to confirm the myelin-induced increase in CL-P1 expression. We further show that mouse primary microglia and bone-marrow derived macrophages (BMDMs), as well as cell lines closely resembling these phagocytes (BV-2, microglia; RAW264.7, macrophages), express CL-P1 and that myelin uptake results in an elevated expression of CL-P1 by these cells (fig 2.1e). Interestingly, CL-P1 expression was not increased on primary mouse microglia after myelin uptake. In addition, we found increased expression of CL-P1 by high granular (SSC^{hi}) myelin-containing mouse primary BMDMs compared to low granular (SSC^{lo}) cells that did not substantially phagocytose myelin (fig. 2.1f). This finding indicates that the intensity of CL-P1 immunoreactivity correlates with the amount of internalized myelin.

In MS lesions, phagocytes are likely to encounter modified forms of myelin such as oxidized myelin (408-410). We demonstrate that oxidized myelin more prominently increases the surface expression of CL-P1 on macrophages compared to unmodified myelin (fig.2.1g). In addition, while CL-P1 surface expression gradually decreased on macrophages treated with unmodified myelin, macrophages treated with oxidized myelin retained a high expression of CL-P1 over time (fig. 2.1h).

Previously, we found that myelin-derived lipids, such as cholesterol metabolites and fatty acids, partially account for the phenotype of phagocytes after myelin uptake (203, 411). Activation of the liver X receptor (LXR) and peroxisome proliferator-activated receptor β/δ (PPARβ/δ) underlies the impact of these lipids on the phenotype of phagocytes. By using synthetic agonists for LXR and PPARβ/δ,

we show that myelin increases the expression of CL-P1 in an LXR- and PPARβ/δ-independent manner (fig. 2.1i). We further demonstrate that inflammatory stimuli, such as IFNγ and LPS, do not impact CL-P1 expression by both untreated and myelin-treated macrophages (fig. 2.1j). Collectively, these data show that myelin uptake increases the surface expression of CL-P1 on phagocytes *in vitro* in an LXR- and PPARβ/δ-independent manner, and that inflammatory stimuli do not impact CL-P1 expression.

2.4.2 CL-P1 is expressed by phagocytes in MS lesions

The observed increase in the expression of CL-P1 on macrophages following myelin internalization *in vitro*, prompted us to determine CL-P1 expression in active MS lesions. We show that CL-P1 is predominantly expressed on brain endothelial cells in the normal-appearing white matter (NAWM) (fig. 2.2a). In MS lesions, a profound increase in the expression of CL-P1 was observed (fig. 2b-d). Immune-double labeling revealed that $CD68^+$ parenchymal and perivascular phagocytes expressed CL-P1 within MS lesions (fig. 2.3a-b). Within the NAWM, $CD68^+$ microglia and perivascular macrophages expressed CL-P1 (fig. 2.3c). Interestingly, within active MS lesions, $GFAP^+$ astrocytes also expressed CL-P1 (data not shown). Control staining did not show any immunoreactivity (data not shown). Oil Red O staining further showed that lipid-containing phagocytes were abundantly present in both the parenchyma and perivascular spaces within these lesions (fig. 2.4a-b). These data indicate that CL-P1 is expressed on astrocytes, and myelin-laden perivascular, and parenchymal phagocytes within active MS lesions.

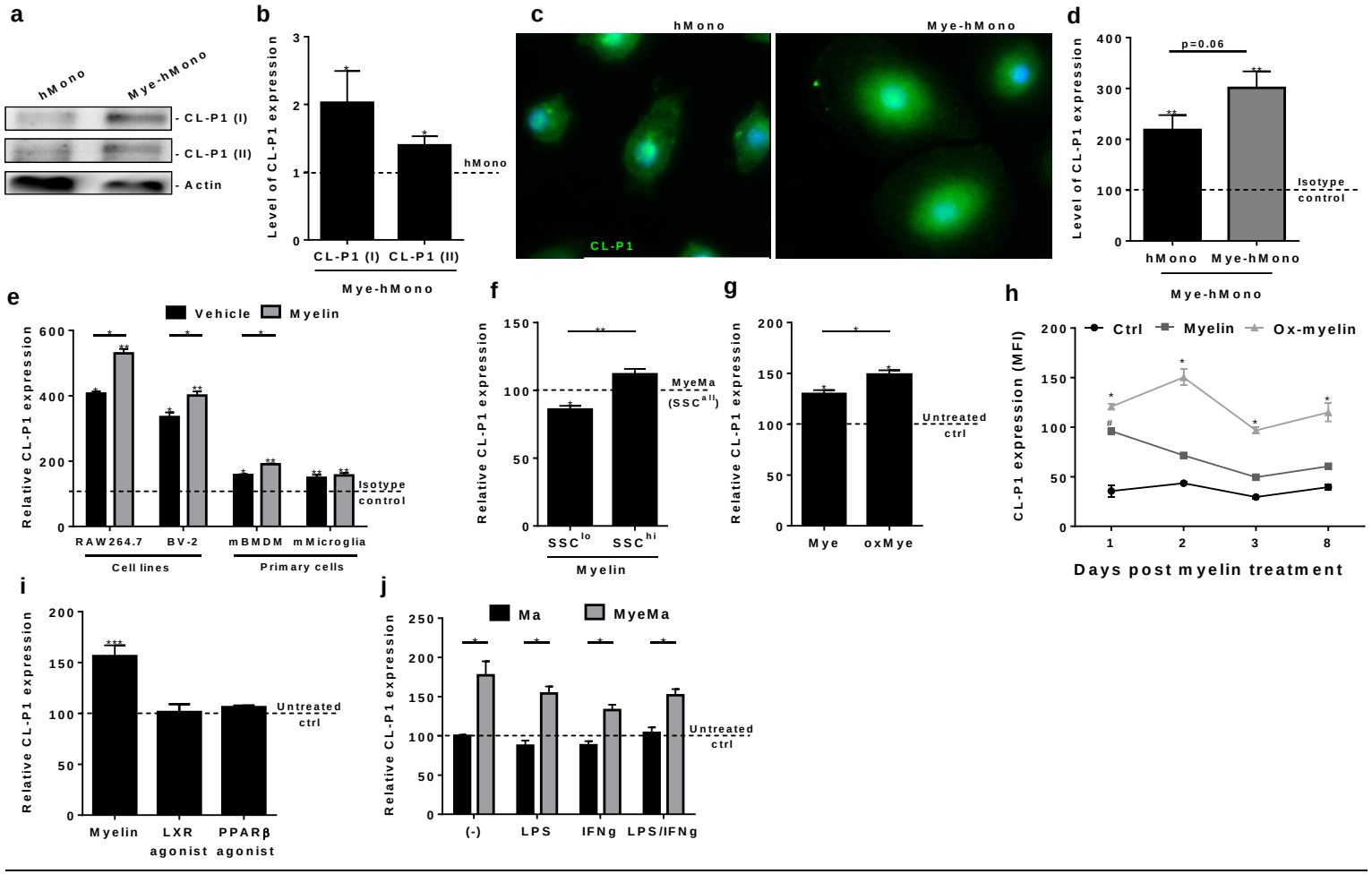
a
hMono
Mye-hMono
- CL-P1 (I)
- CL-P1 (II)
- Actin
b
Level of CL-P1 expression
hMono
CL-P1 (I)
CL-P1 (II)
Mye-hMono
c
hMono
Mye-hMono
CL-P1
d
Level of CL-P1 expression
p=0.06
Isotype control
hMono
Mye-hMono
Mye-hMono
e
Vehicle
Myelin
Relative CL-P1 expression
Isotype control
RAW264.7
BV-2
mBMDM
mMicroglia
Cell lines
Primary cells
f
Relative CL-P1 expression
MyeMa (SSC^all)
SSC^lo
SSC^hi
Myelin
g
Relative CL-P1 expression
Untreated ctrl
Mye
oxMye
h
Ctrl
Myelin
Ox-myelin
CL-P1 expression (MFI)
Days post myelin treatment
i
Relative CL-P1 expression
Untreated ctrl
Myelin
LXR agonist
PPARβ agonist
j
Ma
MyeMa
Relative CL-P1 expression
Untreated ctrl
(-)
LPS
IFNg
LPS/IFNg

Figure 2.1 | **Myelin uptake increases the surface expression of CL-P1 on myeloid cells.** (a,b) Human monocytes (hMono, n = 5), were cultured with or without 100 µg/ml myelin for 24 h. Western blot analysis was used to define CL-P1 expression. Two antibodies were used to define CL-P1 expression. Western blots are displayed in cropped format. (c) Immunohistochemistry (CL-P1, Novus Biologicals) was used to define the expression of CL-P1 by human monocytes cultured with or without 100 µg/ml myelin for 24 h. (d) Human monocytes (n = 7) were cultured with or without 100 µg/ml myelin for 24 h. CL-P1 expression was determined with flow cytometry (CL-P1, R&D). Dotted line represents untreated cells stained with an isotype antibody. (e) RAW264.7 (n = 4), BV-2 (n = 4), mouse BMDMs (n = 4), and mouse microglia (n = 7), were cultured with or without 100 µg/ml myelin for 24 h. CL-P1 expression was determined with flow cytometry (CL-P1, R&D). Dotted line represents untreated cells stained with an isotype control antibody. (f) Mouse BMDMs cultured with 100 µg/ml myelin for 24 h. CL-P1 expression was determined in high granular (SSChi), low granular (SSClo), and all cells (SSCall) using flow cytometry. Dotted line represents myelin-treated cells stained with the CL-P1 antibody (n = 4). (g) RAW264.7 cells were exposed to 100 µg/ml unmodified and CU2-oxidized myelin for 24 h, after which CL-P1 expression was determined. Dotted line represents untreated cells stained with the CL-P1 antibody (n = 4). (h) RAW264.7 cells were cultured with 100 µg/ml unmodified or CU2-oxidized myelin for 1,2,3, and 8 days (n = 3). CL-P1 expression was determined by using flow cytometry. (i) RAW264.7 cells were cultured with a T0901317 (LXR agonist), GW501516 (PPARβ/δ agonist), or 100 µg/ml myelin for 24 h. CL-P1 expression was determined with flow cytometry. Dotted line represents untreated cells stained with the CL-P1 antibody (n = 6). (j) Untreated or myelin treated RAW264.7 cells were exposed to 500 U/ml IFNγ, 100 ng/ml LPS, a combination IFNγ and LPS, or left untreated (n = 4). CL-P1 expression was determined using flow cytometry. Dotted line represents untreated cells stained with the CL-P1 antibody. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

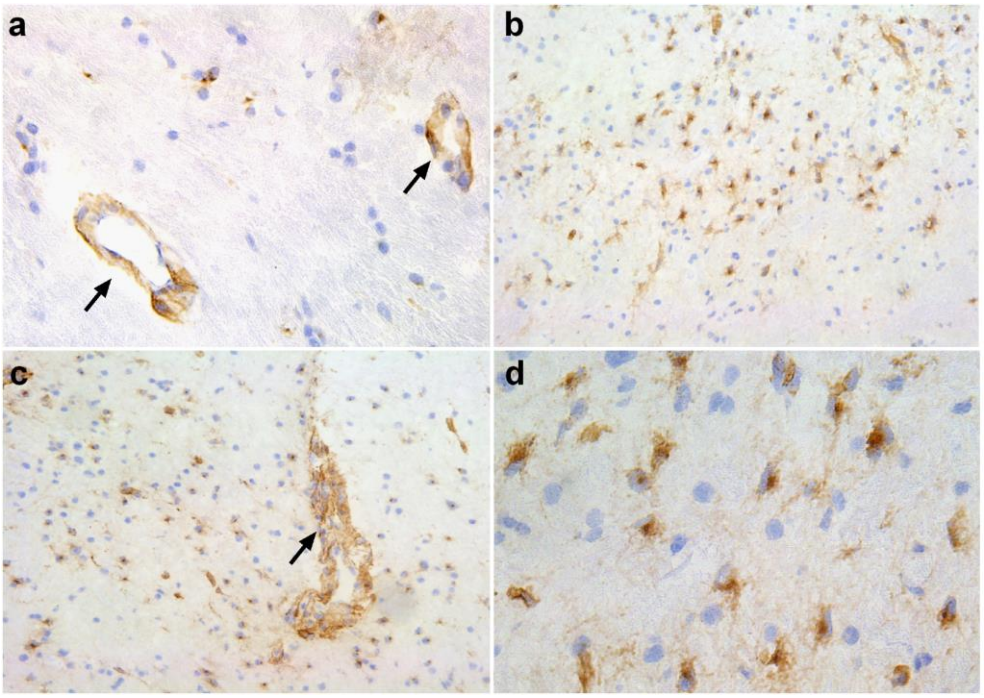

Figure 2.2 | **CL-P1 is highly expressed in MS lesions.** (**a**) Image of normal-appearing matter stained for CL-P1 (40x magnification). Arrows depict blood vessels. (**b–d**) Active MS lesion stained for CL-P1 (**b,c**, 10x magnification; **d**, 40x magnification). Arrow depicts a perivascular cuff filled with infiltrated myeloid cells.

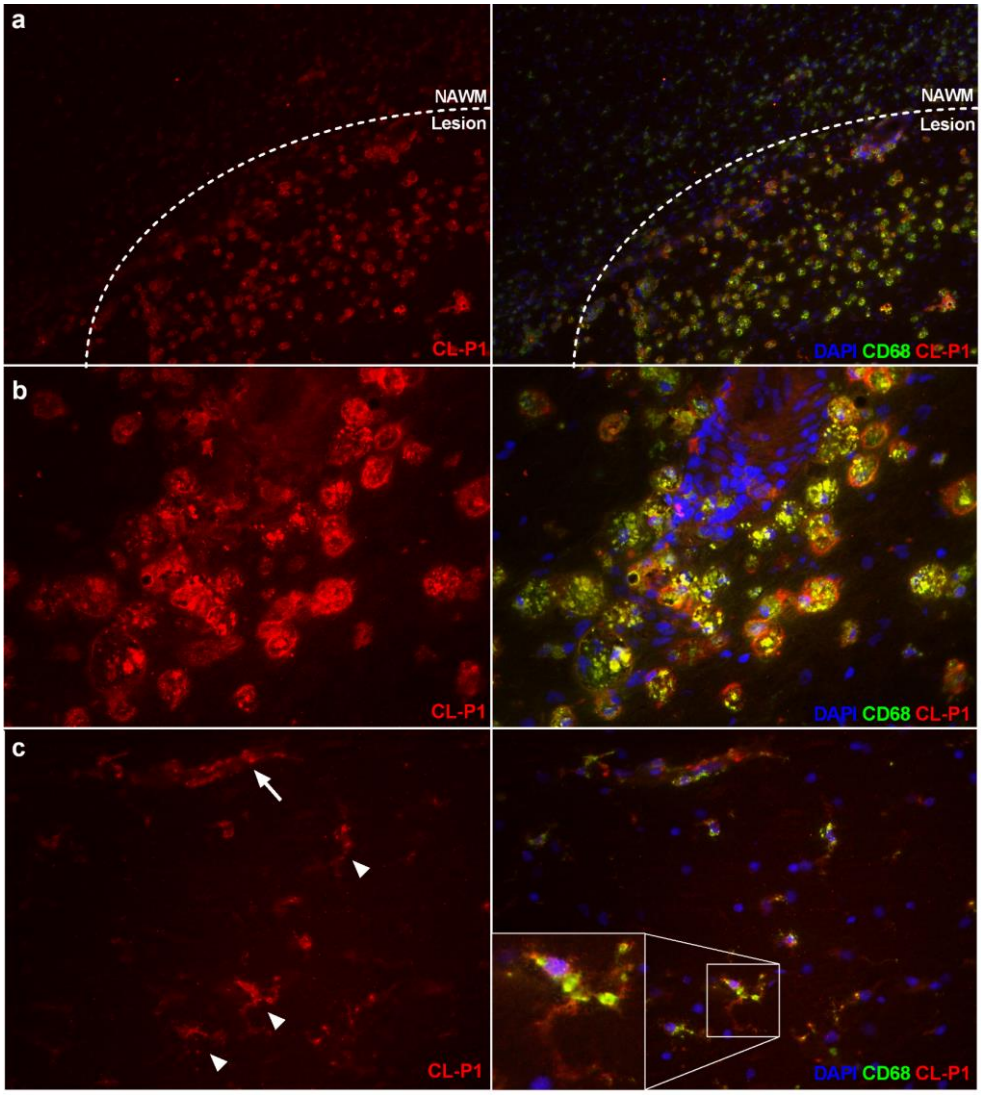

Figure 2.3 | **CL-P1 is expressed by phagocytes in MS lesions.** (**a,b**) Representative images of active MS lesion stained for CD68 and CL-P1 (Novus Biologicals; (**a**), 10x magnification; (**b**), 40x magnification). (**c)** NAWM stained for CD68 and CL-P1 (Novus Biologicals, 40x magnification). Perivascular macrophages and microglia are designated by an arrow and arrowheads, respectively.

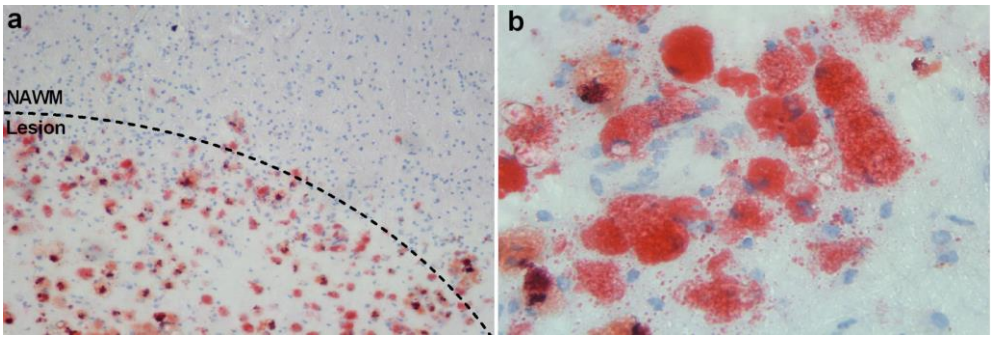

Figure 2.4 | **Abundant lipid-containing phagocytes in perivascular space and parenchyma of active MS lesion.** (**a,b**) ORO staining of active MS lesion showing foamy phagocytes containing neutral intracellular lipids (**a**, 10x magnification; **b**, 40x magnification).

2.4.3 CL-P1 mediates the uptake of myelin

Considering that CL-P1 is structurally related to SRA and that the uptake of myelin by phagocytes is mediated by SRA (100, 392), we determined whether CL-P1 is involved in the internalization of myelin. For this purpose, plasmids expressing *sh*RNA directed against CL-P1 were used. HEK293.1 cells were used as an easy transfectable human cell line with phagocytic properties. Importantly, HEK293.1 avidly endocytosed human myelin debris (fig. 2.5a) and expressed CL-P1 (fig. 2.5b-d). To define the knockdown efficacy and the role that CL-P1 plays in the uptake of myelin, HEK293.1 cells were exposed to a pool of shRNAs directed against CL-P1. We show that the pool of shRNAs (shRNA1-4) completely reduced the cell surface expression of CL-P1 compared to scrambled shRNA (fig. 2.5e). Western blot and qPCR analysis demonstrated a ~60% reduction in CL-P1 expression when cells were exposed to Cl-P1 shRNAs (fig. 2.5c-d). Importantly, we show that silencing of CL-P1 reduced the uptake of myelin by ~50% compared to scrambled shRNA (fig. 2.5e). These data indicate that CL-P1 is involved in the internalization of myelin.

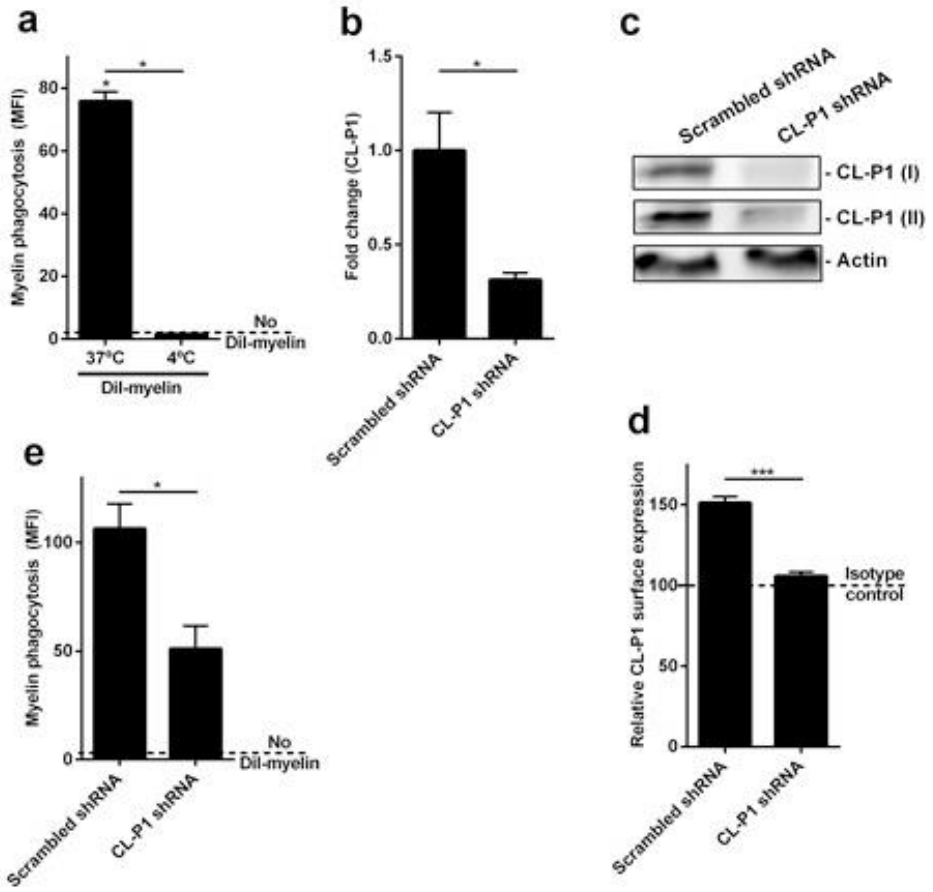

Figure 2.5 | **CL-P1 is involved in the uptake of myelin.** (**a**) HEK293.1 cells were exposed to DiI-labeled myelin for 1.5 h (n = 4). Myelin uptake was assessed using flow cytometry. Cells were exposed to myelin at 4 °C (binding) or 37 °C (binding and uptake). Dotted line represents untreated cells. (**b–d**) HEK293.1 cells were exposed to scrambled shRNA or a pool of shRNA directed against CL-P1 (shRNA1-4) for 48 h. The mRNA and protein expression of CL-P1 was determined using qPCR (**b**, n = 4), western blot (c, CL-P1 I (R&D), CL-P1 II (Novus Biologicals), n = 3), and flow cytometry (**d**, n = 6). Western blots are displayed in cropped format. (**e**) HEK293.1 cells were exposed to scrambled shRNA or a pool of shRNA directed against CL-P1 (shRNA1-4) for 48 h. Next, DiI-labeled myelin was added for 1.5 h (n = 8). Flow cytometry was used to define myelin uptake. Dotted line represents untreated cells. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.5 Discussion

Foamy phagocytes containing myelin debris are the most abundant immune cells in active MS lesions. Our recent transcriptomic analysis demonstrated that CL-P1 is one of the most potently induced genes in macrophages after uptake of myelin. In this study, we sought to determine the dynamics of CL-P1 expression on myelin-phagocytosing phagocytes and unravel what function CL-P1 has on these phagocytes. We show that CL-P1 is expressed by phagocytes in inflammatory MS lesions and that myelin uptake induces cell surface expression of CL-P1 in mouse and human phagocytes *in vitro*. Moreover, we demonstrate that CL-P1 is involved in myelin internalization as knockdown of CL-P1 markedly reduced myelin uptake. These data indicate that CL-P1 is a novel receptor involved in the internalization of myelin by macrophages and likely plays a role in the pathophysiology of MS.

In this study, we show that both mouse macrophages and human monocytes express CL-P1 on their cell surface and that myelin internalization increases the surface expression of CL-P1 on BMDMs in a dose-dependent manner *in vitro*. However, whereas primary mouse microglia expressed CL-P1, myelin internalization did not increase the expression of CL-P1 by these phagocytes. This discrepancy may underline the fact that microglia and infiltrating macrophages react differently to environmental cues (412-414). Ontogenic differences in signaling pathways involved in the regulation of CL-P1 might explain the observed discrepancy between the two phagocyte subsets (111, 118). In active MS lesions, HLA-DR$^+$ phagocytes markedly expressed CL-P1 suggesting that myelin internalization also enhances CL-P1 expression by phagocytes in MS lesions.

Myelin is composed of a variety of lipids and proteins, many of which can alter the physiology of phagocytes upon binding and internalization. Recently, we showed that myelin uptake skews macrophages towards a less-inflammatory phenotype, at least in part, through the activation of the lipid sensing LXR and PPAR (203, 411). Unlike SRAs, such as SPα, MARCO, and CD36, which are well known target genes of LXRs or PPARs (415, 416), we found that the expression of CL-P1 was not regulated by agonists for either of these nuclear receptors. Likewise, inflammatory signaling pathways activated by IFNγ and LPS did not significantly influence the surface expression of CL-P1 on control and myelin-containing phagocytes *in vitro*. Future studies are needed to elucidate how myelin uptake

regulates the expression of CL-P1. Interestingly, oxidized myelin more potently induced and maintained the expression of CL-P1 on phagocytes compared to unmodified myelin. Defining transcriptional differences between phagocytes exposed to unmodified and oxidized myelin may lead to the identification of the biological pathway controlling CL-P1 expression.

Several receptors, such as the complement-receptor 3, SRA I/II, and Fcγ receptors, facilitate the clearance of myelin by macrophages and microglia (100, 391). Our data indicate that CL-P1 also contributes to the internalization of myelin. The phagocytic capacity of SRA largely depends on its collagen-like domain (417). Considering that CL-P1 and SRA share the same collagen-like domain (392), this domain may underlie the role that CL-P1 plays in the internalization of myelin. Future studies are warranted to determine if CL-P1 contributes to myelin uptake *in vivo* and how this affects neuroinflammation and neurodegeneration. As uptake of myelin leads to both demyelination and CNS repair, depending on whether it concerns intact myelin or myelin debris, CL-P1-mediated myelin uptake can be both beneficial or detrimental (115, 116, 139, 418). In our *in vitro* experiments, myelin debris is used to define the impact of CL-P1 on the uptake of myelin. Hence, it is tempting to speculate that CL-P1 might play a role in myelin debris clearance *in vivo*, thereby facilitating remyelination (115, 116, 418).

Aside from a collagen-like domain, CL-P1 contains a C-type CRD that binds with high affinity to glycans bearing Lewis^{x} and Lewis^{a} trisaccharides (397, 398). Interestingly, based on this glycan-specificity, parallels can be drawn between CL-P1 and both DC-SIGN and selectins (419, 420). This suggests that CL-P1 may also play a role in cell migration, cell differentiation, antigen-capture, and T cell priming (421, 422). Interestingly, we found that CL-P1 is markedly expressed on foamy-appearing phagocytes in and near perivascular cuffs in MS lesions. As perivascular cuffs accommodate lymphocytes during active MS, CL-P1 on phagocytes may play a role in T cell priming. Additionally, as myelin-containing phagocytes are located in CNS-draining lymphoid organs (423-425), future studies should determine whether CL-P1 may facilitate lymph node directed migration of these phagocytes.

Increasing evidence indicates that astrocytes actively participate in various processes underlying MS pathogenesis, including neuroinflammation, demyelination, and remyelination (426). We show that astrocytes have increased

expression of CL-P1 in MS lesions. Of interest, CL-P1 immunoreactivity is also increased on reactive astrocytes in AD (384). Follow-up studies should address whether this increased expression of CL-P1 on astrocytes in MS lesions plays a role in the phagocytic capacity of astrocytes, as well as their migration and differentiation.

Based on our findings, we propose a positive feedback model in which CL-P1 mediates the uptake of myelin by phagocytes and subsequently increases its own expression. Considering its role in the uptake of myelin, CL-P1 likely plays an important role in the pathophysiology of MS.

3

Active liver X receptor signaling in multiple sclerosis lesions

Based on:

Active liver x Receptor signaling in phagocytes in multiple sclerosis lesions.

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3 Active liver X receptor signaling in multiple sclerosis lesions

3.1 Abstract

Objective: We sought to determine the LXR ligands present in human macrophages after myelin phagocytosis and whether LXRs are activated in MS lesions.

Methods: We used real-time quantitative PCR and immunohistochemistry to determine expression of LXRs and their response genes in human phagocytes after myelin phagocytosis and in active MS lesions. We used gas chromatographic/mass spectrometric analysis to determine LXR-activating oxysterols and cholesterol precursors present and formed in myelin and myelin-incubated cells, respectively.

Results: Myelin induced LXR response genes ABCA1 and ABCG1 in human monocyte-derived macrophages. In active MS lesions, we found that both gene expression and protein levels of ABCA1 and APOE are upregulated in foamy phagocytes. Moreover, we found that the LXR ligand 27-hydroxycholesterol (27OHC) is significantly increased in human monocyte-derived macrophages after myelin uptake.

Conclusions: LXR response genes are upregulated in phagocytes present in active MS lesions, indicating that LXRs are activated in actively demyelinating phagocytes. In addition, we have shown that myelin contains LXR ligands and that 27OHC is generated in human monocyte-derived macrophages after myelin processing. This suggests that LXRs in phagocytes in active MS lesions are activated at least partially by (oxy)sterols present in myelin and the generation thereof during myelin processing.

3.2 Introduction

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating, and neurodegenerative disease of the CNS (14). Acute inflammatory focal demyelination and the resulting neurodegeneration are among the most prominent pathological hallmarks of MS. Monocyte-derived macrophages and resident microglia secrete inflammatory and toxic mediators that negatively impact axonal and myelin integrity (91, 139, 427). Moreover, phagocytes contribute to MS lesion formation by phagocytosing myelin, apoptotic cells, and cellular debris. Although degeneration of myelin is considered detrimental, its clearance is essential prior to initiation of remyelination. Myelin debris contains proteins that inhibit both axonal re-growth and differentiation of oligodendrocyte precursor cells into mature oligodendrocytes during remyelination (428, 429). This emphasizes the cardinal role of phagocytes in stimulating CNS repair.

Cholesterol is the most abundant lipid within myelin (430) and plays a key role in myelination, illustrated by perturbed myelin formation in mice with defective cholesterol synthesis in the myelin-forming oligodendrocytes (301). Virtually all cholesterol within the CNS is synthesized *in situ* and a stable turnover is necessary to maintain cholesterol homeostasis (431). Breakdown of myelin results in a disturbed CNS cholesterol metabolism in MS patients, demonstrated by altered plasma levels of CNS-specific cholesterol metabolites 24(S)-hydroxycholesterol (24SOHC) and 27-hydroxycholesterol (27OHC) (254, 379, 380). Infiltrating macrophages and resident microglia phagocytose myelin leading to an increased uptake of cholesterol. This may lead to the formation of intracellular oxysterols. Oxysterols and other intermediates of the cholesterol biosynthetic pathway, such as desmosterol, are natural liver X receptor (LXR) ligands, fueling cellular sterol efflux (385). Both isoforms, LXRα and LXRβ, are expressed in the CNS and immune cells (415, 432). When activated, LXRs heterodimerize with retinoid x receptors (RXR). They regulate the expression of genes that participate in reverse cholesterol transport, such as ATP binding cassette transporter (*ABC*) *A1*, *ABCG1*, and apolipoprotein E (*APOE*). LXRs have also emerged as suppressors of inflammatory pathways in macrophages through SUMOylation (216, 217). Evidence is emerging that macrophages display an anti-inflammatory phenotype upon myelin phagocytosis (94, 405, 433), suggesting that myelin contains

functional LXR ligands or leads to the formation thereof. Recently, we demonstrated that myelin-derived lipids are able to activate LXRs in rat macrophages and skew these macrophages towards a less inflammatory phenotype *in vitro* (203). However, it is currently unknown if LXRs are activated in phagocytes in active MS lesions. We hypothesize that the uptake of myelin and the subsequent formation of LXR ligands, results in the activation of LXRs in phagocytes in active MS lesions.

We show that phagocytosis of myelin by human monocyte-derived macrophages leads to a significant induction of LXR response genes. In addition, we show that infiltrated macrophages and resident microglia express enhanced levels of LXRα, ABCA1 and APOE in active demyelinating lesions. Lastly, we show that the oxysterol 27OHC is formed after myelin uptake and processing. The formation of this LXR ligand may explain the LXR activation seen in myelin-phagocytosing phagocytes in active demyelinating lesions.

3.3 Methods

3.3.1 Myelin Isolation

Myelin was purified from human brain tissue by density-gradient centrifugation, as described previously (375). Protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Erembodegem, Belgium). Endotoxin content was determined using a LAL Chromogenic endotoxin quantitation kit (Thermo Fisher). The isolated myelin contained a negligible amount of endotoxin (<1.8×10-3 pg/µg myelin).

3.3.2 Cell culture

Human peripheral blood mononuclear cells (PBMCs) were obtained from consented healthy donors using Ficoll (Cedarlane Labs, Canada) density gradient. Monocytes were isolated from the PBMCs by anti-CD14 EasySep microbeads (Stemcell technologies, Grenoble, France) according to manufacturer's instructions. Monocytes (1x106 cells/ml) were cultured in 24-well plates (Greiner Bio-one) in macrophage differentiation medium (RPMI (Lonza, Vervier, Belgium), supplemented with 10% v/v normal fetal calf serum (Life Technologies, Ghent, Belgium) and 50 U/ml v/v penicillin-streptomycin (Sigma-Aldrich, Bornem, Belgium)), at 37°C, 5% CO2. Monocytes matured into macrophages in the course of 7 days. For experiments, myelin (100 µg/ml) was added to macrophages for 24 hours preceding mRNA extraction.

3.3.3 Autopsy material

Brain tissue samples were obtained from the Netherlands Brain Bank. All patients, healthy controls or their next of kin, had given informed consent for autopsy and use of brain tissue for research purposes. Active lesions were immunohistochemically characterized as lesions with abundant immune cell infiltrates and extensive myelin loss (table 1).

Table 3.1 Brain tissue characteristics. (NDC; non demented controls, RR-MS; relapse remitting MS, PP-MS; primary progressive MS; SP-MS; secondary progressive MS). Cases marked * were used for IHC, cases marked + were used for qPCR.

Nr.	Autopsy	Diagnosis	Gender	Age	Type MS
1	**S94-163**$^{+}$	NDC	Female	74y	-
2	**S95-019**$^{+}$	NDC	Male	54y	-
3	**S96-373**$^{+}$	NDC	Male	70y	-
4	**S01-016**$^{+}$	NDC	Female	64y	-
5	**S01-174**$^{+}$	NDC	Female	47y	-
6	**S97-131**$^{+}$	NDC	Female	65y	-
7	**S01-58**$^{+}$	MS	Female	48y	RR-MS
8	**S01-298**$^{+}$	MS	Female	53y	RR-MS
9	**S03-142**$^{+}$	MS	Male	53y	PP-MS
10	**S04-045**$^{+}$	MS	Female	41y	RR-MS
11	**S99-261***	MS	Female	38y	RR-MS
12	**S07-269***	MS	Male	41y	PP-MS
13	**S07-314**$^{+}$	MS	Female	66y	SP-MS
14	**S09-152***	MS	Male	50y	Unknown
15	**S11-008***	MS	Male	54y	RR-MS

3.3.4 Quantitative PCR

Total RNA was prepared using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions with the following modification: Qiazol lysis reagent (Qiagen) with 1% β-mercaptoethanol (Sigma-Aldrich) was used as lysis buffer. RNA concentration and purity was determined using a Nanodrop spectrophotometer (Isogen Life science). RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Biosciences, Boston, USA) according to the manufacturer's instructions. Quantitative PCR (qPCR) was conducted on a StepOnePlus™ Real-Time PCR system (Applied biosystems, Ghent, Belgium). The

SYBR green master mix (Applied biosystems), 10 µM of forward and reverse primers, nuclease free water and 12.5 ng template cDNA in a total reaction volume of 10 µl. Relative quantification of gene expression was accomplished by using the comparative Ct method. Data were normalized to the most stable reference genes. Primers were chosen according to literature or designed using Primer-express (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Details of primers used are shown in table 3.2.

Table 3.2 | Primers used to measure the relative expression of different human genes.

Gene	Sequence (5'–3')	Product size	Accession number
LXRα forward	CGCACTACATCTGCCACAGT	159 bp	NM_001251935.1
LXRα reverse	CTTGCCGCTTCAGTTTCTTC		
LXRβ forward	CAGCAGCAGGAGTCACAGTC	157 bp	NM_001256647.1
LXRβ reverse	GTTCTTGAGCCGCTGTTAGC		
ABCA1 forward	AACGAGACTAACCAGGCAATC	148 bp	NM_005502.3
ABCA1 reverse	ACACAATACCAGCCCAGAAC		
ABCG1 forward	CCAGAAGTCGGAGGCCATC	63 bp	NM_207629.1
ABCG1 reverse	AAGTCCAGGTACAGCTTGGCA		
APOE forward	ACTGGGTCGCTTTTGGGATT	64 bp	NM_000041.2
APOE reverse	CTCCTCCTGCACCTGCTCA		
TBP forward	TATAATCCCAAGCGGTTTGC	170 bp	NM_003194.4
TBP reverse	GCTGGAAAACCCAACTTCTG		
CYCA forward	AGACTGAGTGGTTGGATGGC	142 bp	NM_021130.3
CYCA reverse	TCGAGTTGTCCACAGTCAGC		
YWHAZ forward	CTTGACATTGTGGACATCGG	145 bp	NM_003406.3
YWHAZ reverse	TATTTGTGGGACAGCATGGA		
Rpl13a forward	AAGTTGAAGTACCTGGCTTTCC	171 bp	NM_012423.3
Rpl13a reverse	GCCGTCAAACACCTTGAGAC		

3.3.5 Immunohistochemistry

Snap-frozen brain tissue was sectioned at 5 µm and stained for proteolipid protein (PLP, 1:500, MCA839G, AbD Serotec, Temse, Belgium) and HLA-DR (biotin-labeled clone LN3, eBioscience; 13-9956) to determine MS lesion type. All antibodies were diluted in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA, Sigma-Aldrich).

For DAB-stainings, sections were air-dried and fixed in acetone for 10 minutes. Endogenous peroxidase activity was quenched by incubating the slides in 0.3% hydrogen peroxide. Sections of each tissue sample were incubated either with goat anti-LXRα (1:200, kindly provided by Prof. Dr. Gustafsson), goat anti-LXRβ (1:200, kindly provided by Prof. Dr. Gustafsson), rabbit anti-ABCA1 (1:200, NB400-105, Novus biologicals, Denver, USA), rabbit anti-APOE (1:200, NBP1-19807, Novus biologicals), or rabbit anti-LDLR (1:400, NB110-57162, Novus biologicals) for 60 minutes at room temperature. Then, slides were incubated with EnVision+ Dual Link System-HRP (DAKO, Heverlee, Belgium) for 30 minutes or with HRP labeled rabbit anti-goat (1:100, DAKO) for 60 minutes at room temperature. 3, 3'-diaminobenzidine (DAB+, DAKO) was used as chromogen. Sections were thoroughly washed with PBS between each incubation step. After a short rinse in tap water, sections were incubated with hematoxylin for 1 minute and extensively washed with tap water for 10 minutes. Sections were dehydrated with ethanol followed by xylene and mounted with Entellan (Merck).

For immunofluorescence staining, sections were air-dried and fixed in acetone for 10 minutes. Non-specific binding was blocked using 1% BSA in PBS for 30 minutes. Sections of each tissue sample were incubated either with anti-LXRα, anti-LXRβ, anti-ABCA1, anti-APOE or anti-LDLR overnight at 4°C followed by incubation with Alexa-488-labeled donkey anti-goat (1:150, Molecular Probes) or Alexa-488-labeled goat anti-rabbit (1:400, Molecular Probes). To visualize HLA-DR positive macrophages, an antibody against HLA-DR (biotin-labeled clone LN3, eBioscience; 13-9956) was subsequently applied for 60 minutes. HLA-DR slides were further incubated with Alexa-555-labeled streptavidin (1:400, Molecular Probes). Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 4.20 software (Nikon).

3.3.6 Oil red O staining

Myelin treated human monocyte-derived macrophages were fixed using 4% paraformaldehyde (Sigma-Aldrich) for 20 min. Next, cells were stained with oil red O (ORO) (Sigma-Aldrich) for 10 min and exhaustively rinsed with water. In order to quantify intracellular myelin levels, 1 ml of isopropyl alcohol was added to the stained culture dish, the extracted dye was immediately removed by gentle pipetting and its absorbance determined spectrophotometrically at 510 nm. To delineate active MS lesion tissue for qPCR, cryosections (unfixed) were stained with ORO for 10 min and exhaustively rinsed with water. Next, a hematoxylin counterstain was applied and cryosections were subsequently mounted.

3.3.7 Sterol analysis

Human monocyte-derived macrophages were treated with or without 100µg/ml human myelin for 24 hours. Next, the medium containing myelin was collected and stored for further analysis, while cells were incubated for another four days in fresh medium. Cells were then lysed in 1M NaOH (in 80% ethanol) and sterols were subsequently extracted from cell lysates and medium using chloroform-methanol (2:1). Sterol levels were determined by gas chromatograph mass spectrometer (GC/MS) as described previously (434, 435). The change in amount of sterols was determined by subtracting (condition with myelin) from (condition no myelin), and subsequently dividing it by the amount of sterols present in pure myelin after normalizing with the plant sterol campesterol, which is not metabolized by mammalian cells (436). A flow diagram of the experimental design is included in figure 3.4B.

3.3.8 Statistical Analysis

Data were statistically analyzed with GraphPad Prism 5 for windows and are reported as mean values ± standard error (SEM). D'Agostino and Pearson omnibus normality test was used to test normal distribution. An analysis of variances (ANOVA) or two-tailed unpaired student t-test (with Welch's correction if necessary) was used for normally distributed data sets (t(df); P). The Mann-Whitney U analysis (MWU, n1, n2; P) was used for data sets, which did not pass normality. *P<0,05, **P<0,01 and ***P<0,001.

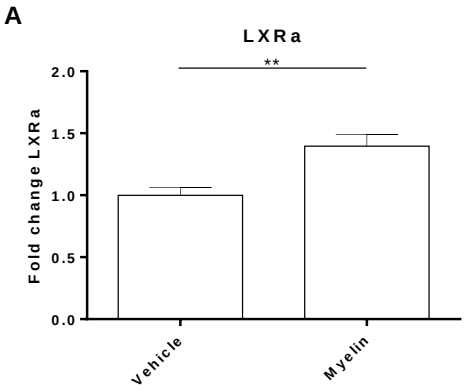
3.4 Results

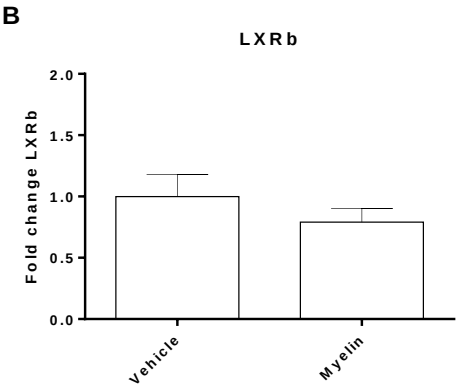
3.4.1 Induction of LXR response gene expression in human monocyte-derived macrophages after myelin uptake

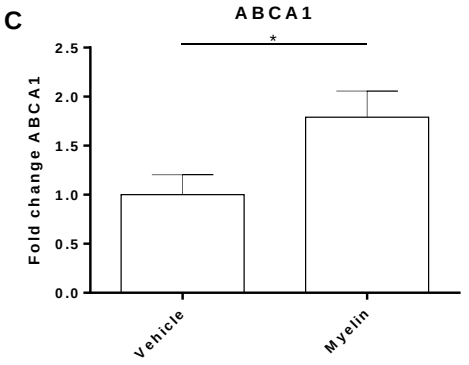
To investigate if LXRs in human monocyte-derived macrophages are activated after myelin uptake, we determined the expression of well-known LXR downstream targets by quantitative real-time PCR (qPCR) analysis. mRNA expression of the autoregulated LXRα and LXR response genes ABCA1 and ABCG1 was increased in myelin-treated cells, compared to non-treated cells, whereas LXRβ and APOE mRNA levels remained unchanged (fig. 3.1). The Ct value of LXRα in unstimulated cells is lower than LXRβ (LXRα:17.90 vs. LXRβ:26.38). The threshold cycle is inversely proportional to the expression level indicating that LXRα mRNA expression is ~250 times higher than LXRβ in human macrophages. This indicates that LXRα appears to be the dominant driver behind LXR signaling in macrophages.

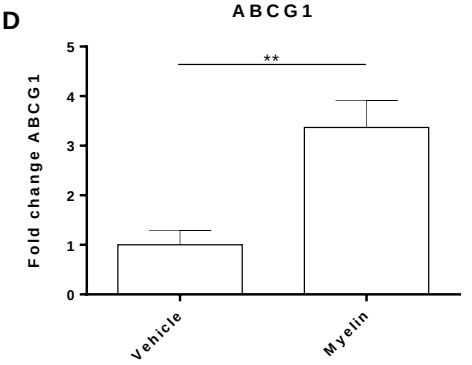
3.4.2 Induction of LXR response gene expression in active MS lesions

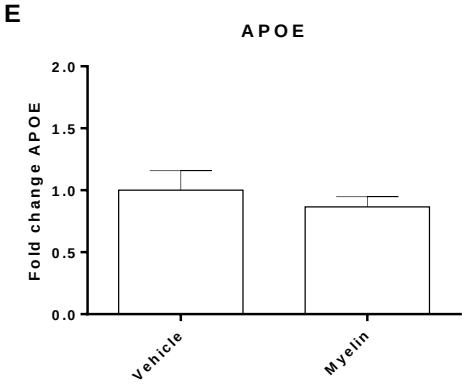
To determine whether LXRs are activated in MS lesions, gene expression levels of LXRs and downstream targets were determined (fig 3.2). RNA was isolated from regions accommodating lipid-containing macrophages and microglia, determined by Oil Red O (ORO) staining. mRNA expression of LXRα and its response genes ABCA1 and APOE were increased in active lesions, compared to white matter tissue from non-neurological controls (fig. 3.2A, C, E). Moreover, LXRβ mRNA expression was significantly reduced in active MS lesions, whereas the expression of ABCG1 remained unchanged. These results show LXR response genes are upregulated in active demyelinating MS lesions.











F

n	*LXRa (Ct)*	*LXRb (Ct)*
1	17,9242	26,5524
2	16,9822	25,9581
3	17,2364	25,734
4	18,4959	27,0704
5	18,9064	26,5815
Average	**17,909**	**26,3793**

Figure 3.1 | **LXR activation after myelin phagocytosis in human monocyte-derived macrophages**. Comparison of fold changes of LXRs and their response genes between untreated (n=11) and myelin-treated (100 µg/ml) human monocyte-derived macrophages (n=11). Relative quantification of LXRα ($t(20)=3.516$; $p<0.003$) (**A**), LXRβ (**B**), ABCA1 (MWU=29; 11; 11; $p<0.039$) (**C**), ABCG1 (MWU=8; 11; 11; $p<0.001$) (**D**) and APOE (**E**) was accomplished by using comparative Ct method. Data were normalized to the most stable reference genes, determined by Genorm (TBP and CYCA).

3.4.3 LXR downstream targets ABCA1 and APOE are upregulated in phagocytes in active MS lesions

To determine whether LXR response genes are also induced at protein level in MS lesions and to establish the cellular location of this induction, we determined the expression of LXRs and LXR response genes in active MS lesions by immunohistochemistry. For this purpose, we selected active demyelinating MS lesions of four MS cases (table 3.1). Identification of active lesions was based on immunohistochemical analysis of phagocytes (anti-HLA-DR) and proteolipid protein (PLP). Active lesions are characterized by extensive myelin loss and abundant myelin-containing phagocytic macrophages and microglia (fig. 3.3A-C). We found that the LXR response genes ABCA1, APOE, LXRα, and LXRβ are abundantly expressed in active lesions (fig. 3.3D-E, G-H, J-K, and M-N). Double immunofluorescence stainings revealed that ABCA1, APOE, and LXRα/β were highly expressed in HLA-DR+ cells with a foamy appearance (fig. 3.3F, I, L, O) suggesting that LXRs are activated in phagocytes in active MS lesions.

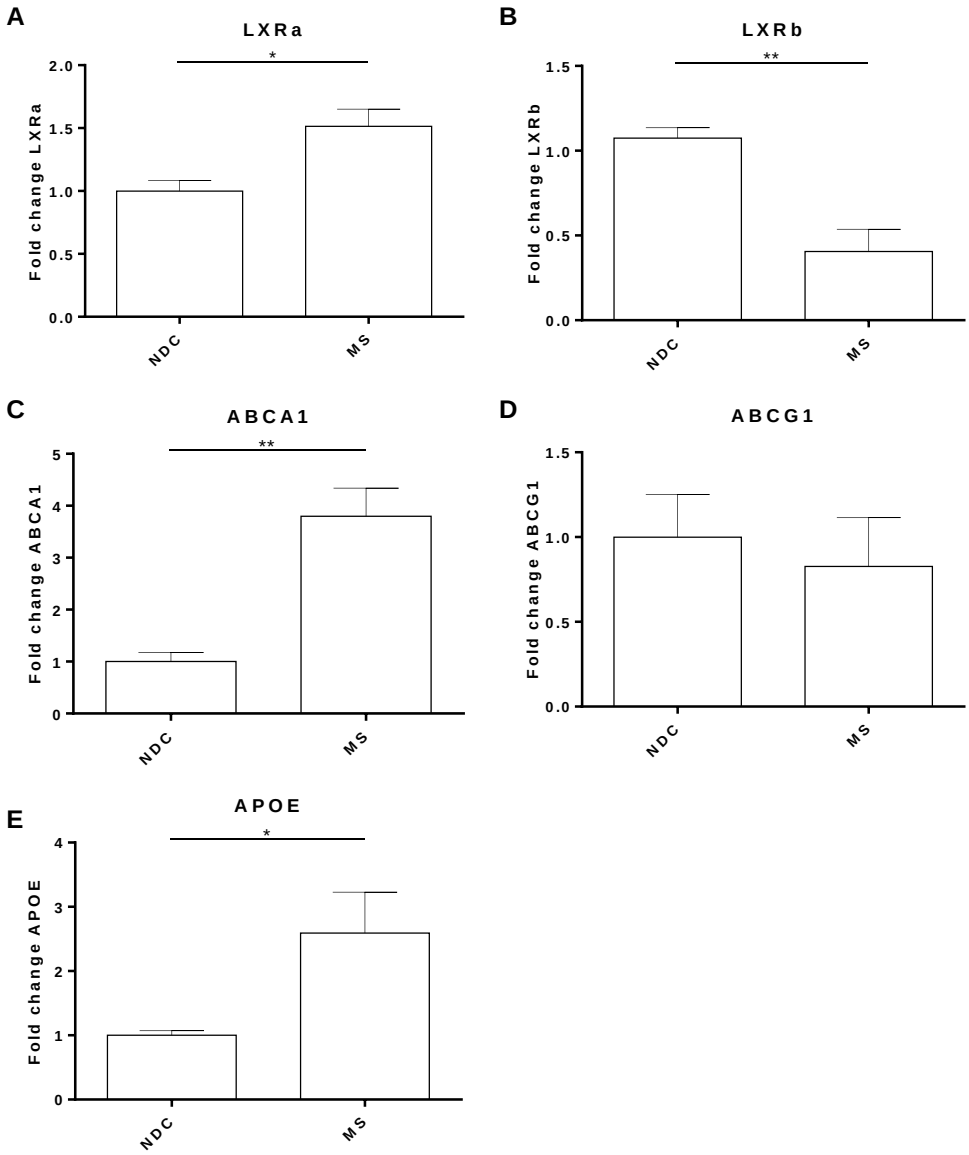


Figure 3.2 | **Active LXR signaling in MS lesions.** Comparison of fold changes of LXRs and their response genes between non-demented controls (n=6, see table 3.1 nr:1-6) and active MS lesions (n=5, see table 3.1 nr:7-11). Relative quantification of LXRα (MWU=6; 6; 5; $p<0.012$) (A), LXRβ ($t(11)=3.620$; $p<0.004$) (B), ABCA1 ($t(12)=4.293$; $p<0.001$) C), ABCG1 (D) and APOE (MWU=9; 6; 5; $p<0.036$) (E) was accomplished by using comparative Ct method. Data were normalized to the most stable reference genes, determined by Genorm (YWHAZ and Rpl13a). NDC; non-demented controls.

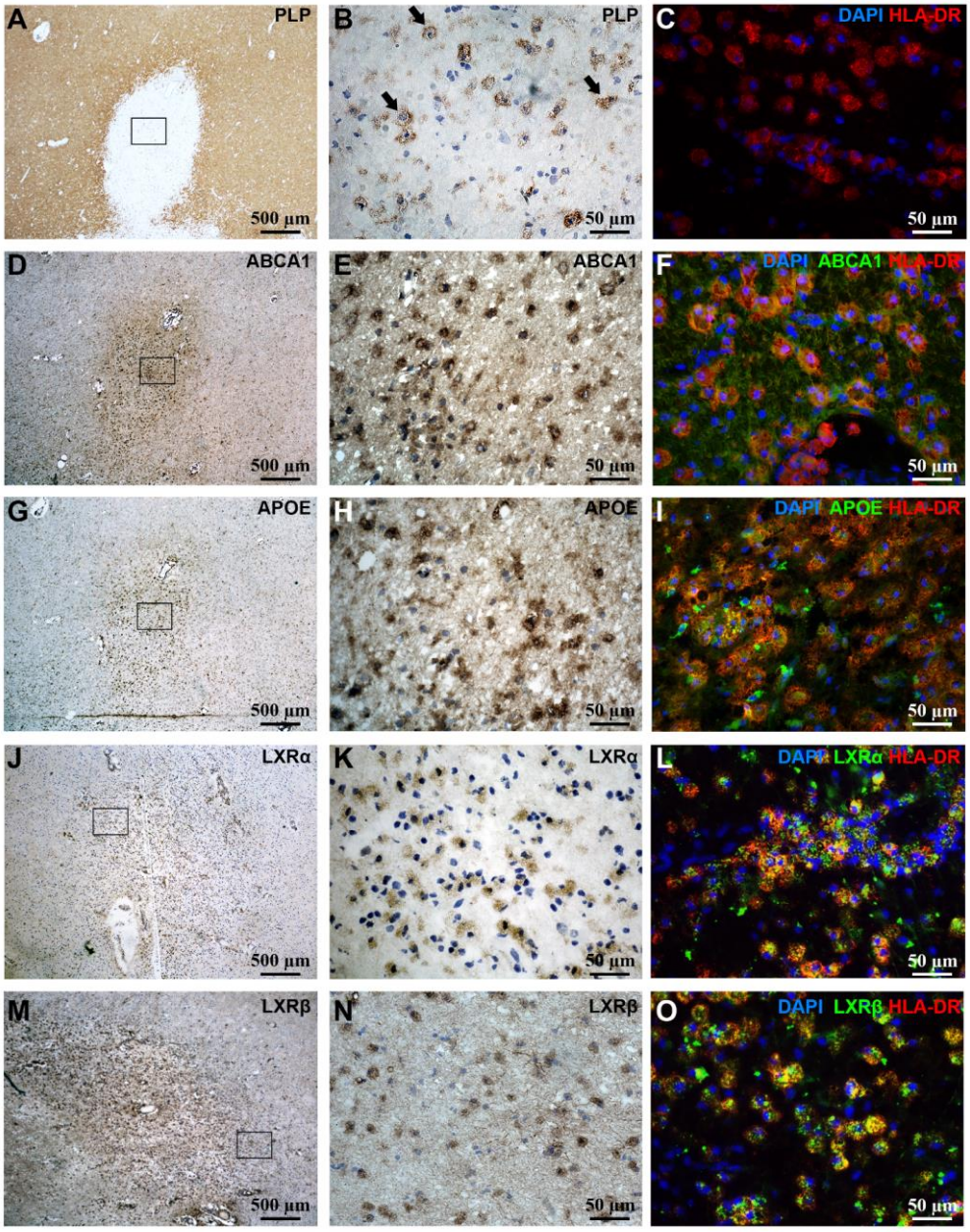
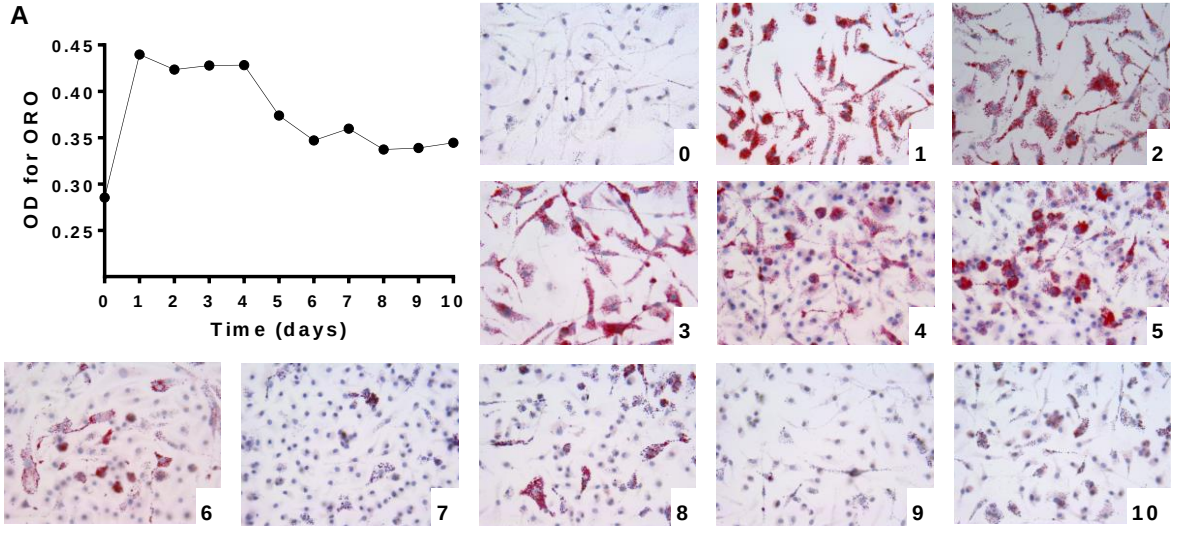


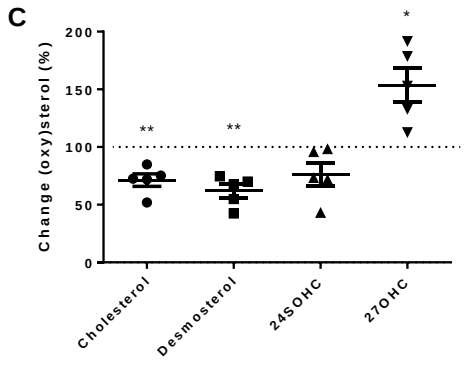
Figure 3.3 | **LXRs are activated in HLA-DR+ phagocytes in active MS lesions.** Extensive demyelination and PLP+ phagocytic cells (black arrows) are observed in active MS lesions (A, B: proteolipid protein). Figure B represents a magnification of the outlined square in A. Active lesions are characterized by infiltrating macrophages and microglia expressing HLA-DR (C). ABCA1 (D, E), APOE (G, H), LXRα (J, K), and LXR β (M, N) were abundantly expressed by phagocytes. Figure E, H and K represent a magnification of the outlined square

in D, G and J respectively. ABCA1 (F), APOE (I) and LXRα/β (L/O) were strongly expressed by HLA-DR+ phagocytes in active lesions. Original magnifications: A, D, G, J and M: 4x; B, C, E, F, H, I, K, L, N and O: 40x.

3.4.4 27-hydroxycholesterol is formed in human monocyte-derived macrophages after myelin ingestion

After myelin is phagocytosed, it is degraded inside the cell (fig. 3.4A). Myelin, visualized by ORO-staining, is rapidly phagocytosed and intracellular levels remain stable until day four. At day five a decline can be seen, indicating processing of myelin and possibly the release of cholesterol oxidation products. Using GC/MS we determined cellular oxysterol levels in human macrophages, treated with or without myelin incubated for five days. We compared these levels with the levels present in human myelin (fig. 3.4B-C). We found that while levels of cholesterol and the LXR ligand desmosterol were decreased after five days of myelin ingestion, 27OHC, but not 24OHC levels, were significantly increased in macrophages.





	Cholesterol		Desmosterol	
	Mean (µg/100µg)	SD	Mean (ng/100µg)	SD
Pure myelin	3,79	0,16	1,96	0,13
Present after 5 days	2,71	0,46	1,21	0,26
Change (oxy)sterol (%)	71,50%		61,73%	
	24SOHC		**27OHC**	
	Mean (ng/100µg)	SD	Mean (ng/100µg)	SD
Pure myelin	1,17	0,11	0,98	0,06
Present after 5 days	0,89	0,26	1,51	0,32
Change (oxy)sterol (%)	76,07%		154,08%	

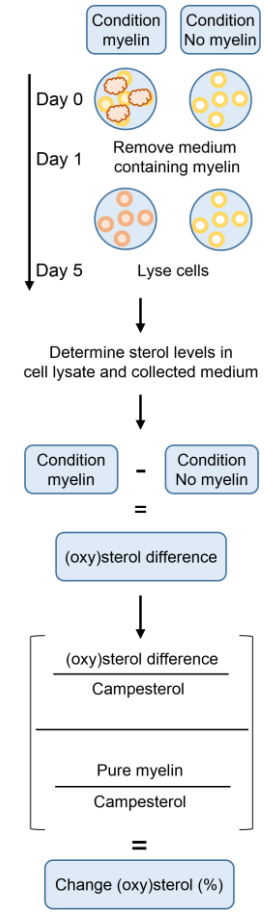


Figure 3.4 | **Degradation of myelin and the formation of 27OHC.** A) Monocyte-derived macrophages (0) were treated with 100 µg/ml myelin (24h) after which the unbound myelin was washed away and incubated for 10 consecutive days (1-10). Intracellular myelin was visualized by ORO staining. Quantification was performed by determining the extracted dye. B) Flow diagram of the experimental design. C) Human monocyte-derived macrophages were treated with 100 µg/ml myelin for 24h, after which the unbound myelin was washed away and cells incubated for another four days. Next, cholesterol, desmosterol and oxysterol levels were determined in the samples using GC/MS. Sterols were normalized using the plant sterol campesterol which is not metabolized. Relative levels of cholesterol ($t(4)=5.318$; $p<0.006$, ●), desmosterol ($t(4)=6.503$; $p<0.003$, ■), 24SOHC (▲) and 27OHC ($t(4)=3.720$; $p<0.021$, ▼) are shown (n=5).

3.4.5 27-hydroxycholesterol induces LXR response gene expression in human monocyte-derived macrophages

We calculated that the concentration of 27OHC present per 1x106 macrophages after five days of myelin incubation is around 5 µM. Therefore, we treated human monocyte-derived macrophages with 27OHC to determine whether the level of 27OHC induced in myelin phagocytosing macrophages is sufficient to induce LXR response gene expression (fig. 3.5). The expression of LXRα (MWU=0; 5; 5; $p<0.008$) and its response genes ABCA1 (MWU=1; 5; 5; $p<0.016$) and ABCG1 (MWU=0; 5; 5; $p<0.008$) was significantly increased in 27OHC-treated cells, compared to non-treated cells whereas LXRβ and APOE mRNA expression remained unchanged. These results suggest that the amount of 27OHC present in vitro after myelin phagocytosis activates LXRs.

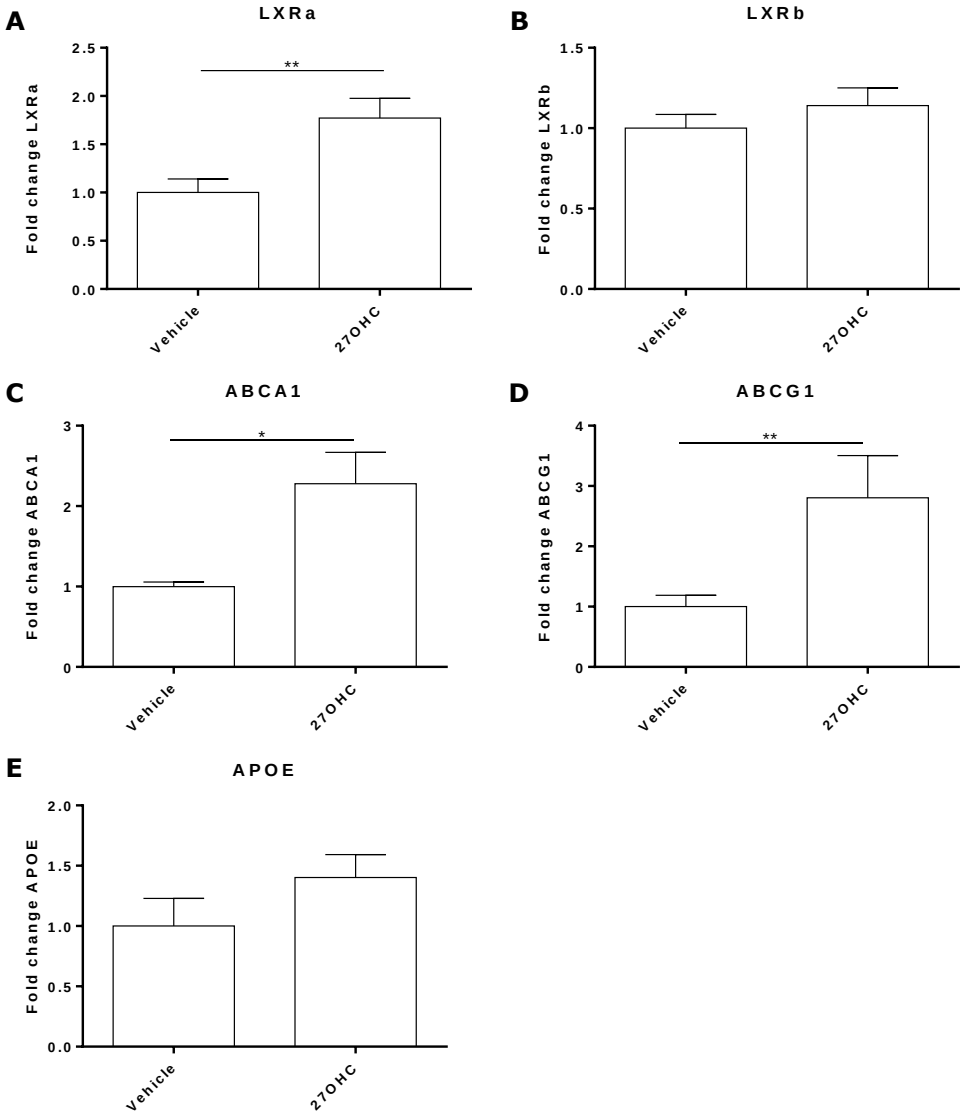


Figure 3.5 | **LXR activation after 27OHC treatment.** Comparison of fold changes between untreated (n=5) and 27OHC-treated (5μM) human monocyte-derived macrophages (n=5) for 24h. Relative quantification of LXRα (A), LXRβ (B), ABCA1 (C), ABCG1 (D) and APOE (E) was accomplished by using comparative Ct method. Data were normalized to the most stable reference genes, determined by Genorm (YWHAZ and RPL13a).

3.5 Discussion

In this study, we show that LXRs and their downstream targets are induced in myelin-laden macrophages in active demyelinating MS lesions, indicative of LXR activation. In active lesions, macrophages and activated microglia contribute to lesion progression by phagocytosing large quantities of myelin. Conceivably, upon myelin processing, LXR ligands are released leading to the upregulation and activation of LXRs, and their response genes. LXRs are master regulators of cholesterol metabolism and inflammatory responses and are critical for brain homeostasis. By activation of LXRs, reverse cholesterol transport is induced which protects myelin-phagocytosing cells from elevated intracellular free cholesterol and oxysterol-induced toxicity (245). In the brain, LXRβ is ubiquitously expressed, whereas LXRα expression is largely restricted to infiltrating macrophages and microglia (415, 432).

We found LXRα expression in active lesions to be mainly present in HLA-DR$^+$ cells. Likewise, LXRβ expression was observed in HLA-DR$^+$ cells and in perilesional astrocytes (data not shown). Though both isoforms are expressed by phagocytes, it appears that LXRα, at least at mRNA level, is the dominant driver behind LXR signaling in macrophages (437) which is also evidenced by our *in vitro* data (fig. 1). However, it is likely that due to translational and/or post-translational regulation the protein expression ratio differs from the gene expression ratio. In line herewith, we show that the difference in the expression level of LXRα and LXRβ is less clear on protein level in acute MS lesions (fig. 3.3). Microarray analysis of myelin-laden rat macrophages showed that the expression of LXR response gene *ABCA1* was significantly upregulated compared to non-myelin treated cells (203). In concordance, we found that *ABCA1* is induced in both myelin-treated human monocyte-derived macrophages and active MS lesions. In summary, based on the observation that downstream targets of LXRs are highly upregulated in myelin-laden phagocytes it is likely that LXRs are activated upon myelin digestion in active MS lesions.

Myelin contains various lipids and oxysterols that can influence macrophage function. Recently, we demonstrated that myelin phagocytosis alters macrophage phenotype in rat macrophages through activation of LXRs (203). Therefore, we hypothesized that the uptake of myelin and the subsequent formation of LXR

ligands results in the activation of LXRs in phagocytes in active MS lesions. We measured (oxy)sterols present in myelin such as desmosterol, 24SOHC and 27OHC that are known to potently activate LXRs (438-441). After myelin phagocytosis, myelin is processed by the phagocyte, possibly generating cholesterol oxidation products, such as oxysterols. We found that five days after uptake of myelin by macrophages, intracellular myelin levels start to decrease and the levels of the cholesterol metabolite 27OHC, but not 24SOHC and desmosterol, are significantly elevated. Taken together, these results indicate that LXR ligands are present in human myelin, and that after myelin processing, 27OHC is one of the major LXR ligands that is formed.

The amount of 27OHC present *in vitro* in macrophages after myelin phagocytosis activates both LXR-isoforms with a higher affinity for LXRα (439, 442). Exposing macrophages to 27OHC resulted, very similarly to myelin, in upregulation of *LXRα*, and LXR-response genes *ABCA1* and *ABCG1*. Based on these data, we speculate that initially LXRs are activated by ligands present in myelin (e.g. 24SOHC, 27OHC and desmosterol), while during later stages also LXR ligands that are generated after myelin processing contribute to LXR activation.

Collectively, based on the observation that downstream targets of LXRs are upregulated in myelin-laden phagocytes in active MS lesions it is conceivable that LXRs are activated by ligands present in myelin. Moreover, 27OHC is the major LXR ligand that is formed after myelin processing and the levels of 27OHC that are generated are capable of upregulating LXR downstream targets in phagocytes indicating that 27OHC is, at least in part, responsible for activation of LXRs in MS lesions. Although myelin is very stable in content, it cannot be excluded that differences in myelin composition between and within individuals (e.g. different anatomical CNS regions) may modulate the conversion of cholesterol to 27OHC. LXRs are of great importance in regulating cholesterol homeostasis and protect cells from elevated intracellular free cholesterol and oxysterol-induced toxicity (245). More importantly, LXRs have been shown to inhibit inflammatory gene expression and stimulate remyelination (216, 217, 443, 444) underscoring their significance as important regulators in MS pathogenesis. This notion is highlighted in a recent study in which a mutation was found in the LXRα gene in familial MS (445). The clinical disease progression and severity for mutant LXRα carriers is

evidenced by its rapid progression and severity. In this study, the authors suggest that the p.Arg415Gln mutation, located in the ligand-binding domain, prevents LXR activation by abrogating heterodimerization between LXR and RXR, resulting in loss of function. Because of the oxysterols present in myelin and the formation of cholesterol oxidation products, it is likely that myelin-laden phagocytes in MS are at least in part modulated by a myelin-mediated activation of LXRs, providing an intriguing hypothesis for the LXR-controlled self-limiting nature of MS lesion development.

4

Low-density lipoprotein receptor deficiency attenuates neuroinflammation through the induction of apolipoprotein E

Based on:

Low-density lipoprotein receptor deficiency attenuates neuroinflammation through the induction of apolipoprotein E.

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4 Low-density lipoprotein receptor deficiency attenuates neuroinflammation through the induction of apolipoprotein E

4.1 Abstract

Objective: We aimed to determine the role of the low-density lipoprotein receptor (LDLr) in neuroinflammation by inducing experimental autoimmune encephalomyelitis (EAE) in *ldlr* knock out mice.

Methods: MOG_{35-55} induced EAE in male and female *ldlr*$^{-/-}$ mice was assessed clinically and histopathologically. Expression of inflammatory mediators and apolipoprotein E (apoE) was investigated by qPCR. Changes in protein levels of apoE and tumor necrosis factor alpha (TNFα) were validated by western blot and ELISA respectively.

Results: *Ldlr*$^{-/-}$ attenuated EAE disease severity in female, but not in male EAE mice, marked by a reduced proinflammatory cytokine production in the central nervous system (CNS) of female *ldlr*$^{-/-}$ mice. Macrophages from female *ldlr*$^{-/-}$ mice showed a similar decrease in proinflammatory mediators, an impaired capacity to phagocytose myelin and enhanced secretion of the anti-inflammatory apoE. Interestingly, *apoE/ldlr* double knock out abrogated the beneficial effect of *ldlr* depletion in EAE.

Conclusion: Collectively, we show that *ldlr*$^{-/-}$ reduces EAE disease severity in female but not in male EAE mice, and that this can be explained by increased levels of apoE in female *ldlr*$^{-/-}$ mice. Although the reason for the observed sexual dimorphism remains unclear, our findings show that LDLr and associated apoE levels are involved in neuroinflammatory processes.

4.2 Introduction

Multiple sclerosis (MS) is an inflammatory autoimmune demyelinating disease of the central nervous system (CNS). Infiltrated macrophages and resident microglia are considered the dominant effector cells in MS and its animal model, experimental autoimmune encephalomyelitis (EAE) (446, 447). Effector mechanisms include phagocytosis of myelin and the secretion of inflammatory and toxic mediators (91, 97, 139, 427, 448). However, increasing evidence indicates that phagocytes can also acquire a disease-resolving phenotype in the CNS. For instance, we recently defined that ingestion of myelin by macrophages alters the inflammatory phenotype by activating the cholesterol- and fatty acid-sensing nuclear liver X receptors (LXRs) and *peroxisome proliferator-activated receptors* (PPARs) (203, 449, 450). These studies stress the pleiotropic role that phagocytes play in the pathophysiology of MS and indicate that lipid-signaling pathways drive the phenotype of phagocytes in MS lesions.

The impact of cholesterol and its metabolites on the pathophysiology of MS is a topic of interest in MS-research (254, 301, 303, 380, 451, 452). In the MS brain, marked alterations in both myelin cholesterol and other lipid metabolites are found (451). Furthermore, plasma and cerebrospinal fluid (CSF) (oxy)sterol levels are disturbed and closely correlate to neurodegenerative processes (254, 380, 452, 453). Additionally, we showed that that lipoprotein levels and function are altered in relapsing-remitting MS patients (RRMS), which may advance disease progression in these patients (184). More specifically, we found smaller low-density lipoprotein (LDL) particles in RRMS patients compared to healthy controls. Interestingly, the average LDL particle size was smaller in male RR-MS patients compared to female RRMS patients, which suggests gender differences in lipid metabolism in MS. LDL size is described to be important for LDL function indicating the lipoprotein may be involved in the pathophysiology of disease. Smaller particles have an increased susceptibility to oxidation and a decreased LDL receptor (LDLr) affinity, which may promote their proinflammatory properties (185-187).

The LDLr is a 160kDa cell surface glycoprotein that plays a key role in plasma cholesterol homeostasis (192). It binds two physiologically important ligands, apolipoprotein B-100 (apoB) and apolipoprotein E (apoE). The only known ligand

for the LDLr in the CNS is apoE since apoB is not synthesized in the CNS and, in contrast to apoE, cannot cross the blood-brain barrier (192). Malfunctioning of the receptor in humans results in familial hypercholesterolemia (FH) and is an important risk factor for cardiovascular disease (454). FH is caused by genetic mutations that directly or indirectly affect the function of the LDLr. It is characterized by defective catabolism of LDL, which results in increased plasma cholesterol concentrations and lipid accumulation in macrophages and other immune cells. This promotes proinflammatory responses, including enhanced NF-κB signaling, inflammasome activation, and increased production of neutrophils and monocytes in the bone marrow and spleen (194-198). The ability of lipoproteins to modulate neuroinflammation has been suggested in recent studies (387, 388). In addition, elevated LDL plasma levels were found to increase neurotoxicity in a mouse model for Alzheimer's disease (388). The above findings indicate that lipoproteins modulate neuroinflammation and neurodegeneration. However, the direct contribution of LDL and LDLr to these processes remains unclear.

Here, we determined the role of the LDLr in neuroinflammation by using the EAE model and *ldlr* knock out (*ldlr*$^{-/-}$) mice. We show that *ldlr* deficiency reduces disease severity in female, but not in male EAE mice. In line with this finding, the inflammatory burden was significantly lower in the CNS of female *ldlr*$^{-/-}$ mice compared to *ldlr*$^{-/-}$ male mice. Macrophages isolated from female *ldlr*$^{-/-}$ mice show an altered inflammatory profile and an impaired capacity to phagocytose myelin. Interestingly, apoE release was enhanced in macrophages derived from females and the beneficial effect of *ldlr* deficiency in EAE was absent in *apoE/ldlr* double knock out mice. This shows that elevated apoE levels likely attenuate EAE severity in female *ldlr*$^{-/-}$ mice. Collectively, our findings indicate that changes in *ldlr* expression contribute to the progression of neuroinflammatory diseases in a gender specific manner.

4.3 Methods

4.3.1 Animals

ldlr$^{-/-}$ and *apoE*$^{-/-}$ mice on a C57BL/6 background and C57BL/6 wild type (WT) mice were purchased from Harlan Laboratories. Animals were fed a regular diet and housed in the animal facility of the Biomedical Research Institute of Hasselt University. All experiments were performed according to institutional guidelines and were approved by the ethical committee for animal experiments of Hasselt University.

4.3.2 Induction of EAE

Eleven-week-old mice were immunized subcutaneously at the base of the tail with 200 µg of recombinant human myelin oligodendrocyte glycoprotein MOG_{35-55} emulsified in 100µl complete Freund's adjuvant supplemented with 4 mg/ml of Mycobacterium tuberculosis (H37RA strain) according to manufacturer's guidelines (Hooke Laboratories, Lawrence, USA). Within two hours and after 22-26 hours, mice were intraperitoneally injected with 0.1 ml pertussis toxin. Immunized mice were weighed and scored daily by following a five-point standardized rating of clinical symptoms: 0, no signs; 1, loss of tail tonus; 2, flaccid tail; 3, hind limb paresis; 4, hind limb paralysis; 5, death. Mice were sacrificed at day 18 (peak) and 27 post immunization.

4.3.3 Peritoneal macrophage cultures

Mice were injected intraperitoneally with 1.5 ml phosphate-buffered saline (PBS, Sigma-Aldrich) supplemented with 5 mM ethylenediamine teraacetic acid (EDTA, VWR, Leuven, Belgium). Peritoneal cells were cultured for 3 hours in RPMI-1640 medium (Lonza, Verviers, Belgium), enriched with 10% fetal calf serum (FCS; Hyclone, Erembodegem, Belgium), 0.5% penicillin/streptomycin (P/S Invitrogen, Merelbeke, Belgium), in a 24-well plate ($5x10^4$ cells/well) at 37°C with 5% CO2. Cells were stimulated for 18h with 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) to assess their gene expression. Medium was stored for protein measurement.

4.3.4 T cell proliferation

Spleen and inguinal lymph nodes (LN) were isolated from mice 9 days after EAE induction. T cells were isolated from LN and spleen and cultured in RPMI medium containing 20 µM β-mercaptoethanol, 2% mouse serum, 1% non-essential amino acids (NEAA), 1% sodium pyruvate, 0.5% P/S. Cells were plated in a 96 well plate at a density of $3x10^5$ cells/well and subsequently stimulated with 10µg/ml MOG for 48 hours. Next, 1µCi [^{3}H] thymidine (Amersham biosciences, UK) was added for 18 hours after which cells were collected using an automatic cell harvester (Pharmacia, Uppsala, Sweden). A β plate liquid scintillation counter (Perkin Elmer, lifesciences, Wessesly, USA) was used to quantify radioactivity and results are expressed as stimulation index (SI). The SI shows the relative proliferation of MOG-stimulated T cells compared to non-stimulated T cells (SI = 1).

4.3.5 Quantitative PCR

RNA was extracted from tissue or cells using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). In short, lysis was performed with Qiazol Lysis reagent (Qiagen) supplemented with 1% β-mercaptoethanol (Sigma-Aldrich). RNA concentration and quality were determined with a Nanodrop spectrophotometer (Isogen Life Science, The Netherlands). cDNA synthesis was conducted using the Quanta qScript cDNA synthesis kit (Quanta Biosciences, Boston, USA) per manufacturer's instructions. Quantitative PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR system (Applied biosystems, Ghent, Belgium) using the SYBR green method (Applied Biosystems). The master mix contained 1x SYBR green, 10 µM primers, 12,5ng cDNA, and nuclease free water. Data were normalized to the most stable reference genes and the ΔΔCt method was used to determine relative quantification of gene expression. Primers were designed using NCBI's Primer-blast (details are shown in table 4.1).

Table 4.1 | Mouse primers for RT-qPCR.

Gene	Sequence (5'–3')	Product size	Accession number
apoE forward	ACTGGGTCGCTTTTGGGATT	64 bp	NM_000041.2
apoE reverse	CTCCTCCTGCACCTGCTCA		
Tnfα forward	CCAGACCCTCACACTCAG	79 bp	NM_013693.3
Tnfα reverse	CACTTGGTGGTTTGCTACGAC		
iNOS forward	GGCAGCCTGTGAGACCTTTG	150 bp	NM_010927.3
iNOS reverse	GCATTGGAAGTGAAGCGTTTC		
IL-6 forward	TGTCTATACCACTTCACAAGTCGGAG	79 bp	NM_001314054.1
IL-6 reverse	GCACAACTCTTTTCTCATTTCCAC		
Ywhaz forward	GCAACGATGTACTGTCTCTTTTGG	149 bp	NM_001253807.1
Ywhaz reverse	GTCCACAATTCCTTTCTTGTCATC		
Pgk1 forward	GAAGGGAAGGGAAAAGATGC	138 bp	NM_008828.3
Pgk1 reverse	GCTATGGGCTCGGTGTGC		
Rpl13a forward	GGATCCCTCCACCCTATGACA	131 bp	NM_009438.5
Rpl13a reverse	CTGGTACTTCCACCCGACCTC		
Ywhaz forward	GCAACGATGTACTGTCTCTTTTGG	149 bp	NM_001253807.1
Ywhaz reverse	GTCCACAATTCCTTTCTTGTCATC		
T-bet forward	CCATGTGACCCAGATGATCG	87 bp	NM_019507.2
T-bet reverse	TCTGGCTCTCCATCATTCAC		
GATA3 forward	CTGCGGACTCTACCATAA	114 bp	NM_001355111.1
GATA3 reverse	GTGGTGGTCTGACAGTTC		
RORγt forward	GGATGAGATTGCCCTCTA	141 bp	NM_011281.3
RORγt reverse	CCTTGTCGATGAGTCTTG		
Foxp3 forward	CCCAGGAAAGACAGCAACCTT	133 bp	NM_001199347.1
Foxp3 reverse	CTGCTTGGCAGTGCTTGAGAA		
IL-12 forward	TCATCAGGGACATCATCAAACC	210 bp	NM_001303244.1
IL-12 reverse	CGAGGAACGCACCTTTCTG		
Arg1 forward	GTGAAGAACCCACGGTCTGT	132 bp	NM_007482.3
Arg1 reverse	GCCAGAGATGCTTCCAACTG		
IL-17 forward	ATCAGGACGCGCAAACATGA	125 bp	NM_010552.3
IL-17 reverse	TTGGACACGCTGAGCTTTGA		
IL-4 forward	CTCACAGCAACGAAGAACACCA	148 bp	NM_021283.2
IL-4 reverse	AAGCCCGAAAGAGTCTCTGCA		
IFNγ forward	TGAGGTCAACAACCCACAGGT	104 bp	NM_008337.4
IFNγ reverse	GACTCCTTTTCCGCTTCCTGAG		

4.3.6 NO production

Peritoneal macrophages in culture were stimulated with 100 ng/ml LPS (Sigma-Aldrich) prior to the assay. NO production was determined in the supernatant of peritoneal macrophages using a Griess-reagent assay (Promega, Leuven, Belgium) according to the manufacturer's instructions.

4.3.7 Immunohistochemistry

Animals were perfused transcardially through the ventricular catheter with ringer (containing heparin). Spinal cord tissue was isolated, snap-frozen, and sectioned with a Leica CM1900UV cryostat (Leica Microsystems, Wetzlar, Germany) to obtain 8 µm slices. Dried cryosections were fixed in acetone for 10 minutes and subsequently blocked for 0,5h with 10% normal serum. Cryosections were incubated overnight at 4°C with primary antibodies. Next, secondary antibodies were added for 1h. Primary antibodies used were rat anti-mouse CD3 (1:150; AbD Serotec) and rat anti-mouse F4/80 (1:100; AbD Serotec). Goat anti-rat Alexa Fluor®555 (1:400; Invitrogen) was used as a secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:2500; Sigma-Aldrich). Stained sections were visualized under a Nikon Eclipse 80i fluorescence microscope (Nikon, Kingston, UK). Sections were analyzed using NIS Elements viewer software 4.0 (Nikon).

4.3.8 Western blot

ApoE secretion by peritoneal macrophages was determined using western blot. The culture medium was collected and separated via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were transferred to a PVDF-membrane (VWR, Leuven, Belgium) and blots were blocked for 1 hour in TBS-Tween 5% non-fat dry milk. Membranes were probed with mouse anti-apoE (Abbiotec, Antwerpen, Belgium). After washing steps with TBS-Tween, blots were incubated with horseradish peroxidase-labeled anti-mouse antibody (Dako, Heverlee, Belgium). Immunoreactive signals were detected with Enhanced Chemiluminiscence (ECL Plus, GE Healthcare, Diegem, Belgium).

4.3.9 Phagocytosis assay

Myelin was isolated from brain tissue of healthy adult WT C57BL/6 OlaHSD mice (Harlan) by means of sucrose density gradient centrifugation, as previously described (375). Next, myelin was labeled with the lypophilic dye 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) (Thermofisher Scientific, Erembodegem, Belgium). Peritoneal macrophages were incubated with DiI labeled myelin (25 µg/ml) for 90 minutes at 37°C and 5% CO_2. Next, cells were rinsed with PBS (Sigma-Aldrich), detached with PBS/EDTA and resuspended in FACS buffer containing 1x PBS, 2% FCS (Hyclone) and sodium Azide. The fluorescent internalized myelin was measured using the FACS Calibur flow cytometer (BD biosciences, Erembodegem, Belgium). Results are expressed as mean fluorescence.

4.3.10 ELISA

Peritoneal mouse macrophages were stimulated with 100 ng/ml LPS (Sigma) for 18h prior to the assay. TNFα concentration in peritoneal macrophage culture supernatant was determined using the TNFα Mouse Uncoated ELISA Kit (Thermofisher), following the manufacturer's instructions. Absorption was measured at 450 nm using a microtiterplate reader (Biorad, Temse, Belgium).

4.3.11 Statistical analysis

Data were statistically analyzed using GraphPad Prism for windows (version 5.0) and are reported as mean ± SEM. D'Agostino and Pearson omnibus normality test was used to test Gaussian distribution. A two-tailed unpaired student t-test was used for normally distributed data sets (t(df); P). The Mann-Whitney analysis ((MWU, n1, n2; P) was used for data sets that did not pass normality. EAE scores were analyzed using 2-way ANOVA(Bonferroni's post hoc Multiple Comparison test). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.4 Results

4.4.1 *Ldlr* deficiency reduces EAE severity in female, but not in male mice

To elucidate whether the LDLr contributes to neuroinflammation, we induced EAE in male and female *ldlr*$^{-/-}$ mice. We show that *ldlr* deficiency has a sex-specific effect on the EAE course. Female *ldlr*$^{-/-}$ mice exhibited a decreased EAE disease severity compared to WT mice, whereas in male mice *ldlr* deficiency did not affect EAE disease severity (fig. 4.1A-B). In female animals, the mean peak of disease symptoms was reached around day 17. The mean disease score was attenuated in LDLr$^{-/-}$ (1.504 ± 0.1995) compared to WT (2.318 ± 0.1782) mice (fig. 4.1C). Accordingly, the maximum disease score was lower in *ldlr*$^{-/-}$ (1.70 ± 0.30) compared to WT (2.68 ± 0.29) mice (fig. 4.1D). The *ldlr*$^{-/-}$ and WT groups displayed a similar day of onset (14.29 ± 1.04 and 12.41 ± 0.34, respectively, fig 1E).

4.4.2 *Ldlr* deficiency has no significant influence on immune cell infiltration into the CNS

EAE is characterized by the infiltration of peripheral immune cells into the CNS leading to a local inflammatory response. To determine whether this process is altered by *ldlr* deficiency in female mice, the accumulation of T cells (CD3) and macrophages (F4/80) in the CNS of WT and *ldlr* deficient female EAE mice was assessed by immunohistochemistry at day 18 and day 33 post immunization (fig. 4.2). Despite a reduced disease severity, no significant differences in the number of infiltrated macrophages and T cells into the spinal cord tissue were observed comparing female WT EAE mice and *ldlr* deficient EAE mice, although a trend on day 18 is visible.

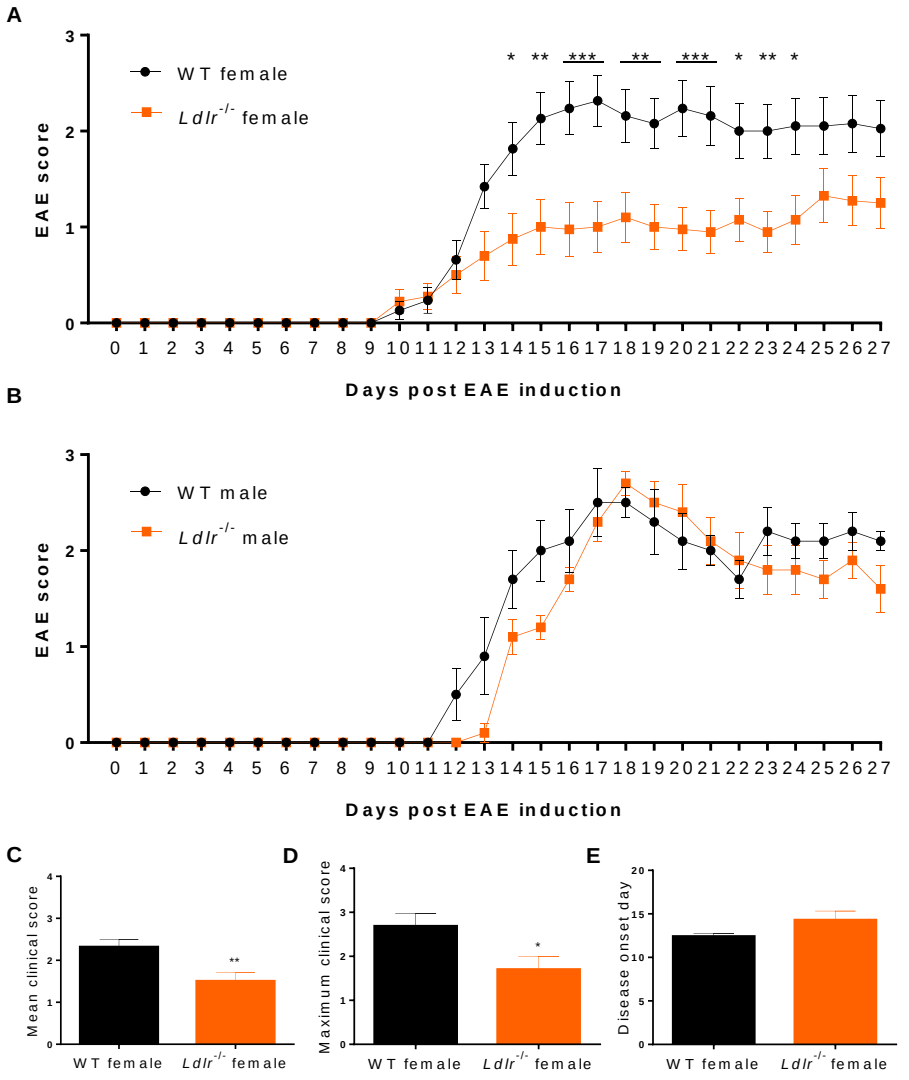

Figure 4.1 | *Ldlr*$^{-/-}$ reduces EAE severity in female but not in male mice. Daily EAE score of female ($p<0.0079$; WT vs *ldlr*$^{-/-}$ by two-way ANOVA) (n=19-20, pooled from three independent experiments) (**A**) and male ($p<0.2221$; WT vs *ldlr*$^{-/-}$ by two-way ANOVA) (n=5) (**B**) mice. The mean score ($t(31)=3.050$; $p<0.0048$) (**C**), the maximum score ($t(37)=2.349$; $p<0.0244$) (**D**), and the day of onset of the disease ($t(29)=1.848$; $p<0.0748$) (**E**).

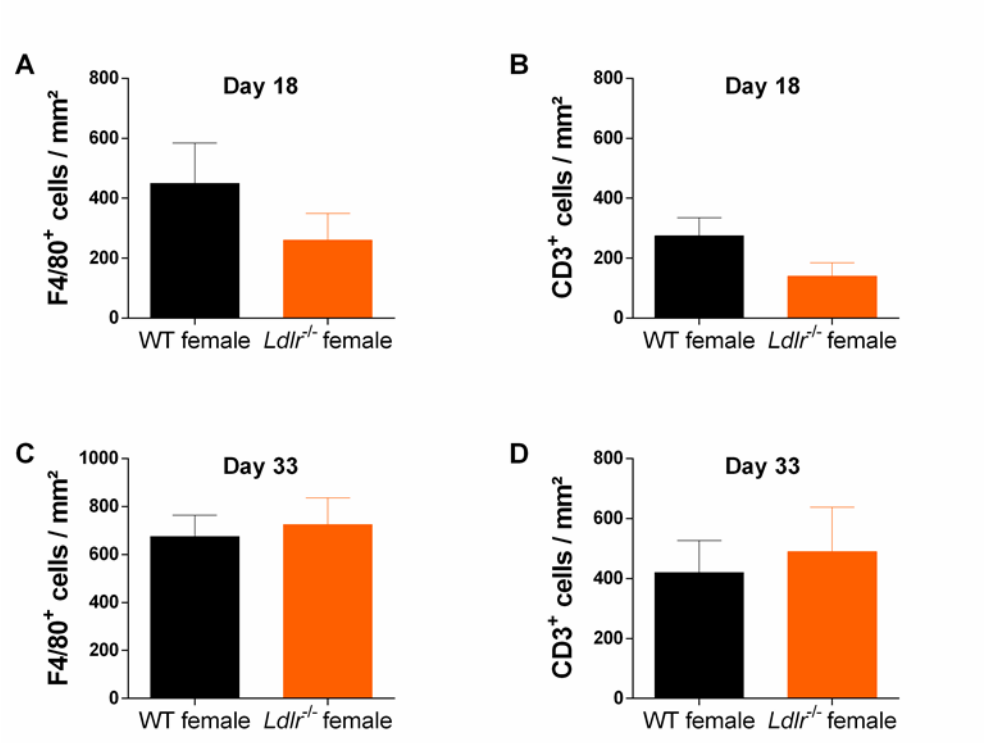

Figure 4.2 | *Ldlr* deficiency has no significant influence on macrophage and T cell infiltration into the CNS. Immunohistological sections of spinal cord tissue from WT and *ldlr*$^{-/-}$ mice at day 18 (**A, B**) and day 33 (**C, D**) post immunization. Sections were stained against F4/80 for macrophages (**A**, **C**) and CD3 for T cells (**B**, **D**). At least six sections per mouse were quantified (n=5).

4.4.3 *Ldlr* deficiency in female mice has no influence on T cell proliferation

T cell proliferation is an important hallmark of EAE and is essential for the initiation of EAE pathogenesis. Since T cells are dependent on cholesterol in order to proliferate (455), we investigated the influence of *ldlr* on T cell proliferation during EAE. T cells from both *ldlr*$^{-/-}$ and WT mice were isolated on day 9 post immunization and a thymidine assay was used to determine their proliferation capacity. No significant differences in T cell proliferation between *ldlr*$^{-/-}$ and WT mice induced with EAE (fig. 4.3A). Additionally, we determined mRNA expression of transcription factors such as T-bet, GATA3, RORγt and Foxp3, which are involved in T cell differentiation into Th1, Th2, Th17 and Treg subsets, in LN and

spleen but found no difference between *ldlr*$^{-/-}$ and WT female mice (fig. 4.3B-C). Lastly, we analyzed the mRNA expression levels of cytokines important in T cell differentiation (IFNγ, IL4, IL17 and IL10) in T cells isolated from both spleen and LN. No differences in the gene expression of these cytokines were observed. The mRNA levels of IL-4 and IL-17 were not detectable (fig. 4.3D-E). Together, these results indicate that altered T cell proliferation and differentiation do not account for the reduced EAE in *ldlr* deficient mice.

4.4.4 *Ldlr* deficiency reduces inflammation in the spinal cord of female mice compared to male mice

Our data indicate that *ldlr* deficiency in female mice attenuates EAE severity without affecting T cell proliferation and the level of immune cell infiltration into the CNS. To further define the inflammatory burden in the CNS, whole spinal cord tissue of male and female *ldlr*$^{-/-}$ mice was analyzed for mRNA expression of proinflammatory genes. Female *ldlr*$^{-/-}$ mice exhibited a significantly lower expression of tumor necrosis factor alpha (*Tnfa*), Interleukin-6 (*IL-6*), and nitric oxide synthase (*iNOS*) compared to male *ldlr*$^{-/-}$ mice and female WT mice (fig. 4.4A-B), whereas the expression of these inflammatory genes is not changed in male *ldlr*$^{-/-}$ mice (fig. 4.4C). These data indicate that female *ldlr*$^{-/-}$ mice have decreased inflammation in the spinal cord compared to male *ldlr*$^{-/-}$ and female WT mice.

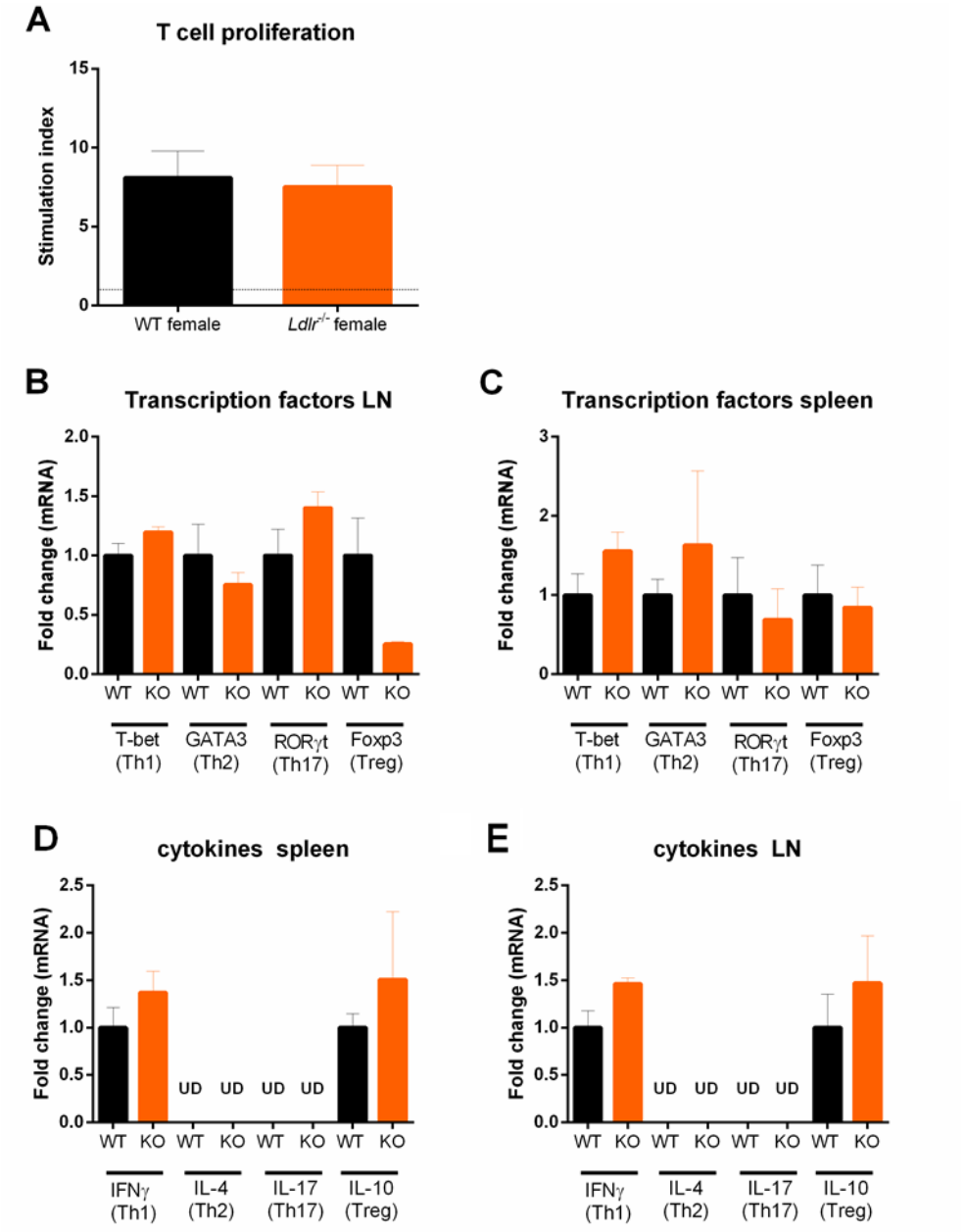

Figure 4.3 | *Ldlr* deficiency has no influence on T cell proliferation. Stimulation index (SI) of T cells isolated from lymph nodes of WT and LDLR deficient mice (n=3). Cells were collected on day 9 post immunisation and proliferation was analysed by means of a thymidine assay. Unstimulated cells an SI of 1 (**A**). Gene expression of transcription factors (T-bet, GATA3, RORγt AND Foxp3) in T cells isolated from lymph nodes (**B**) and spleen (**C**) from WT and *ldlr*$^{-/-}$ female mice on day 9 post immunisation (n=4). mRNA expression levels

of cytokines important in T cell differentiation (IFNγ, IL4, IL17 and IL10) in T cells isolated from both spleen and lymph nodes from WT and *ldlr*$^{-/-}$ female mice (n=4) (**D**, **E**). No differences in the gene expression of these cytokines were observed. The mRNA levels of IL-4 and IL-17 were not detectable.

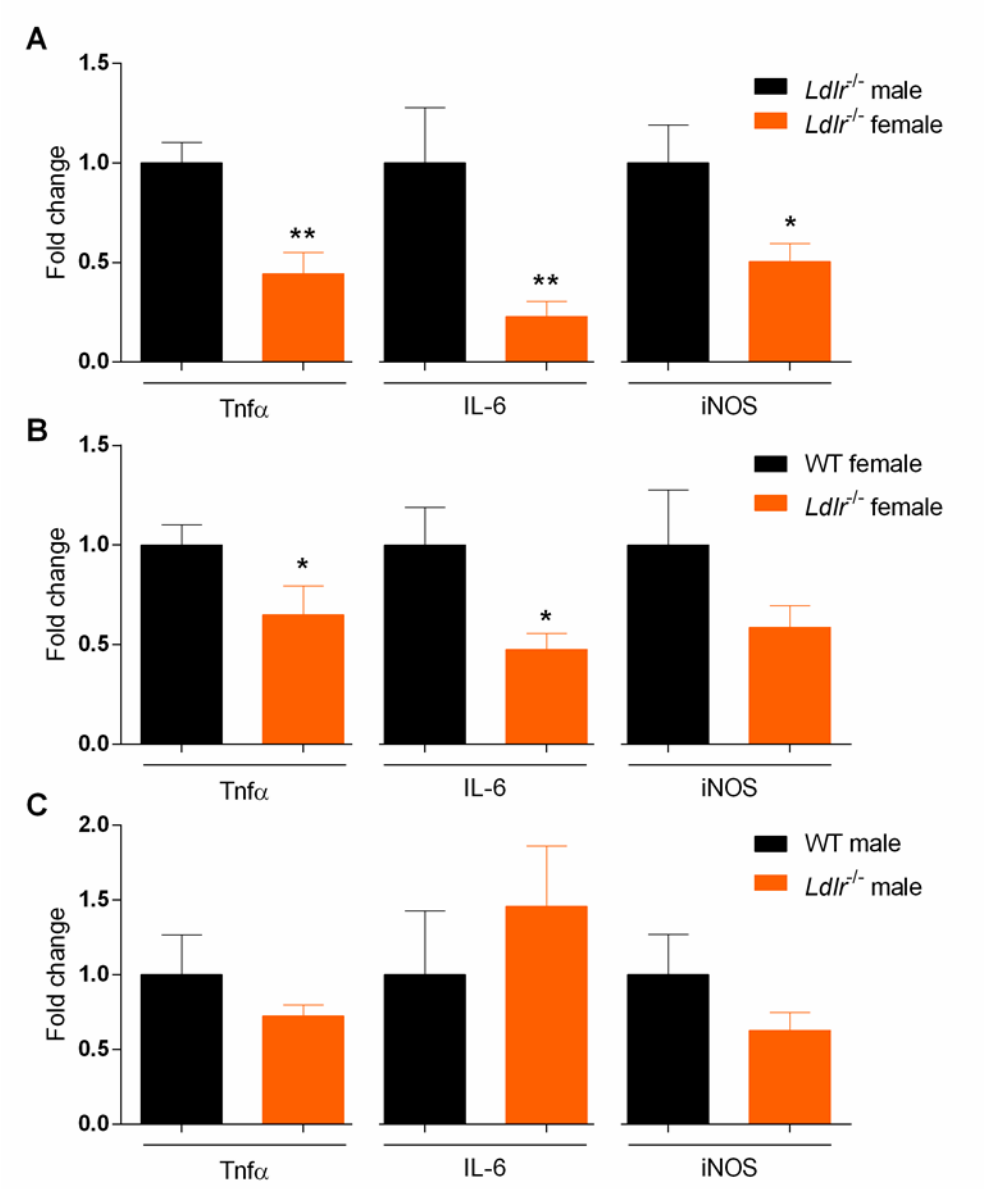

Figure 4.4 | Relative expression of proinflammatory genes in the spinal cord of *ldlr*$^{-/-}$ mice. Expression of *Tnfα* (t(10)=3.668; p<0.0044), *IL-6* (MWU=0; 5; 7; p<0.0026) and *iNOS* (t(10)=2.588; p<0.0271) for male and female *ldlr*$^{-/-}$ mice at EAE peak (n=5-7) (**A**).

Expression of *Tnfα* (t(13)=2.467; p<0.0284), *IL-6* (t(12)=2.553; p<0.0254) and *iNOS* (t(13)=1.313; p<0.2118) for WT and *ldlr*$^{-/-}$ female mice at EAE peak (n=5-7) (**B**). Expression of *Tnfα* (t(7)=1.106; p<0.3054), *IL-6* (t(7)=0.7745; p<0.4641) and *iNOS* (t(7)=1.370; p<0.2129) for WT and *ldlr*$^{-/-}$ male mice at EAE peak (n=5-7) (**C**). Data were normalized to the most stable reference genes, determined by Genorm (*Ywhaz* and *Pgk-1*).

4.4.5 *Ldlr* deficiency suppresses the inflammatory phenotype of macrophages in female mice

As the *ldlr* is highly expressed on macrophages and we observed a reduced expression of cytokines typically released by macrophages in female *ldlr*$^{-/-}$ EAE mice, we investigated the influence of *ldlr* deficiency on the inflammatory phenotype of macrophages *in vitro*. We found that female LPS-stimulated *ldlr*$^{-/-}$ macrophages produce less TNFα and NO compared to macrophages isolated from WT females (fig. 4.5A-B). Additionally, we show that macrophages isolated from *ldlr*$^{-/-}$ female mice less efficiently internalize myelin compared to macrophages isolated from WT females (fig. 4.5C). Interestingly, expression of *lrp1*, a major receptor involved in the phagocytosis of myelin (108), was reduced as well (fig. 4.5D). The expression of the pro-inflammatory genes *Tnfα, IL-6 and Il-12* is reduced in *ldlr*$^{-/-}$ female mice whereas the anti-inflammatory gene *Arg1* is increased in macrophages isolated from *ldlr*$^{-/-}$ female mice compared to WT mice (fig. 4.5E-I). These data indicate that macrophages isolated from female *ldlr*$^{-/-}$ mice have an attenuated inflammatory response upon stimulation and have a reduced phagocytosis capacity compared to their WT counterparts.

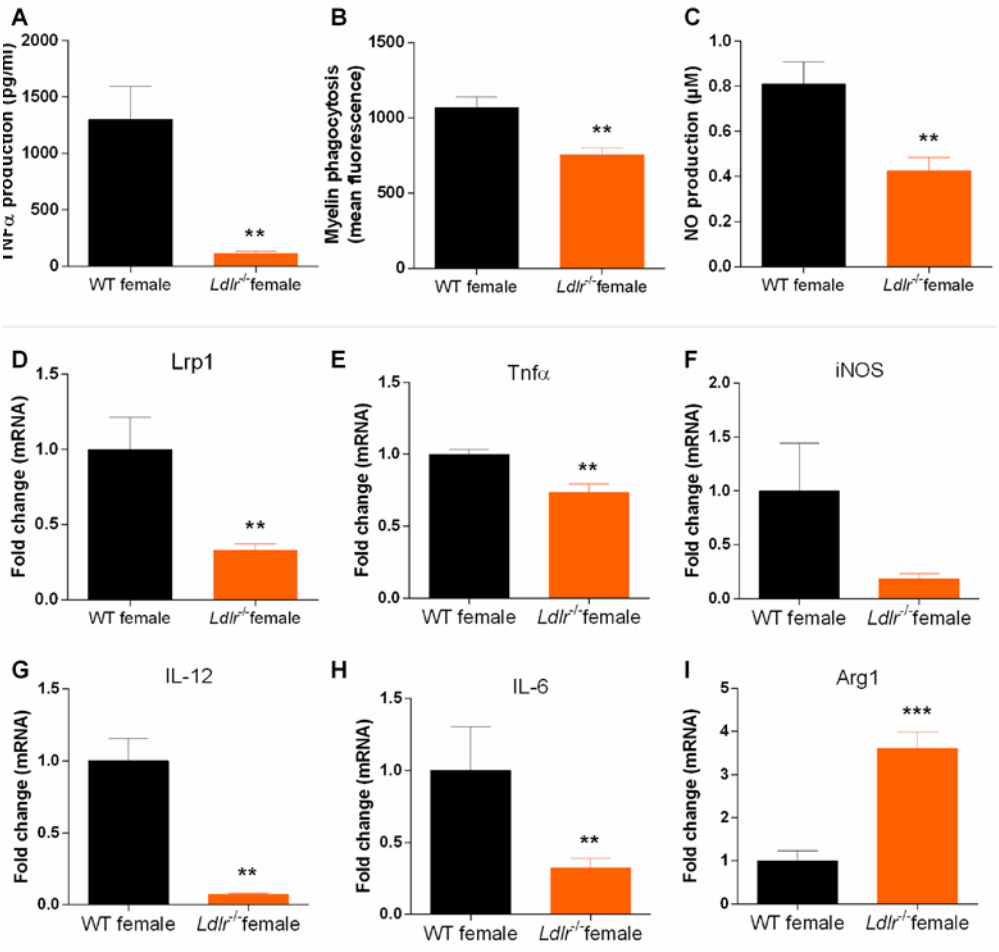

Figure 4.5 | *Ldlr* deficiency influences the production of inflammatory mediators by macrophages. TNFα production (t(17)=3.444; p<0.0032) (n=6) (**A**), NO production (t(22)=3.368; p<0.0029) (n=6) (**B**), phagocytosis (mean fluorescence) (t(28)=3.638; p<0.0012) (n=6). (**C**), *lrp1* expression (MWU=2; 5; 8; p<0.0063) (n=5-8) (**D**), *Tnfα* expression ((t(9)=3.630; p<0.0055) (**E**), i*NOS* expression ((t(9)=2.020; p<0.0742) (**F**), *IL-12* expression (MWU=0; 4; 6; p<0.0095) (**G**), *IL-6* expression ((t(9)=2.391; p<0.0405) (**H**) and *Arg1* expression ((t(8)=5.846; p<0.0004) (**I**) in peritoneal macrophages isolated from WT and *ldlr*$^{-/-}$ female mice (n=6).

4.4.6 ApoE expression is increased in activated peritoneal macrophages isolated from *ldlr*$^{-/-}$ female mice

ApoE has been known to induce an anti-inflammatory phenotype in macrophages (230). Interestingly, we show that ApoE mRNA expression is significantly increased in activated peritoneal macrophages from *ldlr*$^{-/-}$ female mice compared to both male *ldlr*$^{-/-}$ mice and female WT mice (fig. 4.6A-B), whereas apoE mRNA expression is not significantly different in male *ldlr*$^{-/-}$ mice or between male and female WT mice (fig. 4.6C-D). Next, we used Western blot to determine the amount of apoE secreted into the culture medium. We found that only LPS-stimulated macrophages isolated from female *ldlr*$^{-/-}$ mice secreted significant levels of apoE (fig. 4.6E). These data suggest that *ldlr* deficiency in female mice is responsible for the increase and subsequent secretion of apoE by macrophages.

4.4.7 Lack of apoE abrogates the reduced neuroinflammatory response in female *ldlr*$^{-/-}$ mice

To define to what extend an increase in apoE accounts for the observed reduction in EAE disease severity in *ldlr*$^{-/-}$ female mice and the observed less-inflammatory phenotype of macrophages isolated from these mice, we crossbred *apoE*$^{-/-}$ *and ldlr*$^{-/-}$ mice. In contrast to female *ldlr*$^{-/-}$ mice, female *apoE*$^{-/-}$*ldlr*$^{-/-}$ mice did not exhibit an attenuated EAE disease course compared to WT mice (fig. 4.7A). Additionally, the observed decreased capacity to phagocytose myelin and produce TNFα by macrophages from *ldlr*$^{-/-}$ female mice was counteracted by depletion of apoE (fig. 4.7B-C). Interestingly, the impairment in NO production observed in *ldlr*$^{-/-}$ macrophages was also present in *apoE*$^{-/-}$*ldlr*$^{-/-}$ macrophages indicating apoE is not accountable for this effect (fig. 4.7D). *ApoE*$^{-/-}$ alone did not alter EAE severity in female mice compared to WT mice (fig. 4.7E). These data indicate that the lack of apoE largely restores the impaired inflammatory response induced by *ldlr* deficiency in female mice and that the effect of *ldlr*$^{-/-}$ on NO production is not responsible for the ameliorated disease course in these mice.

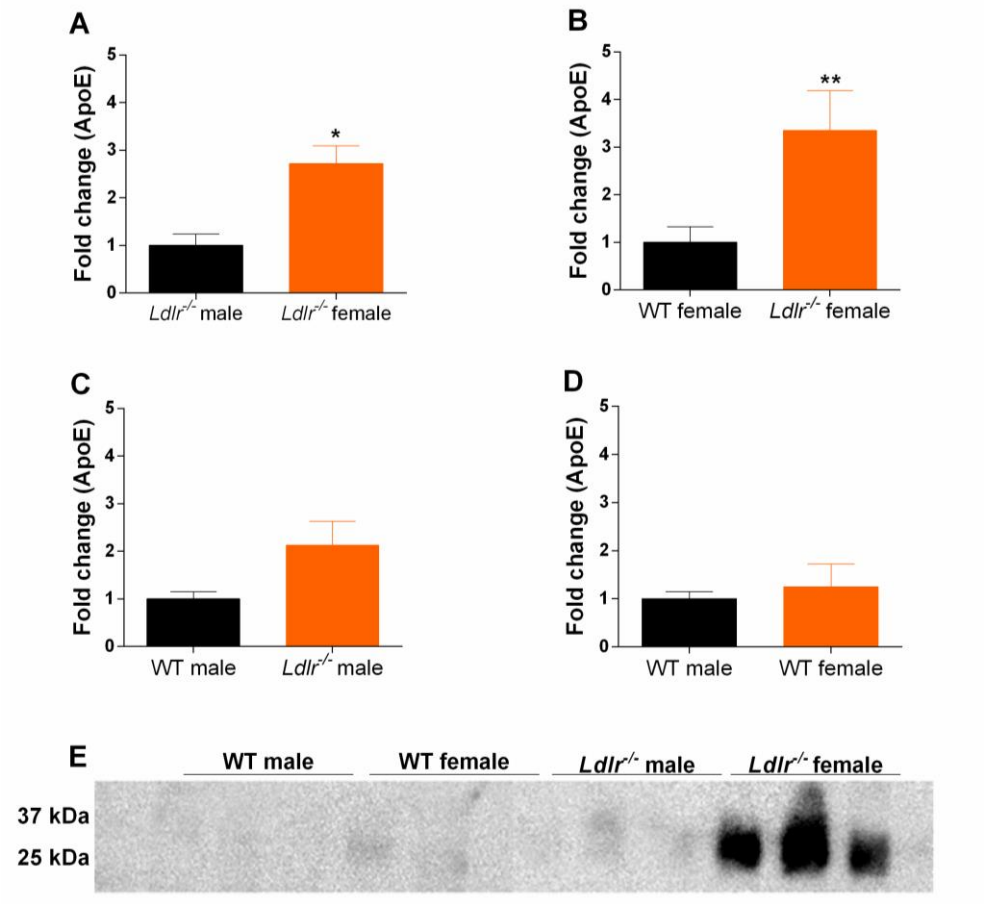

Figure 4.6 | *ApoE* expression by peritoneal macrophages. Comparison of *apoE* gene expression between male and female *ldlr*$^{-/-}$ mice (MWU=1; 6; 6; p<0.0476) (n=6) (**A**), WT and *ldlr*$^{-/-}$ female mice (MWU=1; 6; 6; p<0.0044) (n=6) (**B**), and WT and *ldlr*$^{-/-}$ male mice (n=6) (**C**). Western blot analysis of cell culture medium from LPS-stimulated peritoneal macrophages, stained for apoE (n=3) (**D**).

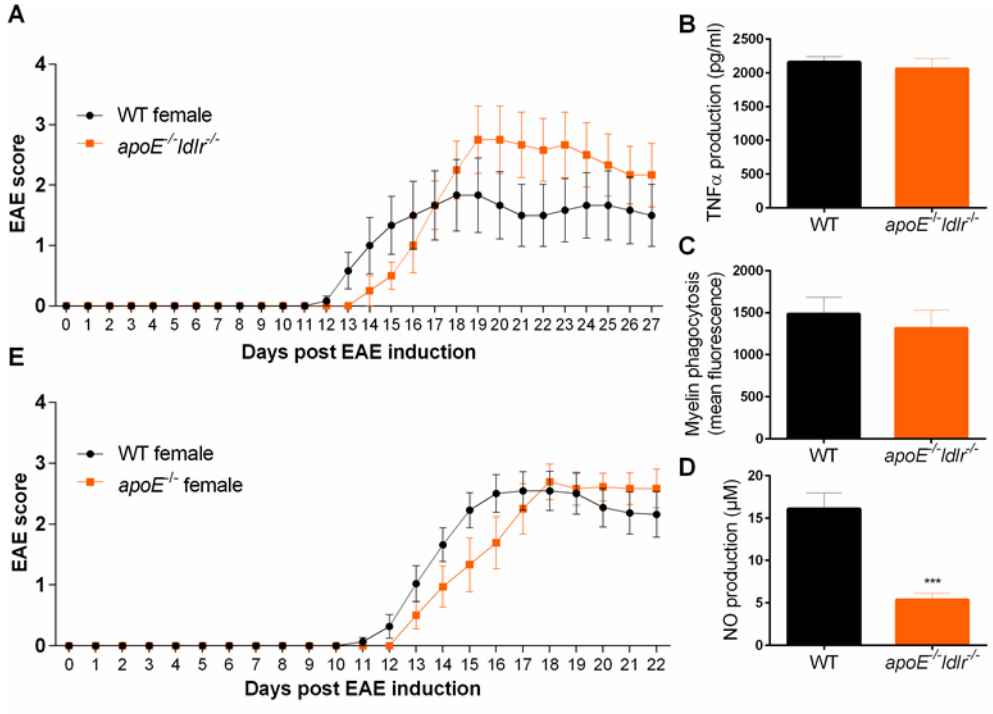

Figure 4.7 | Lack of apoE abrogates the reduced neuroinflammatory response in female *ldlr*$^{-/-}$ mice. Daily clinical EAE score of female WT and *apoE*$^{-/-}$*ldlr*$^{-/-}$ mice (n=6) (**A**). ELISA to measure TNFα production (n=6) (**B**), myelin phagocytosis assay (n=6) (**C**), Griess assay to measure NO production ($t(15)=5.091$; $p<0.0001$) (n=6) (**D**) for WT and *apoE*$^{-/-}$*ldlr*$^{-/-}$ female mice. ApoE$^{-/-}$ female mice show a similar EAE disease score compared to WT female mice (n=9-11) (**E**).

4.5 Discussion

In this study, we show that *ldlr*$^{-/-}$ attenuates neuroinflammation in female but not in male EAE mice, and that macrophages isolated from female mice have an attenuated inflammatory response and reduced phagocytic capacity. Interestingly, apoE deficiency counteracts the impact of *ldlr* deficiency in females on the course and pathology of EAE. These findings indicate that an increased apoE expression in macrophages in female *ldlr*$^{-/-}$ mice is at least partially responsible for the observed reduction in EAE severity.

We show that *ldlr* deficiency in female mice decreases the inflammatory burden in the CNS but does not affect T cell proliferation and differentiation, and immune cell infiltration into the CNS. However, the levels of proinflammatory mediators in the CNS were reduced in these mice and isolated macrophages showed an impaired inflammatory response. These findings suggest that *ldlr* deficiency affects the inflammatory and phagocytic properties of macrophages, thereby altering EAE disease severity. In concordance, levels of the pro-inflammatory markers *Tnfa, IL-6 and Il-12* were reduced while an increased gene expression of the anti-inflammatory marker *Arg1 was observed* in macrophages isolated from *ldlr*$^{-/-}$ female mice compared to WT macrophages. Moreover, *ldlr* deficiency decreases macrophage mediated myelin phagocytosis and expression of *lrp1*. LRP1 is a member of the LDLr family that also functions as a scavenger receptor and has been shown to be directly involved in myelin phagocytosis (108). Myelin degradation and phagocytosis by macrophages enhances demyelination which leads to neurological symptoms in MS (91). Although speculative, it is possible that lack of *ldlr* results in decreased *Lrp1* expression, thereby indirectly impairing myelin phagocytosis. Together, an altered macrophage response at least partially accounts for the beneficial effects of *ldlr* deficiency on EAE severity.

In contrast to the reduction in EAE severity in *ldlr*$^{-/-}$ mice, the clinical outcome was not altered in *apoE*$^{-/-}$*ldlr*$^{-/-}$ mice. Activated peritoneal macrophages isolated from *ldlr*$^{-/-}$ female mice significantly increased *apoE* expression compared to WT or their male counterparts and, the beneficial effect of *ldlr* deficiency on myelin phagocytosis and TNFα production was absent in *apoE*$^{-/-}$*ldlr*$^{-/-}$ macrophages. Although the LDLr is described to directly regulate apoE levels in the CNS (386), we found no elevated apoE gene expression in the CNS of *ldlr*$^{-/-}$ EAE mice (data

not shown). Studies have reported that increased expression of apoE leads to a less inflammatory phenotype in macrophages by downregulating M1-like and upregulating M2-like markers. Additionally, apoE suppresses microglial activation and the release of TNFα (230, 456, 457). Furthermore, treatment of EAE animals with apoE-derived peptides ameliorates the clinical course of EAE and apoE deficiency exacerbates EAE by increasing immune reactivity and hampering CNS repair mechanisms (458, 459). These findings suggest that the LDLr suppresses ApoE expression thereby exacerbating neuroinflammatory responses in females.

The reason for the sexual dimorphism in apoE expression remains elusive. It is reported that apoE exerts moderate sex-specific effects on neuroinflammation. Schrewe and co-workers found that the EAE disease course was slightly attenuated in male *apoE*$^{-/-}$ mice, whereas EAE was more severe in female *apoE*$^{-/-}$ mice compared to WT mice. In our hands *apoE*$^{-/-}$ did not alter EAE disease outcome in female mice compared to WT mice. Methodological differences (e.g. immunization protocol) may explain these discrepancies. Moreover, in Schrewe's study differences between WT and *apoE*$^{-/-}$ female mice were only visible after 30-36 days post-immunization, whereas our experiment lasted for up to 22 days post-immunization. Nevertheless, the question remains why only macrophages from *ldlr*$^{-/-}$ female mice abundantly secrete apoE. Macrophages express receptors for sex hormones such as estrogen and testosterone and these hormones modulate macrophage activity (460, 461). Interestingly, administration of estrogens has been shown to directly regulate hepatic LDLr activity in human cells *in vitro* and 17β-estradiol increases the expression of apoE during the maturation stage of human monocytes to macrophages (462-464). As lack of LDLr has been shown to increase apoE levels also (386), both mechanisms may contribute to the increased apoE levels observed in female *ldlr*$^{-/-}$ mice.

However, it remains to be elucidated whether sex hormones are exclusively responsible for the observed effects. Studies investigating plasma lipid and lipoprotein concentrations across the menstrual cycle in women report virtually no variation (465-467), and those that do, report only small reductions in LDL during the luteal phase and no changes in plasma triglyceride or HDL concentrations (465, 466). Therefore, the sexual dimorphism in lipid metabolism and its impact on neuroinflammation appears to be the result of an intricate network of sex hormones in combination with other modulators of lipid metabolism. The key

pathways and mediators of this network remain to be elucidated, but there are many other factors involved such as insulin and adipokines (468), and gene expression and imprinting (469), that need to be explored in the future.

In addition to apoE, differences in female and male mice may be the result of differences in LDLr levels and function. However, there are few studies about sex-related differences in LDLr behavior. Segatto and coworkers determined age- and sex-related differences in extra-hepatic LDLr expression and found that LDLr protein expression was decreased in 12-month-old (onset of menopause) female rat brains, which was completely restored by estrogen treatment. In skeletal muscle, LDLr levels were increased in both male and female aged rats, whereas in the heart no modifications were observed either in aged rats or rats of a specific gender (470). These data illustrate the complex age- and sex-related and tissue-specific regulation of LDLr expression, and warrant the need of more research to clarify the implications of these differences for neuroinflammatory diseases and other conditions involving the LDLr.

Collectively, we show that *ldlr*$^{-/-}$ attenuates EAE disease severity in female but not in male EAE mice. Although the nature of the observed sexual dimorphism remains unclear, our findings show that LDLr and associated apoE levels are involved in neuroinflammatory diseases such as multiple sclerosis, which may have implications for future treatment strategies.

5

The impact of dietary plant sterols on remyelination

Based on:

The impact of dietary plant sterols on remyelination.

Mailleux J[1], Bogie JFJ[1], Montes Diaz G[1], Schepers M[1], Struys T, Lambrichts I[1], Lütjohann D[2], van Horssen J[3], Vanmierlo T[1*], Hendriks JJA[1*].

In preparation

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5 Dietary plant sterols may promote remyelination after cuprizone-induced demyelination

5.1 Abstract

Objective: We sought to determine whether LXR-activating plant sterols promote remyelination in the cuprizone model.

Methods: Primary mouse oligodendrocytes were treated with or without plant sterols. Expression of myelin-associated genes was investigated by qPCR and western blot. The cuprizone model was used to determine the impact of a 5% plant sterol diet. Electron microscopy, qPCR, and immunohistochemistry were used to assess *in vivo* remyelination. The complex wheel assay was used to detect motor function in mice with cuprizone-induced demyelination and during remyelination.

Results: Plant sterols stimulate gene expression of integral myelin proteins and significantly induce myelin basic protein levels in primary mouse oligodendrocytes. Using the cuprizone model, we found no effect of dietary plant sterols on remyelination in the corpus callosum and motor function measured in the *in vivo* model

Conclusion: Collectively, although plant sterols induce myelin basic protein gene expression *in vitro* they did not stimulate remyelination after cuprizone-induced demyelination. We conclude that four weeks of plant sterol treatment is insufficient to promote remyelination in the CNS. Future experiments should include longer treatment periods and plant sterols that selectively induce transcriptional pathways involved in oligodendrocyte differentiation and maturation.

5.2 Introduction

In MS, demyelination in the CNS is widespread though reversible, which is evidenced by transient phases of recovery during which remyelination, the regeneration of damaged myelin sheaths around demyelinated axons, occurs. Failure of remyelination can be partially attributed to insufficient capacity of oligodendrocyte precursor cells (OPCs) to differentiate to mature myelin-forming oligodendrocytes (115, 471). Interestingly, several research groups have recently demonstrated links between OPC differentiation and the cholesterol metabolism (381, 390, 472). Sterols, particularly cholesterol, are essential components of myelin, illustrated by the perturbed myelin formation in mice with defective cholesterol synthesis in the myelin-forming oligodendrocytes (301). In addition, Saher *et al.* demonstrated that dietary cholesterol promotes remyelination and repair of demyelinated lesions, in the adult mouse brain (381). Results from this study point to a role of cholesterol in promoting myelin repair after demyelinating episodes.

Plant sterols are plant analogues of mammalian cholesterol. They structurally and functionally resemble cholesterol except for an additional methyl or ethyl group at C24 and/or a double bond at C22 (Δ22) (436). The most prevalent plant sterols are sitosterol (ethyl group at C24), stigmasterol (ethyl group at C24, Δ22) and campesterol (methyl group at C24). In contrast to cholesterol, plant sterols are exclusively derived from diet and cannot be synthesized in mammals (282). They can however cross the blood-brain barrier, accumulate in the brain (282) and are suggested to activate liver X receptors (LXRs) (261, 263, 265). LXR activation involves heterodimerization with one of the retinoid X receptor isotypes (RXRs) and activation of specific RXR subtypes was shown to be critically involved in CNS remyelination (304, 390). Therefore, we hypothesize that plant sterols promote remyelination *in vivo*.

Here, we show that plant sterols stimulate gene expression of myelin basic protein (*Mbp*) in differentiating primary mouse OPCs. In addition, we investigate the effects of increased dietary plant sterols on remyelination using the cuprizone model. In this model, behavioral parameters are assessed using a complex wheel assay. Using the cuprizone model we found no effect of dietary plant sterols on remyelination in the corpus callosum (CC) and motor skills.

5.3 Methods

5.3.1 OPC cultures

Primary mouse OPC cultures were prepared as previously described (473). In short, mixed glial cultures were prepared from postnatal day 2 mouse cerebral cortices of C57BLl/6JOlaHsd animals and used to generate OPC-enriched glial cultures by separating the OPCs from the astrocyte monolayer by orbital shaking followed by purification by differential adhesion to plastic. Purified OPCs were seeded on poly-L-lysine (PLL) (5 µg/ml; Sigma-Aldrich, Bornem, Belgium) coated plates. For immunohistochemistry, OPCs (20,000 cells/well) were plated PLL-coated 12mm glass coverslips (Corning, Tewksbury, USA). Isolated OPCs were plated in 24-well plates (100,000 cells/well; Greiner Bio-One, Frickenhausen, Germany) and induced to differentiate for 6 days with a plant sterols mixture (25 µM; 50% β-sitosterol, 33% stigmasterol and 17% campesterol; kindly provided by Ingmar Wester, Raiso) or vehicle (EtOH) in SATO-medium supplemented with 2% horse serum (Sigma-Aldrich).

5.3.2 Quantitative PCR

Total RNA was prepared using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions with the following modification: Qiazol lysis reagent (Qiagen) with 1% β-mercaptoethanol (Sigma-Aldrich) was used as lysis buffer. RNA concentration and purity was determined using a Nanodrop spectrophotometer (Isogen Life science).

RNA was converted to cDNA using qScript cDNA SuperMix (Quanta Biosciences, Boston, USA). According to the manufacturer's instructions. Quantitative PCR (qPCR) was conducted on a StepOnePlus™ Real-Time PCR system (Applied biosystems, Ghent, Belgium). The SYBR green master mix (Applied biosystems), 10 µM of forward/reverse primers, nuclease free water and 12.5 ng template cDNA in a total reaction volume of 10 µl. Relative quantification of gene expression was accomplished by using the comparative C_t method. Data were normalized to the most stable reference genes. Primers were chosen according to literature or designed using Primer-express (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Details of primers used are shown in table 5.1.

Table 5.1 | Forward and reverse primers used to measure the relative expression of different mouse genes.

Gene	Sequence (5'–3')	Product size
Mbp forward	TCA CAG AAG AGA CCC TCA	110 bp
Mbp reverse	GAG TCA AGG ATG CCC GTG TC	
Plp forward	TTG TTT GGG AAA ATG GCT AGG A	265 bp
Plp reverse	GCA GAT GGA CAG AAG GTT GGA	
Abca1 forward	CCC AGA GCA AAA AGC GAC TC	102 bp
Abca1 reverse	GGT CAT CAT CAC TTT GGT CCT TG	
Tnfa forward	CCA GAC CCT CAC ACT CAG ATC A	79 bp
Tnfa reverse	CAC TTG GTG GTT TGC TAC GAC	
Igf1 forward	GGT GGA TGC TCT TCA GTT	178 bp
Igf1 reverse	TGC TTT TGT AGG CTT CAG	
Cyca forward	GCG TCT CCT TCG AGC TGT T	145 bp
Cyca reverse	AA GTC ACC ACC CTG GCA	
Hmbs forward	GAT GGG CAA CTG TAC CTG ACT G	168 bp
Hmbs reverse	CTG GGC TCC TCT TGG AAT G	
Rpl13a forward	GGA TCC CTC CAC CCT ATG ACA	131 bp
Rpl13a reverse	CTG GTA CTT CCA CCC GAC CTC	
Ywhaz forward	GCAACGATGTACTGTCTCTTTTGG	149 bp
Ywhaz reverse	GTCCACAATTCCTTTCTTGTCATC	

5.3.3 Immunohistochemistry

OPCs were fixed with 4% paraformaldehyde (PFA) for 20 minutes. Non-specific binding was blocked using 1% BSA in PBS for 30 minutes. Cells were then incubated with anti-MBP (1:500, MAB386, EMD Millipore) and anti-O4 (1:1000, MAB1326, R&D systems) for 2 hours at room temperature followed by incubation with Alexa-488-labeled goat anti-rabbit (1:400, Molecular probes) and Alexa-555-labeled goat anti mouse IgM (1:1000, Molecular probes) for 1 hour at room temperature. Analysis was carried out using a Nikon eclipse 80i microscope and NIS Elements BR 4.20 software (Nikon).

5.3.4 Animals

Male C57BLl/6JOlaHsd mice were single housed in a 12h light/dark cycle. Water and normal chow diet were provided *ad libitum*. Wellbeing and weights were assessed daily and weekly respectively. All animal experiments were approved by the local Ethical committee for Animal Experiments of Hasselt University and executed in adherence with institutional guidelines.

5.3.5 Cuprizone

Male C57BLl/6JOlaHsd mice (six weeks old) were fed *ad libitum* a diet of 0.3% (w/w) cuprizone (oxalic bis-(cyclohexylidenehydrazide); Sigma-Aldrich) mixed in normal chow, which can be administered for specific periods to induce patterns of demyelination and remyelination of the corpus callosum (CC) (474). To induce prolonged 'chronic' demyelination, mice were fed cuprizone continuously for 10 weeks. Controls were aged-matched male C57BLl/6JOlaHsd mice fed normal chow. To measure bilateral motor coordination, we used the complex running wheel. A running assay using wheels with irregularly spaced rungs has been shown to detect functional differences in mice with cuprizone-induced demyelination (475). The experimental setup is depicted in figure 5.1. In brief, two weeks prior to the start of the experiment running behavior is normalized using both a training (all rungs intact) and complex running wheel (irregularly spaced rungs). Based on the complex wheel data obtained in the second week of normalization (-1w/0w) a baseline is established, and animals are randomized. Next, 0.3% cuprizone is administered (week 0-10). Upon withdrawal of the cuprizone diet spontaneous remyelination will occur and a 5% plant sterol enriched diet is provided. Data are collected at various time points as indicated by black arrows. Data (maximum velocity, average speed, distance and time spent running on the wheel) are collected automatically every 24h.

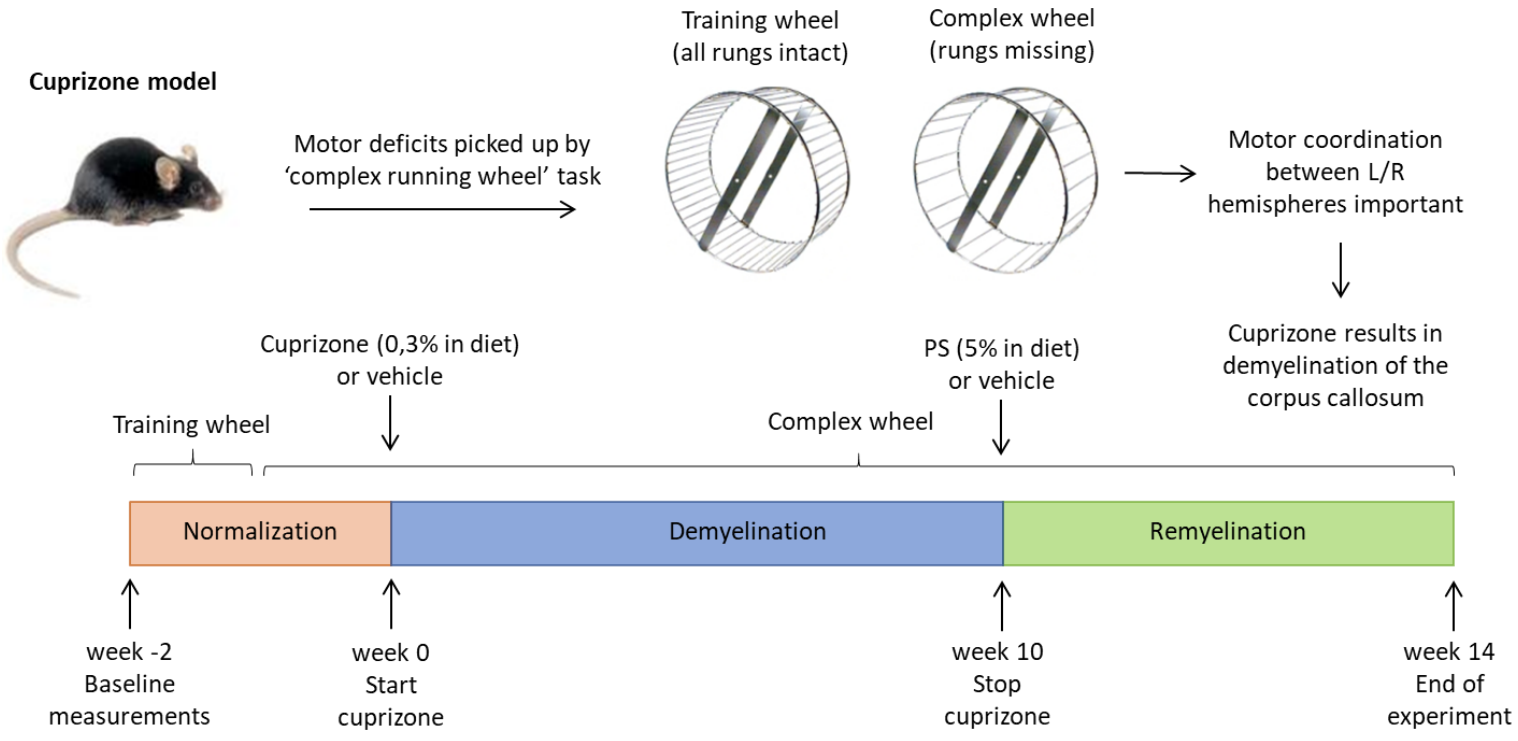

Figure 5.1 | Experimental setup of the complex running wheel. The complex running assay is used to measuring the bilateral motor coordination to detect functional differences in mice with cuprizone-induced demyelination.

5.3.6 Transmission Electron microscopy

The sample preparation for TEM was performed as previously described (476) with minor modifications. Briefly, mice were transcardially perfused with lactated Ringer's solution under deep anesthesia. A coronal brain block (1 mm thick) within the anteroposterior coordinates from -0.3 to -1.5 mm was cut in the midsagittal plane. Next, tissue was fixed with 2% glutaraldehyde and post fixated with 2% osmiumtetroxide in 0.05M sodium cacodylate buffer (pH=7.3) for 1h at 4°C. The tissue was then stained with 2% uranyl acetate in 10% acetone for 20 min, dehydrated through graded concentrations of acetone and embedded in epoxy resin (araldite). Semithin sections (0.5 µm) were stained with a solution of thionin and methylene blue (0.1% aqueous solution) for light microscopic examination to delineate the region of interest. Subsequently, ultrathin sections (0.06 µm) were cut and mounted on 0.7% formvar-coated grids and contrasted with uranyl-acetate followed by lead citrate and examined on a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV.

5.3.7 Statistical Analysis

Data were statistically analyzed with GraphPad Prism 6 for windows and are reported as mean values ± standard error (SEM). D'Agostino and Pearson omnibus normality test was used to test normal distribution. An analysis of variances (ANOVA) or two-tailed unpaired student t-test (with Welch's correction if necessary) was used for normally distributed data sets (t(df); P). The Mann-Whitney U analysis (MWU, n1, n2; P) was used for data sets that did not pass normality. *P<0,05, **P<0,01 and ***P<0,001.

5.4 Results

5.4.1 Plant sterols induce *Plp* gene and MBP protein expression in primary mouse oligodendrocytes.

To investigate whether plant sterols induce the stimulation of myelin-associated genes, we determined gene expression of the two major myelin proteins *Mbp* and *Plp* in primary mouse oligodendrocytes treated with or without cholesterol and plant sterols. mRNA expression of *Mbp* was not significantly increased compared to non-treated cells whereas *Plp* was increased in plant sterol- and cholesterol-treated oligodendrocytes compared to non-treated cells (fig. 5.2A-B). Next, we performed immunohistochemistry to validate these results on protein level. We found that the expression of MBP is significantly increased after plant sterol treatment compared to both cholesterol and non-treated cells (fig. 5.2C). These results suggest that plant sterols induce myelin gene expression in primary mouse oligodendrocytes and that on protein level plant sterols induce expression of MBP more potently than cholesterol.

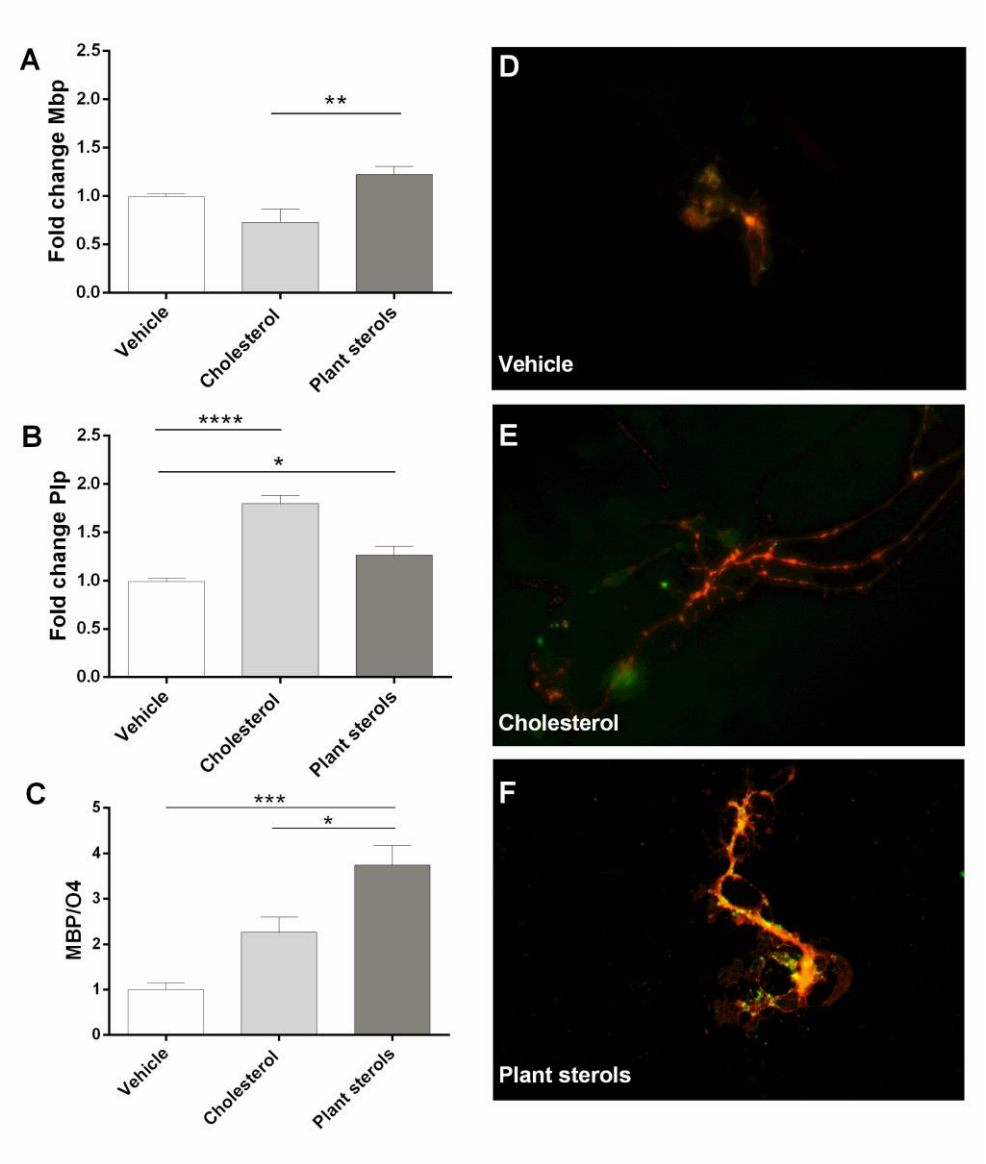

Figure 5.2 | Plant sterols induce expression of MBP and *Plp* in primary mouse oligodendrocytes. Comparison of fold changes of Mbp (**A**) and Plp (**B**) genes between untreated (n=11) and PS-treated (25 μM) oligodendrocyte precursor cells (n=11) after three days of incubation. Relative quantification of *Mbp* ($t(20)=2{,}675$; $p<0.015$) and *Plp* (MWU=28; 11; 11; $p<0.034$) was accomplished by using comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (*Cyca* and *Hmbs*). OPCs (n=7) were stained for MBP (green) and O4 (red) after six days of incubation (**D**-**F**).

The MBP/O4 ratio (**C**) was determined to calculate the percentage of O4+ cells expressing MBP.

5.4.2 Plant sterols do not stimulate remyelination in the corpus callosum.

To assess the effect of a plant sterol diet on remyelination after cuprizone-induced demyelination, we performed electron microscopy on the CC to measure g-ratios (fig. 5.3A-C). The g-ratio represents the ratio of the diameter of the axon to the diameter of the myelinated fiber and allows comparison of myelin thickness for different axon sizes. The average g-ratio did not differ significantly between normal chow and plant sterol fed mice (fig. 5.3A-B) and neither did the percentage of myelinated axons (data not shown). We also assessed the distribution of g-ratios among the myelinated axons but found no difference between cuprizone-treated groups (fig. 5.3C). These data indicate that a plant sterol enriched diet had no impact on ultrastructural remyelination after cuprizone-induced demyelination.

5.4.3 Plant sterols do not increase gene expression of *Mbp* and *Plp* in the corpus callosum.

In order to determine if plant sterols influence myelin-associated, LXR-response and inflammatory pathways after cuprizone treatment, we determined gene expression of *Mbp*, *Plp*, *Abca1*, *Apoe* and *Tnfα* (fig. 5.4). Expression of *Mbp* and *Plp* was significantly decreased after ten weeks of cuprizone treatment. Plant sterols failed to stimulate expression of *Mbp in vivo* after four weeks of recovery (fig. 5.4A). However, *Plp* expression in the CC is significantly increased in plant sterol fed animals compared to animals sacrificed after ten weeks of cuprizone (fig. 5.4B). An increase of *Plp* levels corresponds to the appearance of remyelination axons, indicating ongoing remyelination (477). Inflammation (*Tnfα*) and LXR-response gene (*Abca1* and *Apoe*) expression was also elevated after ten weeks of cuprizone treatment (fig. 5.4C-E). However, plant sterols did not induce LXR-driven gene expression and neither affected TNFα gene expression in the CC after four weeks of recovery.

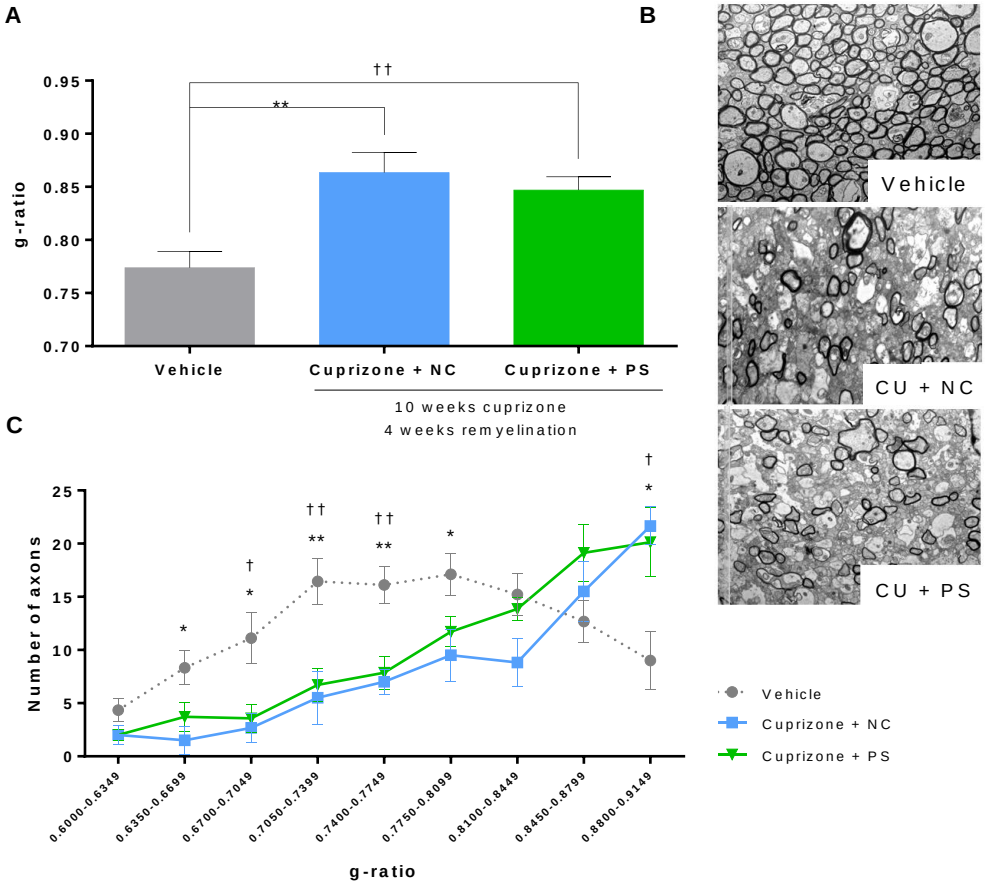

Figure 5.3 | Ultrastructural analysis of the CC. (**A)** Quantification of the average g-ratio of myelinated axons of healthy mice (n=9), cuprizone + normal chow (n=6) and cuprizone + plant sterols fed mice. (n=7). (**B)** Representative electron micrographs of the caudal CC. (**C)** Number of axons per g-ratio value. *p < 0.05, **p < 0.01 with respect to cuprizone + NC; †p < 0.05, ††p < 0.01 with respect to cuprizone + PS. CU; cuprizone, NC; normal chow, PS; 5% plant sterol diet.

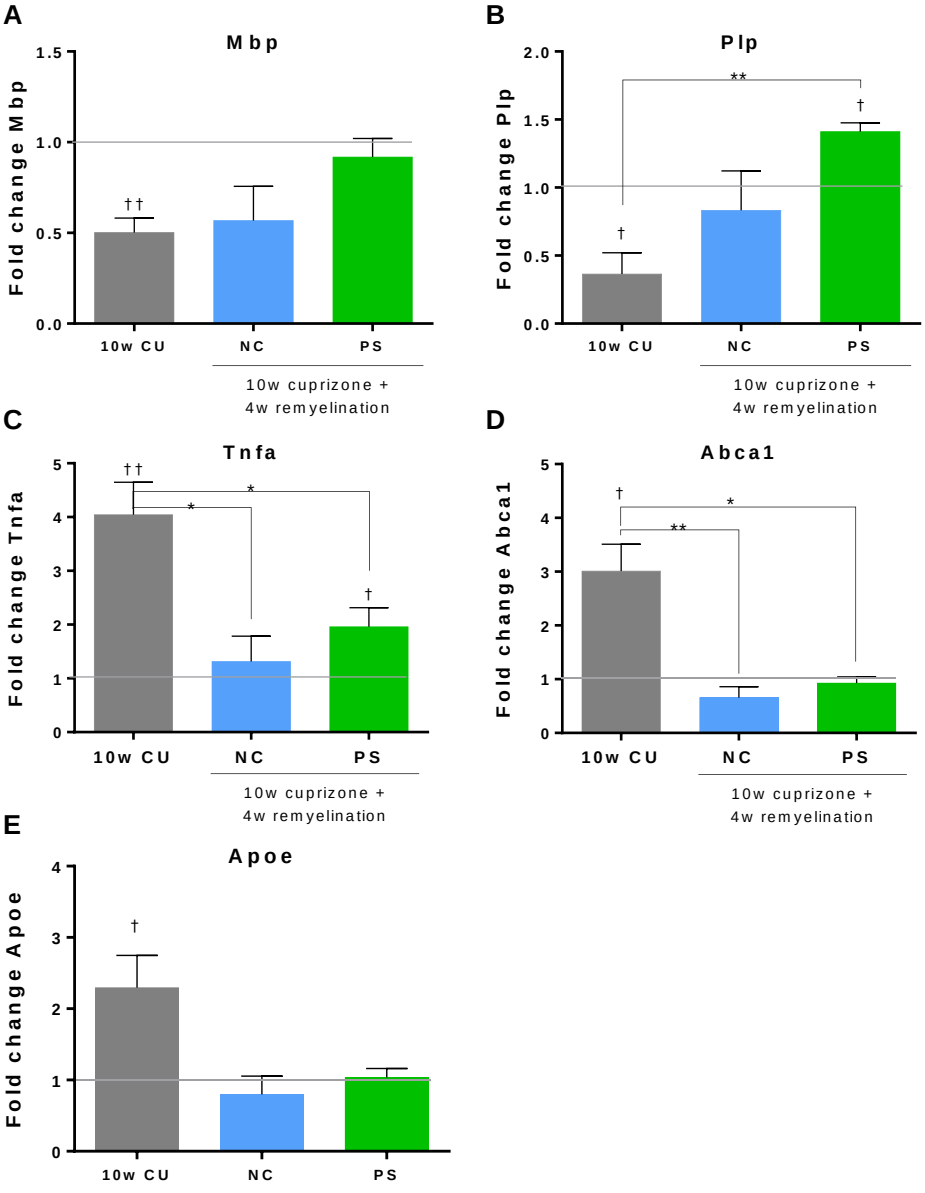

Figure 5.4 | Impact of plant sterols on gene expression in the CC. Comparison of fold changes of selected genes in the CC. Animals that did not receive cuprizone (vehicle, gray line) are indicated by the gray line (n=10). Animals that received ten weeks of cuprizone treatment are indicated by the dark gray column (n=6). Animals after four weeks of recovery are indicated by the blue (NC, n=7) or green (PS, n=7) column. Relative quantification of *Mbp* (A), *Plp* (B), *Abca1* (C), *Apoe* (D) and *Tnfa* (E) was accomplished by

using the comparative C_t method. Data were normalized to the most stable reference genes, determined by Genorm (*Hmbs* and *Rpl13a*). *p < 0.05, **p < 0.01 with respect to the condition indicated; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ with respect to vehicle (fold change = 1). CU; cuprizone, NC; normal chow, PS; 5% plant sterol diet.

5.4.4 Plant sterols do not improve motor coordination after cuprizone-induced chronic demyelination.

To study the impact of cuprizone and plant sterols on CC key functions, we used the complex running wheel paradigm (475). The parameters recorded daily from the running behavior include the total distance, maximum velocity (Vmax), average speed and time spent on the wheel. The Vmax appeared to be the most sensitive parameter for detecting differential motor coordination between cuprizone and non-treated mice (data not shown). To investigate if plant sterol uptake stimulates motor coordination after cuprizone-induced chronic demyelination, we administered cuprizone-fed mice a diet with or without plant sterols for four weeks. 0.3% cuprizone significantly decreased performance on the complex running wheel after 10 weeks (fig. 5.5A). However, a 5% plant sterol diet failed to improve motor coordination after four weeks of recovery, despite showing a trend after two weeks (fig. 5.5B).

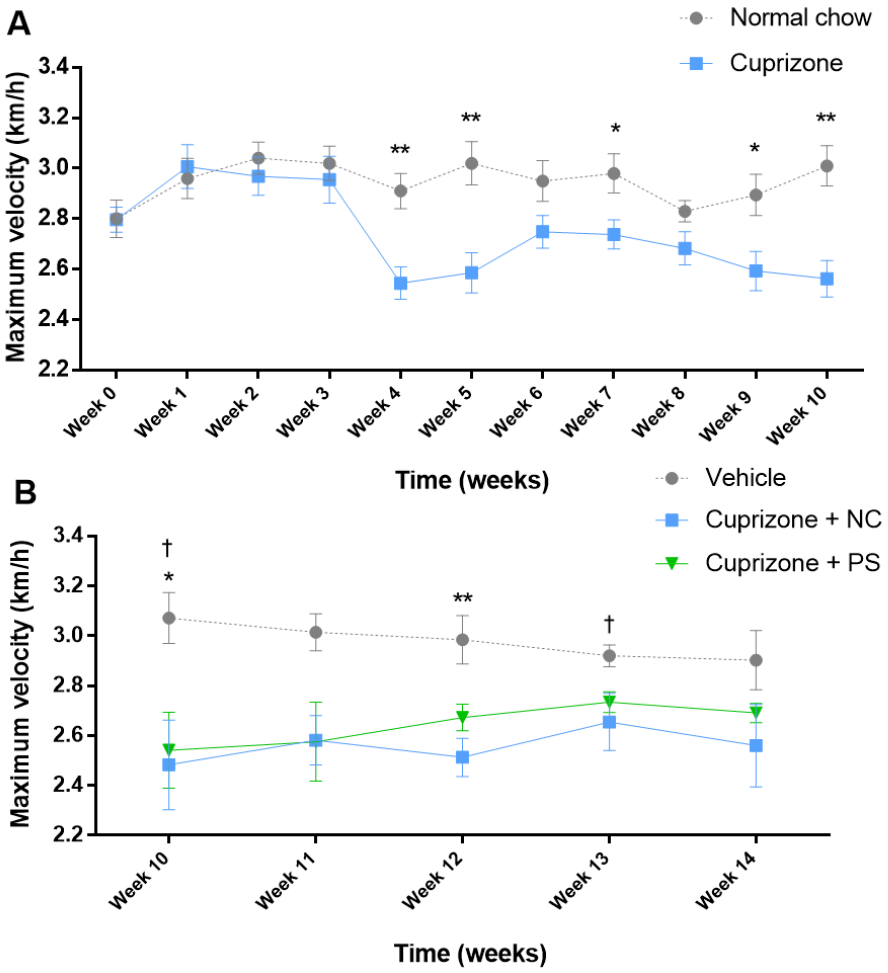

Figure 5.5 | Plant sterols do not improve motor coordination after cuprizone-induced chronic demyelination. The Vmax recorded is shown for each week (7-day average). **(A)** Vmax during 10 weeks cuprizone demyelination. Mice fed normal chow (n=10) are shown in gray circles, mice that received cuprizone (n=19) are shown in blue squares. *p < 0.05, **p < 0.01 by Student's t-test. Two-way ANOVA testing also distinguishes mice on normal chow and cuprizone. **(B)** Vmax during 4 weeks of remyelination. Mice fed normal chow (n=7) are shown in gray circles, cuprizone fed mice on a normal diet (n=5) are shown in blue squares, and cuprizone mice fed 5% enriched PS diet (n=7) are shown in green triangles. *p < 0.05, **p < 0.01 with respect to cuprizone + NC; †p < 0.05 with respect to cuprizone + PS. CU; cuprizone, NC; normal chow, PS; 5% plant sterol diet.

5.5 Discussion

In this study, we evaluated the effects of dietary plant sterols using an *in vivo* mouse model of de/remyelination. We showed that plant sterols increased MBP and PLP expression in primary oligodendrocyte precursor cells (OPCs), but failed to boost remyelination. In line with these findings, we did not observe a beneficial effect of plant sterols on motor coordination measured by the complex running wheel.

Failure of remyelination in chronic MS lesions is regarded to be a result of a block of OPCs differentiation rather than an impairment of OPC proliferation (364, 471, 478, 479). Therefore, we investigated the effects of plant sterols on myelin-associated gene expression in primary mouse OPCs. Treating OPCs with plant sterols significantly upregulated *Plp* gene expression. Moreover, the ratio of MBP-expressing maturated OPCs increased significantly upon plant sterol treatment, demonstrating that plant sterols can promote OPC maturation *in vitro*. These results prompted us to investigate the role of plant sterols in an *in vivo* model for remyelination.

To determine the effect of plant sterols on remyelination *in vivo*, we used the cuprizone model. No significant differences in myelin, inflammatory or LXR response gene expression were detected comparing normal chow and plant sterol fed mice. We cannot exclude the possibility that 10 weeks of cuprizone treatment may have completely depleted the OPC pool although previous studies have shown that remyelination is still possible after applying an even longer treatment protocol (480). Interestingly, *Plp* expression in the CC is significantly increased in plant sterol fed animals compared to animals sacrificed after ten weeks of cuprizone. An increase of *Plp* levels corresponds to the appearance of remyelination axons, indicating ongoing remyelination (477).

Using the complex running wheel, we assessed bilateral sensorimotor coordination as a functional measure for de- and remyelination in the CC. Upon cuprizone administration, maximal oligodendrocyte cell loss is observed after three weeks, with a spontaneous recovery period occurring after week five (481). This was reflected by our running wheel data, which showed a sharp decrease in the mean Vmax after three weeks and increase at week five. Although cuprizone-impaired

motor coordination was detectable by the complex running wheel, we could not distinguish sustained differences in CC function during plant sterol treatment.

Recently, LXRs have been implicated in CNS remyelination (444). Although plant sterols are known to activate LXRs *in vitro* (261, 263, 265), we could not detect LXR activation in the CC. Plant sterols did not increase the expression of LXR target genes *Abca1* and *ApoE*. Our plant sterol-based diet consisted out of 50% β-sitosterol, 33% stigmasterol and 17% campesterol. Although campesterol is a relatively weak LXR agonist, both β-sitosterol and stigmasterol are potent LXR agonists *in vitro* (482). Whether these plant sterols indeed activate LXRs *in vivo* has yet to be decisively determined.

Although our *in vitro* data showed that plant sterols increased the number of MBP^+ oligodendrocytes more potently than cholesterol, they did not improve CNS remyelination. Interestingly, Saher and co-workers recently showed that dietary cholesterol supplementation (2% w/w) enhanced remyelination upon cuprizone exposure (381). Cholesterol supplementation dramatically increased OPC proliferation and differentiation. Moreover, compared to normal fed mice, one week of cholesterol supplementation following four weeks of cuprizone-induced demyelination significantly improved performance on the complex running wheel (381). Once dietary sterols have entered the CNS, they tend to accumulate in lipid rich membranes of neurons and likely also oligodendrocytes (282, 284). We previously showed that feeding mice a plant sterol enriched diet for six weeks resulted in a two-fold increase in lipid raft-associated plant sterol concentration (~3.5 ng/mg protein to ~7 ng/mg protein), whereas cholesterol concentration in the lipid raft remained stable (~2,300 ng/mg protein) (282). It remains to be determined whether this 1:300 plant sterol-to-cholesterol ratio is sufficient to modulate membrane and lipid raft properties. Although plant sterols induce myelin gene expression *in vitro* we observed no beneficial effects of plant sterol supplementation on CNS remyelination *in vivo*. Future studies should include longer treatment periods to improve the plant sterol-to-cholesterol ratio. In addition, future experiments should include selective plant sterols that more potently active LXRs *in vivo*, such as the oxidized plant sterol 24(S)-Saringosterol.

6

Summary and general conclusions

6 Summary and general conclusions

MS is a chronic, inflammatory, neurodegenerative disorder, in which phagocyte-mediated myelin destruction is considered one of the key pathological hallmarks. Nonetheless, when phagocytes ingest myelin they have been described to obtain a more anti-inflammatory phenotype. As cholesterol is the most abundant lipid in myelin, its ingestion and breakdown by phagocytes might produce LXR-activating oxysterols that affect phagocyte phenotype. The upregulation of the LXR-target apoE could stimulate the lipid-supplying function of glial cells and provide the necessary sterols needed for remyelination by oligodendrocytes. However, it is unclear how changes in the sterol metabolism contribute to repair mechanisms in the CNS. In this chapter, the main questions and results of this thesis are summarized and discussed. Furthermore, some suggestions for future research are presented. An overview that summarizes these findings is shown in figure 6.1.

What is the role of CL-P1 on phagocytes in MS lesions?

Our transcriptomic analysis of myelin phagocytosing macrophages demonstrated that CL-P1 is one of the most potently induced genes in rat macrophages after phagocytosis of myelin (203). It is structurally similar to scavenger receptor A (SRA), which is one of the key receptors involved in myelin clearance by both macrophages and microglia (99-101). As demonstrated in **chapter 2**, both mouse macrophages and human monocytes express CL-P1 on their cell surface and myelin phagocytosis increases the surface expression of CL-P1 on bone-marrow derived macrophages (BMDMs) in a dose-dependent manner *in vitro*. However, despite its resemblance to scavenger receptors, myelin internalization did not increase the expression of CL-P1 in primary mouse microglia. Ontogenic differences in signaling pathways involved in the regulation of CL-P1 may explain the observed discrepancy between macrophages and microglia (111, 118). Interestingly, in active MS lesions HLA-DR$^+$ foamy phagocytes strongly expressed CL-P1 suggesting that myelin phagocytosis enhances CL-P1 expression by phagocytes in MS lesions. The main constituents of myelin are lipids and proteins, many of which can alter the phenotype of phagocytes upon binding and internalization. In **chapter 3,** we show that the lipid-sensing LXRs are activated in phagocytes in active MS lesions. This LXR activation is at least partially

mediated by (oxy)sterols present in myelin and the generation thereof during myelin processing. Unlike scavenger receptors CD36 and SPα, which are well-known targets of LXRs (415, 483), we found that activation of nuclear receptors PPAR and LXR did not affect CL-P1 mRNA expression. These results indicate that myelin increases the expression of CL-P1 in an LXR-and PPAR-independent manner. However, future experiments should confirm this statement by using shRNA to silence LXR/PPAR expression and determine if CL-P1 expression is indeed unaffected after myelin stimulation.

Interestingly, oxidized myelin more potently induced and maintained CL-P1 expression on phagocytes compared to unmodified myelin. Scavenger receptors are known to have a high affinity for oxidized targets such as oxLDL. For example, SRA plays a key role in atherosclerosis by binding oxLDL with a very high affinity (484). In atherosclerosis, increased uptake of oxLDL leads to the deposition of esterified cholesterol in the cytoplasm of macrophages and generation of foam cells (485). Likewise, different studies have reported altered myelin oxidation in MS patients. Lipid peroxidation-derived malondialdehyde (MDA) is increased in and around active MS lesions and oxidized MOG is phagocytosed more efficiently than its unmodified version (408, 409). Another product of lipid peroxidation, 4-hydroxynonenal, was increased in myelin isolated from NAWM of MS patients compared to myelin from healthy donors (410). In active MS lesions, phagocytes produce large amounts of reactive oxygen species (ROS) and other detrimental factors that can oxidize and damage myelin. Moreover, a large proportion of the plasma LDL which enters the CNS of MS patients is oxidatively modified in the lesions (486). During oxidation, a wide variety of chemical and physical changes occurs leading to the generation of oxidation-specific neoepitopes. These epitopes are important targets of scavenger receptors (487). Although the phagocytosis of myelin leads to demyelination, the clearance of myelin debris is an important prerequisite for proper CNS remyelination. Depending on whether CL-P1 mediates the uptake of intact myelin or myelin debris, its function may be both beneficial as detrimental (115, 116, 139, 418). Because myelin is damaged by oxidation, presumably by the generation of free radicals, and we used myelin debris in our *in vitro* experiments, it is tempting to speculate that CL-P1 may play a role in myelin debris clearance *in vivo*, thereby facilitating CNS remyelination (115, 116, 418).

To determine whether CL-P1 is involved in the internalization of myelin, we used plasmids expressing *s*hRNA directed against CL-P1. However, as phagocytes are difficult to transfect, we used HEK293 cells as they similarly express CL-P1 and are often used as a model system to study phagocytic receptors (402, 403, 488). We found that CL-P1 is involved in myelin internalization as knockdown of CL-P1 markedly reduced myelin uptake in HEK293 cells. It is likely that a similar mechanism is also present in phagocytes, as both cell types express CL-P1 and can phagocytose myelin. However, future studies are needed to confirm if CL-P1 contributes to myelin uptake in phagocytes by using new methods to successfully knock-down CL-P1 in phagocytes, or by using lysophosphatidylcholine (LPC) to demyelinate cerebellar brain slices obtained from microglia-specific CL-P1 KO mice.

How can myelin activate liver X receptors in human phagocytes in MS?

In **chapter 3** we showed that LXRs in phagocytes in active MS lesions are activated at least partially by 27-hydroxycholesterol (27OHC) present in myelin and the generation thereof upon myelin processing. Myelin contains several LXR-activating oxysterols of which 24(S),25-epoxycholesterol (24S25-EC), 24SOHC and 27OHC are the most potent (438-441, 489). We speculated that after phagocytosis, cholesterol oxidation products such as oxysterols are generated during the breakdown of myelin. We found that five days after uptake of myelin by macrophages, intracellular myelin levels started to decrease and the levels of the cholesterol metabolite 27OHC, but not 24SOHC and desmosterol, significantly increased. Although 24S25-EC is the strongest oxysterol LXR agonist, we did not determine its levels in myelin-laden macrophages. 24S25-EC present in myelin likely contributes to LXR activation, however it is not generated in human monocyte-derived macrophages (439), and is, in contrast to 24OHC and 27OHC not derived from cholesterol, but via a shunt in the cholesterol biosynthetic pathway (490-492). Since cholesterol synthesis is inhibited by rising intracellular cholesterol levels, myelin-induced cholesterol overload would suppress any formation of 24S25-EC (490, 491). Taken together, these results indicate that LXR ligands are present in human myelin, and that after myelin processing, 27OHC is one of the major LXR ligands that is formed.

The generation of LXR-activating oxysterols protects the myelin-phagocytosing macrophages from elevated intracellular free cholesterol and oxysterol-induced toxicity (245). Also, enzymatic oxidation mechanisms involving the formation of side-chain oxidized oxysterols reduce cellular cholesterol levels as they are able to cross cell membranes at orders of magnitude higher than the rate of cholesterol (493, 494), without the need of specific receptors and transporters. Conversion of 24SOHC or 27OHC allows expulsion of excess cholesterol from macrophages. It seems that 27-hydroxylation of cholesterol is of particular importance in macrophages (495). When macrophages were treated with cyclosporine, a potent 27-hydroxylase inhibitor, the secretion of 27OHC from macrophages was reduced during 24h by more than 90%, with a concomitant increase of 40% of intracellular cholesterol (496). Since 27-hydroxylase is an evolutionary old enzyme, it is possible that oxidative processes may have been more important for elimination of cholesterol at an earlier stage of evolution (497). Perhaps this process is currently still more relevant in macrophages than the LXR-induced reversed cholesterol transport. Although 27OHC is known to be an LXR agonist *in vitro*, its regulatory role *in vivo* is controversial. Björkhem *et al.* showed that under normal basal conditions side-chain oxidized oxysterols such as 24SOHC and 27OHC are not important regulators of LXR activity (498). However, the situation may be different under pathological conditions. For example, fibroblasts from patients with Tangier's disease, a genetic condition in which patients have non-functioning ABCA1 transporters leading to intracellular cholesterol overload, contain markedly increased levels of side-chain oxidized oxysterols with a parallel downregulation of cholesterol synthesis (499). Collectively, oxysterols present and the generation thereof in myelin-laden phagocytes in MS are probably at least in part modulated by a myelin-mediated activation of LXRs, providing an intriguing hypothesis for the LXR-controlled self-limiting nature of MS lesion development. In the future, studies using triple knockout of CYP46A1, CYP27A1, and CH25H, enzymes responsible for the generation of 24SOHC, 27OHC and 25OHC respectively, could determine the *in vivo* relevance of these oxysterols in the involvement of LXR activation, and if a myelin-mediated activation of LXRs in phagocytes may dampen neuroinflammation in MS.

Is liver X receptor activation beneficial in MS?

Recently, we demonstrated that myelin-derived lipids activate LXRs in rat macrophages and skew these macrophages towards a less inflammatory phenotype *in vitro* (203). However, it was not known if LXRs are activated in phagocytes in active MS lesions and which myelin constituents are responsible for this activation. In **chapter 3**, we show that LXRs and their downstream targets are induced in myelin-laden macrophages in active demyelinating MS lesions indicative of LXR activation. We show an upregulation of LXR response genes, LXRα, ABCA1 and apoE in macrophages in active MS lesions, indicating that these phagocytes have an enhanced capacity to mediate cholesterol efflux, thereby preventing a toxic intracellular cholesterol overload.LXR Additionally, activation of LXRs causes SUMOylation of specific residues in their ligand-binding domain, leading to the transrepression of inflammatory pathways in CNS infiltrating and resident immune cells (216). For example, several research groups have demonstrated that SUMOylated LXRs are required for the suppression of NF-κB-dependent inflammatory responses by LXRs (216-218). LXRs can also induce the expression of several genes involved in the elongation and unsaturation of fatty acids, which ultimately leads to the formation of long-chain PUFAs (222). The increase of long-chain PUFAs alters histone acetylation in enhancer and/or promotor regions of NF-κB target genes, thereby suppressing inflammatory responses (222). In EAE, treatment with a synthetic LXR agonist T0901317 significantly suppressed EAE symptoms. This was marked by a decrease in CNS inflammation, demyelination, and immune cell infiltration into the CNS (334). Similarly, other groups showed that LXR activation ameliorated EAE disease activity by suppressing IL-23 signalling and immune cell infiltration into the CNS (333, 500). These studies demonstrate a major role for LXRs in the inhibition of autoimmunity. Interestingly, LXR activation has also been shown to be beneficial in other CNS pathologies. In stroke patients, LXRs promote synaptic plasticity and axonal regeneration (501). Moreover, it is required for dopaminergic neuron differentiation, providing possible treatment options for Parkinson's disease (502, 503). In Alzheimer's disease (AD), LXR activation improves the cognitive phenotype in animal models of AD, decreases synaptic compensatory mechanisms, and stimulates the proteolytic degradation of Aβ by microglia (504-509).

Both LXR and LDLr are key regulators of cholesterol homeostasis. While LXRs promote the efflux of cholesterol, the LDLr promotes its uptake. Interestingly, both receptors are molecularly entwined as LXR activation can decrease LDLr protein levels, thereby limiting cholesterol uptake. LXRs bind to an LXR response element in the promotor of Inducible Degrader Of the Low-density lipoprotein receptor (IDOL), and as the name suggests, trigger the ubiquitination of the LDLr subsequently marking it for degradation (539). As we showed in **chapter 4**, lack of *ldlr* ameliorates EAE and skews macrophages towards a more anti-inflammatory phenotype and it provides an additional mechanism by which LXR activation, under certain conditions, can induce a protective phenotype by decreasing LDLr.

We previously showed that LXRs also regulate cholesterol homeostasis in oligodendrocytes. More specifically, we showed that treatment of oligodendrocytes with the synthetic LXR-agonist T0901317 resulted in an enhanced cholesterol efflux in the presence of Apo-AI or HDL particles (304). Based on the notion that cholesterol is rate limiting for myelin formation in the CNS (510), LXR activation in oligodendrocytes would be considered undesirable. However, Meffre *et al.* recently demonstrated that LXR activation enhanced remyelination after lysolecithin-induced demyelination (444). The effect seems to be mediated by LXR activation in oligodendrocytes, as treatment with T0901317 and the oxysterol 25OHC can stimulate expression of myelin-associated genes MBP and PLP. However, the high dose of 25OHC used in these experiments has been shown to be toxic to oligodendrocytes after 24h of treatment (511), warranting caution in interpreting these results. Altogether, given their cholesterol-regulating and anti-inflammatory properties it is likely that LXRs play an important role in dampening neuroinflammation in MS.

What is the role of apoE in MS?

In the healthy CNS, apoE is predominantly synthesized and secreted by astrocytes to generate apoE-containing lipoprotein-like particles that supply neurons with cholesterol (512). Since virtually all cholesterol in the CNS is synthesized locally, astrocytes are the main hubs of cholesterol distribution throughout the CNS (251, 252) and activation of LXRs directly orchestrates cholesterol turnover in astrocytes (251, 253). These findings are in line with the vital role of the LXR pathway in controlling the biogenesis of lipoprotein-like particles. Therefore, disturbances in

the formation of lipoprotein-like particles by astrocytes and a consequent imbalance in cholesterol transport may contribute to the development of MS. Moreover, apoE has been suggested to be involved in CNS remyelination as MS patients with the apoE ε2 allele showed reduced remyelination compared to people lacking the apoE ε2 allele, although results should be interpreted with caution as the study's sample size was relatively small (513). In addition, apoE is expressed by CNS macrophages and microglia, which have led to the investigation of apoE in MS and its animal model EAE. Several controversial results have been reported for apoE in EAE including both beneficial and detrimental effects on disease progression (459, 515). Discrepancies may be due to methodological differences such as the immunization protocol or due to other factors that have not been probed in the respective studies. In parallel, the association of the apoE genotype and MS has been inconsistent. Some studies suggest that apoE4 may modulate MS progression by increasing damage to the brain and worsening cognitive dysfunction and disease severity (516, 517). However, the conclusions remain controversial (518). So far, apoE association studies in MS have yielded mostly negative results with some studies reporting significant effects and others being unable to confirm them. Two polymorphisms, rs429358 (ε4, Cys130Arg) and rs7412 (ε2, Arg176Cys), have been extensively scrutinized for their role in MS. However, a recent study based on 29.000 MS patients found no association with disease susceptibility (519). Divergent findings may be due to confounders or effect modifiers such as sex or age. A small study using 221 patients suggested a beneficial association between apoE2 and female MS patients, while there was no association in men (520). However, these results were challenged by another group claiming a detrimental and not beneficial association between apoE2 and female MS patients (521).

In this study, we show in **chapter 3** that apoE is highly expressed in phagocytes in active MS lesions where it may be involved in the clearance of myelin (514). In **chapter 4**, we assessed the role of LDLr on EAE severity in both male and female mice. Interestingly, we found that female *ldlr*$^{-/-}$ mice exhibited reduced EAE symptoms compared to male EAE mice and that macrophages isolated from female *ldlr*$^{-/-}$ mice abundantly secreted apoE, whereas this was not the case in male or WT female mice. These data suggest that apoE in female *ldlr*$^{-/-}$ mice is responsible for the observed reduction in EAE severity. The immunomodulatory

properties of apoE on immune function are well established. Studies have demonstrated that both apoE-containing lipoproteins and synthetic apoE peptides can inhibit antigen-stimulated T cell proliferation by modifying the function of IL-2R, reducing phospholipid turnover and downregulating DNA synthesis (522-525). In addition, stimulated macrophages from apoE-deficient mice more effectively upregulate the expression of pro-inflammatory mediators, MHC class II molecules, and costimulatory molecules *in vitro* (229). Moreover, in macrophages apoE has been shown to suppress the production of pro-inflammatory cytokines such as TNFα and IL-1β in an isoform-specific manner (E2 > E3 > E4) (526). In concordance, we found that TNFα production by macrophages was reduced by almost 90%. Studies have reported that increased expression of apoE leads to a less inflammatory phenotype in macrophages by downregulating M1-like and upregulating M2-like markers. Additionally, apoE suppresses microglial activation and release of TNFα (230, 456, 457). The activity of these cells can be modulated by sex hormones such as estrogen and testosterone (460). Both estrogen and androgen receptors are expressed on immune cells and have immunomodulatory effects (460, 461). Interestingly, administration of estrogens has been shown to directly regulate hepatic LDLr activity in human cells *in vitro* and 17β-estradiol has been shown increase the expression of apoE during the maturation stage of human monocytes to macrophages (462-464). As lack of LDLr has been shown to increase apoE levels (386), it is possible that both mechanisms contribute to the increased apoE levels observed in female *ldlr*$^{-/-}$ mice, which may explain the observations described in this study. Although the reason for this sexual dimorphism remains unclear, our findings indicate that apoE may modulate the progression of neuroinflammatory diseases such as MS. Epidemiological studies spanning decades have demonstrated that females are more susceptible to MS than men (527). Future studies should elucidate this sexual apoE dimorphism to better understand pathological factors involved in MS. For example, experiments using female mice that have undergone oophorectomy, may elucidate if sex hormones indeed underlie the observed effects in this study.

What is the role of the low-density lipoprotein receptor in MS?

The LDLr is localized on the outer surface of many types of cells, where it binds to LDL particles and transports them into the cell. The LDLr is ubiquitously

expressed and genetic mutations result in a condition with extremely elevated serum LDL levels and early onset atherosclerosis known as familial hypercholesterolemia (FH) (193). FH is characterized by defective catabolism of LDL, which results in elevated plasma cholesterol and lipid accumulation in immune cells. Though LDLr is the predominant regulator of cholesterol and apoE homeostasis in the periphery (528, 529), its role in the CNS remains elusive (530). Some LDLr polymorphisms show a sex-dependent increased risk for developing AD (531, 532), and in mice lacking LDLr in the CNS apoE is increased but has no effect on cholesterol levels (386).

In **chapter 4**, we demonstrated an important role for the LDLr in EAE disease progression as *ldlr*$^{-/-}$ deficiency significantly ameliorated EAE disease severity. Considering the LDLr is involved in myelin phagocytosis (108), we investigated if *ldlr* deficiency impacts macrophage mediated myelin phagocytosis. Myelin phagocytosis was significantly decreased in macrophages isolated from *ldlr*$^{-/-}$ female mice compared to WT mice. We found that *ldlr* deficiency greatly reduced expression of LRP1. LRP1, or CD91 is a scavenger receptor involved in the removal of myelin debris, as well as necrotic and apoptotic cells (108, 533, 534). Together with CL-P1 discussed in **chapter 2**, these scavenger receptors mediate clearance of myelin debris generated during demyelination, which is critical for the regenerative capacity of the CNS. Impaired myelin debris clearance has been shown to delay recovery in a cuprizone mouse model (535). Additionally, LRP1 can also directly modulate inflammatory pathways as it has been shown to suppress NF-κB activity and the production of proinflammatory mediators (536, 537). Moreover, LRP1 expression is increased in phagocytes and astrocytes present in active demyelinating lesions, and its activity in microglia has been shown to be protective during EAE (538), suggesting LRP1 plays an important role in modulating disease progression. Taken together, our findings indicate that changes in LDLr activity may contribute to the progression of neuroinflammatory diseases such as MS. However, exactly how LDLr modulates LRP1 expression remains unclear. Future studies should therefore unravel the molecular dynamics between LDLr and LRP1 and determine the impact on neuroinflammation.

Are dietary plant sterols able to promote CNS repair?

Plant sterols are naturally occurring compounds that structurally and functionally resemble cholesterol in mammals (436). They are known to lower plasma cholesterol levels, although the underlying mechanism and long-term perspectives remain poorly understood. Even though the transport of circulating sterols across an intact BBB is very limited (282, 285), recent studies have indicated that dietary plant sterols stably accumulate in the CNS (282, 284, 325). This finding has prompted researchers to explore its physiological roles in healthy and diseased CNS. The mechanism by which plant sterols cross the BBB remains to be determined. It is unlikely that the Abcg5/g8 transporter complex mediates transport across the BBB as it is not detectable within the brain (284). Compared to WT mice, Abcg5$^{-/-}$ and apoE$^{-/-}$ mice display up to 12-fold increase of plasma plant sterols however only Abcg5$^{-/-}$, but not apoE$^{-/-}$ mice, show increased plant sterol levels in the CNS (284). Moreover, plant sterols are predominantly incorporated in VLDL in apoE$^{-/-}$ mice, whereas in WT and Abcg5$^{-/-}$ mice plant sterols are mainly incorporated in HDL (284). This supports, although speculative, the assertion that apoE and HDL are involved in plant sterol transport across the BBB. Interestingly, SR-BI, which is the major receptor for HDL, binds apoE-containing lipoproteins and is expressed at the apical membrane of BBB endothelial cells (285, 540). Aside from the anti-inflammatory properties of apoE discusses in **chapter 4**, this notion provides an interesting perspective on the role of apoE as a modulator of BBB plant sterol transport.

In **chapter 5,** we evaluated the effects of dietary plant sterols on an *in vivo* demyelinating mouse model. Based on the ability of PS to cross the BBB, their anti-inflammatory potential and their LXR agonist activity, we hypothesized that dietary PS boost repair in the CNS by activating LXR signaling. We found that plant sterols increased *Mbp* and *Plp* expression in primary mouse OPCs. Even though our *in vitro* data showed that plant sterols increased the number of MBP^{+} oligodendrocytes more potently than cholesterol, they did not improve CNS remyelination. Additionally, we found no significant differences in myelin, inflammatory or LXR response gene expression between normal chow and plant sterol fed mice. Interestingly, Saher *et al.* showed that dietary cholesterol supplementation (2% w/w) enhances remyelination after cuprizone exposure

(381). Cholesterol supplementation dramatically increased OPC proliferation and differentiation. Moreover, compared to normal fed mice, one week of cholesterol supplementation following four weeks of cuprizone-induced demyelination significantly improved performance on the complex running wheel (381). Once dietary sterols have entered the CNS, they tend to accumulate in lipid rich membranes of neurons and likely also oligodendrocytes (282, 284). Vanmierlo *et al.* previously showed that feeding mice a plant sterol enriched diet for six weeks resulted in a two-fold increase in lipid raft-associated plant sterol concentration. However, this only amounts to a plant sterol-to-cholesterol ratio of 1:300 (~7 ng/mg protein versus ~2,300 ng/mg protein) It remains to be determined whether this 1:300 plant sterol-to-cholesterol ratio is sufficient to modulate membrane and lipid raft properties. However, perhaps the notion that full myelination membrane synthesis requires external lipid supply underlies the benefit of cholesterol supplementation (166, 301). Although speculative, it is possible that the higher molecular side-chain complexity of plant sterols (281-283) prevents PLP-specific myelin targeting. Recent studies have shown that oligodendrocytes enrich myelin membrane cholesterol to a higher level than in normal plasma membranes because PLP chaperones the transfer of cholesterol into the myelin membrane (541). Oligodendrocytes maintain a strict PLP-to-cholesterol ratio in myelin as evidenced by studies using both PLP-overexpressing and –deficient mice (541, 542) without affecting cholesterol biosynthesis (543). In accordance with these findings, we show that *Plp*, but not *Mbp* expression is significantly increased in primary mouse OPCs after cholesterol supplementation.

Recently, LXRs have been implicated in CNS remyelination (444). Our plant sterol-based diet consists out of 50% β-sitosterol, 33% stigmasterol and 17% campesterol. Even though, according to literature, all three are considered LXR agonists *in vitro* (482), the question remains whether plant sterols sufficiently accumulate to exceed the threshold needed to trigger LXR activation in the *in vivo* situation. The ability of plant sterols in a Western diet to activate LXRs is limited compared to synthetic LXR agonists (261, 262, 264-266, 544). In concordance, we could not detect LXR activation in the liver after dietary plant sterol supplementation, whereas treatment with a synthetic LXR agonist significantly increased LXR target *Srebp-1c* expression in the liver. Future research should determine the LXR-activating potential of plant sterols *in vivo*. Correspondingly,

preliminary results from our group suggest that the selective LXRβ-activating oxidized plant sterol 24(S)-saringosterol, significantly improved memory performance and reduced Aβ plaque load in a well-established AD model (unpublished data). The use of 24(S)-saringosterol may be a novel candidate for dietary supplementation to promote CNS repair mechanisms.

What are the implications of the findings of this thesis for MS and other CNS diseases?

Existing therapies for MS focus largely on suppressing or altering the activity of the immune system. However, none of them can halt the neurodegenerative changes that are associated with the progressive phase of the disease. In this thesis, I have demonstrated that key players in the sterol metabolism are involved in immunomodulatory and possibly CNS repair mechanisms. In the CNS, clearance of myelin debris is an important prerequisite to remyelination. CL-P1 may be a new therapeutic target due to its discovery as a novel receptor involved in the uptake of myelin. By stimulating expression of CL-P1, it may be possible to improve myelin clearance, which is essential prior to remyelination. Additionally, its C-type CRD domain strongly resembles both DC-SIGN and selectins, suggesting that CL-P1 may also play a role in cell migration, differentiation and T cell priming (419-422). CL-P1 is primarily expressed in the center and rim of active MS lesions, especially in perivascular macrophages and parenchymal astrocytes. As perivascular cuffs accommodate lymphocytes during active MS, CL-P1 on phagocytes may play a role in T cell priming. Therefore, a future CL-P1 antibody therapy should focus on RRMS patients where the immune component is strongly present and remyelination is most prominent. Research in CL-P1 agonists could provide relevant therapeutic candidates that potentially improve myelin clearance and limit cell migration, differentiation, and T cell priming. Although LXRs likely do not control expression of CL-P1, I have demonstrated that the uptake of myelin by phagocytes present in active lesions activates LXRs. Combined with previous work from our group, it is likely that the phenotype of these phagocytes is at least in part modulated by a myelin-mediated activation of LXRs. In addition, LXRs control the expression of the LDLr. In EAE, the lack of LDLr ameliorated disease symptoms indicating that suppression of LDLr may be beneficial. However, lack of LDLr also decreased LRP1 expression, which is involved in the clearance of myelin

debris. Moreover, suppression of LDLr would inhibit extracellular uptake of cholesterol by oligodendrocytes, hampering CNS remyelination. This illustrates the need for disease stage dependent treatment or cell-specific delivery. Additionally, I found that lack of LDLr increased the anti-inflammatory apoE in female but not in male mice. Although the reason for this sexual dimorphism remains unclear, it indicates apoE as a therapeutic strategy of MS. Likewise, several studies have already demonstrated that subcutaneously injected apoE-mimetic peptides alleviate effects of inflammation-related insults in EAE, LPS-induced inflammation and traumatic brain injury (545-547). Although more research is needed to determine the effect of apoE genotype in humans, we can speculate about the possible effects of subcutaneous injections of apoE-mimetic in MS patients as we have shown that increased apoE suppresses the inflammatory phenotype of macrophages in mice.

Because of their sterol-regulating and anti-inflammatory properties, LXRs are an interesting therapeutic avenue for the development of new MS drugs. However, the use of synthetic LXR agonists induce aversive side effects such as hypertriglyceridemia and hepatic steatosis (385). These side effects, predominantly driven by LXRα, limit the use of these LXR agonists in the clinic. These side effects can be avoided by using highly specific delivery mechanisms such as antibody-directed nanoparticles containing LXR agonists, or by using highly selective LXRβ agonists such as 24(S)-Saringosterol present in the edible seaweed *Sargassum fusiforme* (262). Additionally, plant sterols can be used as these compounds do not induce these aversive side effects. Although the plant sterols used in this work did not activate LXRs or stimulate CNS remyelination *in vivo*, future research may provide other alternatives that do. There is now substantial evidence to apply therapies that combine the established immunomodulatory treatments of MS with compounds that promote CNS repair mechanisms (548, 549). Because MS is a complex multifactorial disease, it is possible that a single therapeutic option will not suffice. A successful therapeutic strategy for MS would at least include a combination of immunomodulatory and CNS repair-inducing agents to prevent and repair damage early on. Thus far, few attempts have been made to address this therapeutic option. One of the most rigorous studies of combined disease-modifying therapies is CombiRx which combines Glatiramer acetate and interferon β1. However, this study did not find

any benefit from taking both drugs over taking glatiramer acetate by itself (550). Research into combination therapies is complicated by the huge costs associated with these studies. Also, the decision on which drugs to combine is of great importance. One can argue that the combination of two immunomodulating agents does not add anything new to the table. A better strategy may be to combine BBB-crossing molecules targeting both the immune component and stimulating CNS repair mechanisms together with peripherally acting molecules. However, one of the drawbacks of brain drug development is the BBB that significantly decreases CNS drug penetration (551). Even monoclonal antibodies such as ocrelizumab (148 kDa) that have obtained good results in MS patients have difficulties crossing the BBB due to their relatively large size. Molecular weight is a critical factor as BBB penetration is inversely related to the square root of the molecular weight. ApoE-mimetics such as COG1410 (1,4 kDa) that have strong anti-inflammatory properties and a small molecular footprint could be used to effectively target the intrathecal compartmentalization of inflammation resisting immunosuppressive treatments in MS patients, especially those in the progressive phase of the disease.

ApoE-mimetics could be combined with a CNS repair-inducing agent. Various studies have demonstrated that inefficient myelin clearance is the primary factor in inhibiting remyelination and drugs stimulating myelin clearance are lacking. The development of CL-P1 nanobodies could help in stimulating CNS repair mechanisms, as we have shown that it is likely involved in myelin uptake by macrophages. Moreover, nanobodies are based on single-domain antibody fragments that contain the unique and functional properties of conventional antibodies but have a smaller molecular footprint (12-15 kDa).

Although plant sterols used in this project did not stimulate remyelination *in vivo*, promising effects have been observed in relation to Alzheimer's disease. Using apoE as a modulator of BBB plant sterol transport, future therapies may combine dietary supplementation of plant sterols together with an apoE-mimetics to increase their CNS uptake. Collectively, results from this work indicate that targeting key players in cholesterol metabolism may hold promise for future intervention strategies aimed at modulating neuroinflammation and CNS repair.

Conclusion

We hypothesized that the activation of the sterol metabolism can induce repair mechanisms in MS. Our results collectively show that key players in the cholesterol metabolism modulate the inflammatory response and possibly contribute to CNS repair mechanisms.

First, we demonstrate that CL-P1 is likely involved in mediating the uptake of myelin by phagocytes. Considering its potential role herein, CL-P1 may play an important role in the pathophysiology of MS. Second, we show that LXRs and their downstream targets are induced in myelin-laden macrophages in active demyelinating MS lesions, indicative of LXR activation. Moreover, the oxysterol 27OHC is the major LXR ligand that is formed after myelin processing and the levels of 27OHC that are generated are capable of upregulating LXR downstream targets in phagocytes indicating that 27OHC is, at least in part, responsible for activation of LXRs in MS lesions. Third, we show that *ldlr*$^{-/-}$ reduces EAE disease severity in female but not in male EAE mice. Macrophages isolated from female *ldlr*$^{-/-}$ mice show an impaired capacity to phagocytose myelin and produce less proinflammatory mediators, likely induced by the anti-inflammatory apoE. Finally, though plant sterols are known to cross the BBB, have anti-inflammatory properties and activate LXRs, they did not improve CNS remyelination *in vivo*. Future research may provide other plant sterol analogues that do. Collectively, these results indicate that targeting key players in cholesterol metabolism are involved in modulating disease progression and may hold promise for future MS therapeutics.

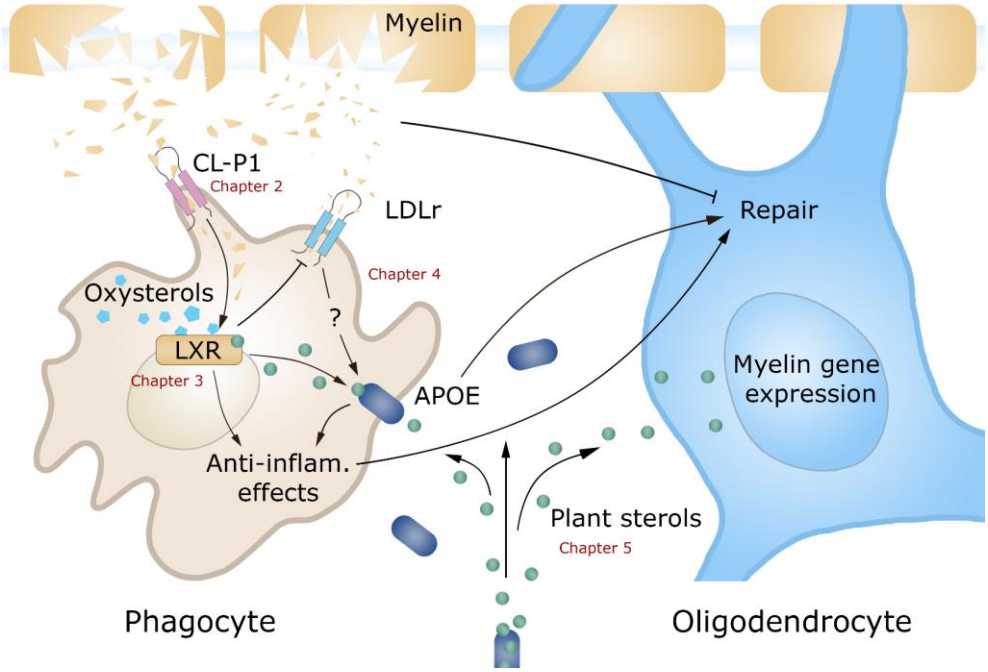

Figure 6.1 | Summary of findings from this thesis. In chapter 2, we identify CL-P1 as a novel receptor likely involved in the uptake of myelin. CL-P1 may be involved in the clearance of myelin debris, which is an important prerequisite for proper CNS remyelination. In chapter 3, we show that when myelin is phagocytosed, LXRs become activated at least partially by (oxy)sterols present in myelin. LXRs are master regulators of the cholesterol metabolism that can downregulate LDLr while inducing expression and secretion of apoE. In chapter 4, we show that lack of LDLr ameliorates EAE symptoms in female mice. Macrophages isolated from female *ldlr*$^{-/-}$ mice show an impaired capacity to phagocytose myelin and produce less proinflammatory mediators, likely induced by the anti-inflammatory apoE. This results in a more anti-inflammatory environment that is favorable for CNS remyelination. In addition, we speculate that apoE is involved in the uptake of plant sterols into the CNS. In chapter 5, we investigate whether plant sterols can induce CNS repair mechanisms. Although they are known to cross the BBB, have anti-inflammatory properties and activate LXRs, dietary plant sterol supplementation did not improve CNS remyelination *in vivo*. Future research may provide other plant sterol alternatives that do.

7

Nederlandse samenvatting

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Multiple sclerose (MS) is een chronische auto-immuunziekte van het centraal zenuwstelsel (CZS). De ziekte treft meer dan 2 miljoen mensen wereldwijd en is de meest voorkomende oorzaak van niet-traumatische neurologische invaliditeit bij adolescenten. Daarnaast worden vrouwen vaker getroffen dan mannen. In België zijn ongeveer 10.000 mensen getroffen door MS. De meest gangbare hypothese stelt dat MS wordt veroorzaakt door het eigen immuunsysteem dat zich tegen het eigen lichaam keert. Immuuncellen, zoals macrofagen en autoreactieve T-cellen, infiltreren het CZS en veroorzaken daar een chronische ontstekingsreactie. Bovendien vallen ze de beschermende isolatielaag (myeline) rondom de zenuwbanen aan waardoor zenuwen bloot komen te liggen. Communicatie tussen hersencellen wordt hierdoor verstoord, hetgeen aanleiding geeft tot symptomen zoals spierzwakte, sensorische en visuele uitval, cognitieve problemen en vermoeidheid. Hoewel de oorzaak van MS nog steeds onbekend is, heeft wetenschappelijk onderzoek aangetoond dat zowel erfelijkheid als omgevingsfactoren een rol spelen.

Verschillende studies hebben aangetoond dat macrofagen en microglia (fagocyten) de belangrijkste immuuncellen zijn die aanwezig zijn in MS-laesies. Fagocyten zijn witte bloedcellen die betrokken zijn bij de opruiming van dode of beschadigde cellen of pathogenen. Aanvankelijk werd gedacht dat fagocyten enkel een agressieve en beschadigende functie (fenotype) hadden in de progressie van MS. Recente studies hebben echter aangetoond dat fagocyten ook demyelinisatie (beschadiging) verminderen en remyelinisatie (herstel) stimuleren door herstel-onderdrukkende factoren te elimineren zoals myeline debris. Interessant is dat verstoringen in het cholesterolmetabolisme geassocieerd zijn met MS en het hiervoor meest gebruikte diermodel, experimentele auto-immune encefalomyelitis (EAE). MS-patiënten en EAE-dieren vertonen veranderde niveaus van verschillende oxysterolen in hun bloed en cerebrospinale vloeistof (CSV). Een veranderend cholesterol evenwicht in het lichaam (homeostase) kan de functie van fagocyten moduleren en hierdoor de voortgang van de ziekte beïnvloeden. Ook is er een positieve correlatie tussen het aantal ontstekingsgebieden in het CZS en cholesterolniveaus in het bloed van MS-patiënten. Cholesterol is het meest voorkomende lipide in myeline en speelt een sleutelrol in remyelinisatie. Omdat

opname van intermediaire cholesterolmoleculen en oxysterolen door fagocyten resulteert in de activatie van de ontsteking onderdrukkende lever-X-receptoren (LXRs), zullen macrofagen een minder agressieve rol aannemen en mogelijk zelfs bijdragen aan het herstelproces. Bovendien zorgt activatie van LXRs voor de productie van apoE, een lipoproteïne dat het aanleveren van vetten controleert. Deze vetten kunnen door oligodendrocyten, cellen die myeline aanmaken, aangewend worden om het herstelproces in de hersenen te stimuleren. **Het is echter momenteel onduidelijk hoe veranderingen in het sterolmetabolisme bijdragen aan het ontstekingsproces en herstelmechanismen in het CZS van MS-patiënten**. In een eerste hoofdstuk concentreren we ons op het onderzoeken van de rol van de CL-P1 receptor die mogelijk betrokken is bij de opname van myeline door fagocyten. Tevens onderzoeken we ook moleculaire mechanismen die betrokken zijn bij de wijzigende functie van fagocyten na myeline opname en bestuderen we de rol van de *low-density* lipoproteïne receptor (LDLr) in neuroinflammatie. In het laatste hoofdstuk wordt onderzocht of plantensterolen, het plantanaloge cholesterol, bijdraagt aan herstel in het CZS door het activeren van LXRs.

Wat is de rol van CL-P1 in macrofagen in MS-laesies?

In hoofdstuk twee hebben we aangetoond dat zowel muis als humane macrofagen CL-P1 tot expressie brengen. Hierbij is het interessant dat opname van myeline door beenmerg-afgeleide macrofagen de expressie van deze receptor op een dosis-afhankelijke manier beïnvloedt. Schuimcellen (macrofagen die veel myeline hebben opgenomen) brengen CL-P1 erg krachtig tot expressie, wat kan betekenen dat myeline opname via de CL-P1 receptor in deze macrofagen die aanwezig zijn in MS-laesies kan stimuleren.

Verschillende studies hebben ook aangetoond dat myeline kan oxideren in MS-patiënten. We hebben gemerkt dat geoxideerd myeline expressie van CL-P1 krachtiger en langer kan stimuleren in vergelijking met niet-geoxideerd myeline. Zogenaamde *scavenger* receptoren (SRs) zijn bekend een sterke affiniteit te hebben voor geoxideerde moleculen zoals geoxideerd LDL (oxLDL). In actieve MS-laesies produceren fagocyten grote hoeveelheden zuurstofmetabolieten (ROS) en andere schadelijke factoren die myeline kunnen oxideren en beschadigen. Tijdens oxidatie vinden verschillende chemische en fysische veranderingen plaats die

herkenningsepitopen van myeline kunnen beïnvloeden. Deze epitopen zijn belangrijk voor SRs. Omdat CL-P1 en SRs eenzelfde collageen domein delen en het fagocytisch vermogen van SRs voornamelijk afhangt van dat domein, is het niet uitgesloten dat dit domein een rol speelt in het opnemen van geoxideerd myeline en LDL door CL-P1.

Hoewel de opname van myeline leidt tot demyelinisatie, is het verwijderen van myeline debris een belangrijke vereiste voor een goed herstel van het CZS. Afhankelijk van of CL-P1 de opname van intact myeline of myeline debris stimuleert, kan deze functie zowel beschadigend of beschermend zijn. Omdat oxidatie myeline beschadigt en we voornamelijk geoxideerd myeline in onze experimenten hebben gebruikt, is het mogelijk dat CL-P1 een rol zou kunnen spelen in het verwijderen van myeline debris. Dit zou betekenen dat CL-P1 een belangrijke rol zou spelen in het CZS-herstelproces van MS-patiënten. Toekomstige studies zullen moeten uitwijzen of de rol van CL-P1 daadwerkelijk gunstig of beschadigend is in MS.

Hoe kan myeline de lever-X-receptoren in humane fagocyten in MS activeren?

Recent werd in rat macrofagen aangetoond dat myeline-afgeleide vetten LXRs activeren, die bijgevolg een minder agressief fenotype gaan vertonen. Het was echter nog niet geweten of LXRs ook geactiveerd werden in fagocyten in actieve MS-laesies en welke myeline componenten hiervoor verantwoordelijk voor zijn. In hoofdstuk drie hebben geprobeerd hierop een antwoord te vinden. We hebben aangetoond dat LXRs in fagocyten aanwezig in actieve laesies op zijn minst gedeeltelijk worden geactiveerd door 27-hydroxycholesterol (27OHC) dat aanwezig is in myeline. Meer nog, we hebben aangetoond dat 27OHC tijdens het afbraakproces van myeline wordt aangemaakt en zodoende ook kan bijdragen aan dit proces. Het feit dat myeline opname mogelijk bijdraagt aan het bepalen van de (beschermende) macrofaag-functie, is een intrigerend gegeven dat mogelijk de zelflimiterende eigenschappen van MS-laesies kan verklaren. De *in vivo* relevantie van oxysterolen zou in toekomstige onderzoeken kunnen worden bepaald door gebruik te maken van *triple-knock out* CYP46A1, CYP27A1, en CH25H enzymen (verantwoordelijk voor de aanmaak van verschillende oxysterolen). Tevens zou dit ook uitsluitsel kunnen geven of myeline-gemedieerde

LXR-activatie in fagocyten ontstekingen in het CZS van MS-patiënten kan beïnvloeden.

Wat is de rol van de LDLr en apolipoproteine E in MS?

In hoofdstuk vier hebben we de rol van LDLr op EAE-onderzoek in zowel mannetjes als vrouwtjes muizen onderzocht. We vonden dat vrouwelijke *ldlr*$^{-/-}$ muizen minder ziek werden en dat macrofagen van deze vrouwtjes meer apoE uitscheidden, hetgeen niet het geval was voor mannelijke *ldlr*$^{-/-}$ of wild-type vrouwelijke muizen. Deze resultaten suggereren dat apoE in deze vrouwelijke *ldlr*$^{-/-}$ muizen verantwoordelijk is voor de vermindering in EAE-symptomen. Recent onderzoek heeft aangetoond dat apoE sterke ontstekingsremmende functies heeft. Zo is het bekend dat het de activatie en proliferatie van T-cellen, de expressie van pro-inflammatoire cytokines en verschillende (type I) ontstekingsprocessen kan onderdrukken. De reden waarom het effect enkel beperkt bleef tot vrouwelijke *ldlr*$^{-/-}$ muizen is nog niet geheel duidelijk. Zowel oestrogeen als androgeen receptoren zouden mogelijk een rol kunnen spelen aangezien ze beiden tot expressie komen op afweercellen waar ze tevens immunomodulerende functies kunnen uitoefenen. Zo werd onlangs aangetoond dat 17β-estradiol de expressie van apoE in macrofagen kan stimuleren.

Hoewel de reden achter deze seksuele discrepantie onduidelijk blijft, tonen deze bevindingen aan dat apoE de voortgang van MS-ontstekingsprocessen kunnen beïnvloeden. Epidemiologische studies hebben aangetoond dat vrouwen vatbaarder zijn voor MS dan mannen. Toekomstige studies moeten dit seksuele apoE dimorfisme bestuderen om de betrokken pathologische factoren in MS beter te kunnen begrijpen.

Kunnen plantensterolen CZS-herstel bevorderen?

Plantensterolen zijn natuurlijke plantaardige componenten die structureel en functioneel sterk lijken op cholesterol. Ze bevinden zich onder andere in groente, fruit en noten. Hun cholesterolverlagende eigenschappen zijn reeds bekend bij het brede publiek, hoewel het precieze mechanisme en langetermijneffecten nog steeds niet volledig zijn begrepen. Hoewel het transport van circulerende sterolen doorheen de bloed-hersen barrière (BBB) erg beperkt is, hebben recente studies aangetoond dat plantensterolen in voeding stabiel accumuleren in het CZS.

Daarnaast heeft onderzoek uitgewezen dat plantensterolen ontstekingsremmende functies hebben die mogelijk gekoppeld zijn aan hun vermogen om LXRs te activeren. Deze eigenschappen hebben er ons toe aangezet om de CZS-herstel bevorderende eigenschappen van plantensterolen te onderzoeken. In een eerste stap hebben we aangetoond dat plantensterolen het aantal myeline vormende oligodendrocyten *in vitro* kan stimuleren. Vervolgens hebben we gebruik gemaakt van een dierenmodel om dit effect *in vivo* te bestuderen. Gebruik makende van het cuprizone model werd eerst demyelinisatie in muizen geïnduceerd. Daarna kregen muizen ofwel een normaal ofwel een 5% plantensterol dieet gedurende een aantal weken. Aan het einde van de proef werden de dieren onderzocht of, en hoeveel, herstel had plaatsgevonden. Echter, plantensterolen hadden geen invloed op CZS remyelinisatie of op myeline-, ontstekings- of LXR-genexpressie. Hoewel plantensterolen wel degelijk in het CZS belanden, is het misschien aangewezen om specifieke plantensterolen met specifieke eigenschappen te gebruiken voor toekomstig onderzoek. Zo zou 24(S)-Saringosterol, een selectieve LXRβ agonist een mogelijke optie kunnen zijn. Hiervan werd reeds aangetoond dat deze plant sterol het geheugen in een dierenmodel voor Alzheimer kon verbeteren. 24(S)-Saringosterol zou dus een nieuwe kandidaat kunnen zijn om herstelmechanismen in het CZS te bevorderen.

Conclusie

Resultaten van deze thesis tonen aan dat sleutelspelers in het cholesterolmetabolisme betrokken zijn bij ontstekingsmechanismen en mogelijk bijdragen aan CZS-herstelmechanismen. Zo werd er aangetoond dat CL-P1 betrokken is bij de opname van myeline door fagocyten in MS en dat myeline in staat is de ontstekingsremmende LXRs te activeren die op hun beurt mogelijk verantwoordelijk zijn voor het beschermende fenotype dat macrofagen aannemen na opname van myeline. De activatie van LXRs na opname van myeline is tenminste gedeeltelijk te wijten aan het oxysterol 27OHC dat gevormd wordt na myeline opname in fagocyten. Daarnaast werden EAE-symptomen sterk onderdrukt in vrouwelijke maar niet mannelijke *ldlr*$^{-/-}$ muizen. Macrofagen geïsoleerd uit deze muizen vertoonden een verminderde capaciteit om myeline op te nemen en produceerden minder ontstekingsfactoren, welke waarschijnlijk te wijten zijn aan het ontstekingsremmende apoE. Hoewel plantensterolen gebruikt

in dit onderzoek doorheen de BBB migreren, ontstekingsremmende functies bevatten en geacht worden LXRs te activeren, hadden ze geen effect op CZS-herstelprocessen. Het gebruik van andere plantensterolen kan mogelijk wel positieve resultaten opleveren. Ontwikkeling van medicijnen die ingrijpen op belangrijke spelers in het cholesterolmetabolisme betrokken bij het moduleren van ziekteprogressie zouden in de toekomst mogelijk kunnen worden aangewend worden om MS tegen te gaan.

Reference list

1. Lublin F. History of modern multiple sclerosis therapy. Journal of neurology. 2005;252 Suppl 3:iii3-iii9.
2. Charcot JM. Histologie de la sclerose en plaque. Gazette des Hopitaux. 1868(41):554-66.
3. Compston A, Coles A. Multiple sclerosis. Lancet. 2008;372(9648):1502-17.
4. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol. 2007;61(4):288-99.
5. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. Ann Neurol. 2007;61(6):504-13.
6. . !!! INVALID CITATION !!! {}.
7. Pugliatti M, Harbo HF, Holmoy T, Kampman MT, Myhr KM, Riise T, et al. Environmental risk factors in multiple sclerosis. Acta Neurol Scand Suppl. 2008;188:34-40.
8. Soldan SS, Jacobson S. Role of viruses in etiology and pathogenesis of multiple sclerosis. Adv Virus Res. 2001;56:517-55.
9. Pugliatti M, Rosati G, Carton H, Riise T, Drulovic J, Vecsei L, et al. The epidemiology of multiple sclerosis in Europe. Eur J Neurol. 2006;13(7):700-22.
10. Hellings N, Raus J, Stinissen P. Insights into the immunopathogenesis of multiple sclerosis. Immunol Res. 2002;25(1):27-51.
11. Pugliatti M, Sotgiu S, Rosati G. The worldwide prevalence of multiple sclerosis. Clin Neurol Neurosur. 2002;104(3):182-91.
12. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Medical progress: Multiple sclerosis. New Engl J Med. 2000;343(13):938-52.
13. Trapp BD, Nave KA. Multiple sclerosis: An immune or neurodegenerative disorder? Annu Rev Neurosci. 2008;31:247-69.
14. Bar-Or A, Oliveira EM, Anderson DE, Hafler DA. Molecular pathogenesis of multiple sclerosis. Journal of neuroimmunology. 1999;100(1-2):252-9.
15. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic Criteria for Multiple Sclerosis: 2010 Revisions to the McDonald Criteria. Annals of neurology. 2011;69(2):292-302.
16. Filippi M, Rocca MA, Ciccarelli O, De Stefano N, Evangelou N, Kappos L, et al. MRI criteria for the diagnosis of multiple sclerosis: MAGNIMS consensus guidelines. Lancet Neurol. 2016;15(3):292-303.
17. Inglese M, Grossman RI, Filippi M. Magnetic resonance imaging monitoring of multiple sclerosis lesion evolution. J Neuroimaging. 2005;15(4):22s-9s.
18. Stys PK, Zamponi GW, van Minnen J, Geurts JJG. Will the real multiple sclerosis please stand up? Nature Reviews Neuroscience. 2012;13(7):507-14.
19. Dutta R, Trapp BD. Mechanisms of neuronal dysfunction and degeneration in multiple sclerosis. Prog Neurobiol. 2011;93(1):1-12.
20. Dutta R, Trapp BD. Pathogenesis of axonal and neuronal damage in multiple sclerosis. Neurology. 2007;68:S22-S31.
21. Weber MS, Hemmer B, Cepok S. The role of antibodies in multiple sclerosis. Biochimica et biophysica acta. 2011;1812(2):239-45.
22. Weber MS, Hemmer B. Cooperation of B cells and T cells in the pathogenesis of multiple sclerosis. Results Probl Cell Differ. 2010;51:115-26.
23. Lehmann-Horn K, Kronsbein HC, Weber MS. Targeting B cells in the treatment of multiple sclerosis: recent advances and remaining challenges. Ther Adv Neurol Disord. 2013;6(3):161-73.
24. Huseby ES, Liggitt D, Brabb T, Schnabel B, Ohlen C, Goverman J. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. J Exp Med. 2001;194(5):669-76.
25. Stys PK, Zamponi GW, van Minnen J, Geurts JJG. Will the real multiple sclerosis please stand up? (vol 13, pg 507, 2012). Nature Reviews Neuroscience. 2012;13(8).

26. Chitnis T. The role of CD4 T cells in the pathogenesis of multiple sclerosis. International review of neurobiology. 2007;79:43-72.
27. Zamvil SS, Steinman L. The Lymphocyte-T in Experimental Allergic Encephalomyelitis. Annu Rev Immunol. 1990;8:579-621.
28. van Noort JM, Verbeek R, Meilof JF, Polman CH, Amor S. Autoantibodies against alpha B-crystallin, a candidate autoantigen in multiple sclerosis, are part of a normal human immune repertoire. Mult Scler. 2006;12(3):287-93.
29. Haider L. Inflammation, Iron, Energy Failure, and Oxidative Stress in the Pathogenesis of Multiple Sclerosis. Oxid Med Cell Longev. 2015;2015:725370.
30. Traka M, Podojil JR, McCarthy DP, Miller SD, Popko B. Oligodendrocyte death results in immune-mediated CNS demyelination. Nat Neurosci. 2016;19(1):65-74.
31. Shaharabani R, Ram-On M, Avinery R, Aharoni R, Arnon R, Talmon Y, et al. Structural Transition in Myelin Membrane as Initiator of Multiple Sclerosis. J Am Chem Soc. 2016;138(37):12159-65.
32. Lassmann H, Bruck W, Lucchinetti CF. The immunopathology of multiple sclerosis: An overview. Brain Pathol. 2007;17(2):210-8.
33. Lassmann H, Raine CS, Antel J, Prineas JW. Immunopathology of multiple sclerosis: Report on an international meeting held at the Institute of Neurology of the University of Vienna. Journal of neuroimmunology. 1998;86(2):213-7.
34. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. New Engl J Med. 1998;338(5):278-85.
35. van der Valk P, De Groot CJ. Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. Neuropathol Appl Neurobiol. 2000;26(1):2-10.
36. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. Brain : a journal of neurology. 2009;132(Pt 5):1175-89.
37. Kutzelnigg A, Lucchinetti CF, Stadelmann C, Bruck W, Rauschka H, Bergmann M, et al. Cortical demyelination and diffuse white matter injury in multiple sclerosis. Brain : a journal of neurology. 2005;128(Pt 11):2705-12.
38. Kornek B, Storch MK, Weissert R, Wallstroem E, Stefferl A, Olsson T, et al. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. The American journal of pathology. 2000;157(1):267-76.
39. Wu GF, Alvarez E. The immunopathophysiology of multiple sclerosis. Neurol Clin. 2011;29(2):257-78.
40. Frohman EM, Racke MK, Raine CS. Multiple sclerosis--the plaque and its pathogenesis. The New England journal of medicine. 2006;354(9):942-55.
41. Lassmann H, van Horssen J, Mahad D. Progressive multiple sclerosis: pathology and pathogenesis. Nature reviews Neurology. 2012;8(11):647-56.
42. Inglese M, Oesingmann N, Casaccia P, Fleysher L. Progressive multiple sclerosis and gray matter pathology: an MRI perspective. Mt Sinai J Med. 2011;78(2):258-67.
43. Bo L, Vedeler CA, Nyland HI, Trapp BD, Mork SJ. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. Journal of neuropathology and experimental neurology. 2003;62(7):723-32.
44. Calabrese M, Gallo P. Magnetic resonance evidence of cortical onset of multiple sclerosis. Multiple sclerosis. 2009;15(8):933-41.
45. Calabrese M, Rocca MA, Atzori M, Mattisi I, Bernardi V, Favaretto A, et al. Cortical lesions in primary progressive multiple sclerosis: a 2-year longitudinal MR study. Neurology. 2009;72(15):1330-6.
46. Calabrese M, Magliozzi R, Ciccarelli O, Geurts JJ, Reynolds R, Martin R. Exploring the origins of grey matter damage in multiple sclerosis. Nature reviews Neuroscience. 2015;16(3):147-58.
47. Calabrese M, Filippi M, Gallo P. Cortical lesions in multiple sclerosis. Nature reviews Neurology. 2010;6(8):438-44.
48. Peterson JW, Bo L, Mork S, Chang A, Trapp BD. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. Annals of neurology. 2001;50(3):389-400.

49. van Horssen J, Brink BP, de Vries HE, van der Valk P, Bo L. The blood-brain barrier in cortical multiple sclerosis lesions. Journal of neuropathology and experimental neurology. 2007;66(4):321-8.
50. Kidd D, Barkhof F, McConnell R, Algra PR, Allen IV, Revesz T. Cortical lesions in multiple sclerosis. Brain : a journal of neurology. 1999;122 (Pt 1):17-26.
51. Bo L, Geurts JJ, Mork SJ, van der Valk P. Grey matter pathology in multiple sclerosis. Acta Neurol Scand Suppl. 2006;183:48-50.
52. Bo L. The histopathology of grey matter demyelination in multiple sclerosis. Acta Neurol Scand Suppl. 2009(189):51-7.
53. Damjanovic D, Valsasina P, Rocca MA, Stromillo ML, Gallo A, Enzinger C, et al. Hippocampal and Deep Gray Matter Nuclei Atrophy Is Relevant for Explaining Cognitive Impairment in MS: A Multicenter Study. AJNR American journal of neuroradiology. 2017;38(1):18-24.
54. Calabrese M, Agosta F, Rinaldi F, Mattisi I, Grossi P, Favaretto A, et al. Cortical lesions and atrophy associated with cognitive impairment in relapsing-remitting multiple sclerosis. Archives of neurology. 2009;66(9):1144-50.
55. Cross AH, Naismith RT. Established and novel disease-modifying treatments in multiple sclerosis. J Intern Med. 2014;275(4):350-63.
56. Montalban X, Hauser SL, Kappos L, Arnold DL, Bar-Or A, Comi G, et al. Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis. N Engl J Med. 2017;376(3):209-20.
57. Kieseier BC. The mechanism of action of interferon-beta in relapsing multiple sclerosis. CNS Drugs. 2011;25(6):491-502.
58. Martinelli V, Radaelli M, Straffi L, Rodegher M, Comi G. Mitoxantrone: benefits and risks in multiple sclerosis patients. Neurol Sci. 2009;30 Suppl 2:S167-70.
59. Ford C, Goodman AD, Johnson K, Kachuck N, Lindsey JW, Lisak R, et al. Continuous long-term immunomodulatory therapy in relapsing multiple sclerosis: results from the 15-year analysis of the US prospective open-label study of glatiramer acetate. Mult Scler. 2010;16(3):342-50.
60. Polman CH, Bertolotto A, Deisenhammer F, Giovannoni G, Hartung HP, Hemmer B, et al. Recommendations for clinical use of data on neutralising antibodies to interferon-beta therapy in multiple sclerosis. Lancet Neurol. 2010;9(7):740-50.
61. Weber MS, Prod'homme T, Youssef S, Dunn SE, Rundle CD, Lee L, et al. Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. Nat Med. 2007;13(8):935-43.
62. Aharoni R, Teitelbaum D, Sela M, Arnon R. Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 1997;94(20):10821-6.
63. Lalive PH, Neuhaus O, Benkhoucha M, Burger D, Hohlfeld R, Zamvil SS, et al. Glatiramer acetate in the treatment of multiple sclerosis: emerging concepts regarding its mechanism of action. CNS Drugs. 2011;25(5):401-14.
64. Filippi M, Rovaris M, Rocca MA, Sormani MP, Wolinsky JS, Comi G, et al. Glatiramer acetate reduces the proportion of new MS lesions evolving into "black holes". Neurology. 2001;57(4):731-3.
65. Bar-Or A. Teriflunomide (Aubagio(R)) for the treatment of multiple sclerosis. Exp Neurol. 2014;262 Pt A:57-65.
66. Wang Q, Chuikov S, Taitano S, Wu Q, Rastogi A, Tuck SJ, et al. Dimethyl Fumarate Protects Neural Stem/Progenitor Cells and Neurons from Oxidative Damage through Nrf2-ERK1/2 MAPK Pathway. Int J Mol Sci. 2015;16(6):13885-907.
67. Hartung HP, Aktas O, Boyko AN. Alemtuzumab: a new therapy for active relapsing-remitting multiple sclerosis. Mult Scler. 2015;21(1):22-34.
68. Bielekova B. Daclizumab therapy for multiple sclerosis. Neurotherapeutics. 2013;10(1):55-67.
69. Gajofatto A, Benedetti MD. Treatment strategies for multiple sclerosis: When to start, when to change, when to stop? World J Clin Cases. 2015;3(7):545-55.
70. Miron VE, Jung CG, Kim HJ, Kennedy TE, Soliven B, Antel JP. FTY720 modulates human oligodendrocyte progenitor process extension and survival. Ann Neurol. 2008;63(1):61-71.

71. Coelho RP, Payne SG, Bittman R, Spiegel S, Sato-Bigbee C. The immunomodulator FTY720 has a direct cytoprotective effect in oligodendrocyte progenitors. J Pharmacol Exp Ther. 2007;323(2):626-35.
72. Chun J, Hartung HP. Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. Clin Neuropharmacol. 2010;33(2):91-101.
73. Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. N Engl J Med. 2010;362(5):387-401.
74. Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med. 2010;362(5):402-15.
75. Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med. 2006;354(9):899-910.
76. Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. Neurodegener Dis. 2008;5(1):16-22.
77. Neuhaus O, Kieseier BC, Hartung HP. Mitoxantrone in multiple sclerosis. Adv Neurol. 2006;98:293-302.
78. Hartung HP, Gonsette R, Konig N, Kwiecinski H, Guseo A, Morrissey SP, et al. Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. Lancet. 2002;360(9350):2018-25.
79. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010;327(5966):656-61.
80. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J Immunol. 2005;175(1):342-9.
81. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008;8(12):958-69.
82. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol. 2011;11(11):723-37.
83. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science. 2007;317(5838):666-70.
84. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009;27:669-92.
85. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med. 2007;204(12):3037-47.
86. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003;19(1):71-82.
87. Leuschner F, Dutta P, Gorbatov R, Novobrantseva TI, Donahoe JS, Courties G, et al. Therapeutic siRNA silencing in inflammatory monocytes in mice. Nat Biotechnol. 2011;29(11):1005-10.
88. Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. J Immunol. 2004;172(7):4410-7.
89. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. The New England journal of medicine. 1998;338(5):278-85.
90. Trebst C, Sorensen TL, Kivisakk P, Cathcart MK, Hesselgesser J, Horuk R, et al. CCR1+/CCR5+ mononuclear phagocytes accumulate in the central nervous system of patients with multiple sclerosis. Am J Pathol. 2001;159(5):1701-10.
91. Hendriks JJ, Teunissen CE, de Vries HE, Dijkstra CD. Macrophages and neurodegeneration. Brain Res Brain Res Rev. 2005;48(2):185-95.
92. Kouwenhoven M, Teleshova N, Ozenci V, Press R, Link H. Monocytes in multiple sclerosis: phenotype and cytokine profile. J Neuroimmunol. 2001;112(1-2):197-205.

93. Vogel DY, Vereyken EJ, Glim JE, Heijnen PD, Moeton M, van der Valk P, et al. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. J Neuroinflammation. 2013;10:35.
94. Boven LA, Van Meurs M, Van Zwam M, Wierenga-Wolf A, Hintzen RQ, Boot RG, et al. Myelin-laden macrophages are anti-inflammatory, consistent with foam cells in multiple sclerosis. Brain : a journal of neurology. 2006;129(Pt 2):517-26.
95. van Rossum D, Hilbert S, Strassenburg S, Hanisch UK, Bruck W. Myelin-phagocytosing macrophages in isolated sciatic and optic nerves reveal a unique reactive phenotype. Glia. 2008;56(3):271-83.
96. Febbraio M, Podrez EA, Smith JD, Hajjar DP, Hazen SL, Hoff HF, et al. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. The Journal of clinical investigation. 2000;105(8):1049-56.
97. Williams K, Ulvestad E, Waage A, Antel JP, McLaurin J. Activation of adult human derived microglia by myelin phagocytosis in vitro. J Neurosci Res. 1994;38(4):433-43.
98. van der Laan LJ, Ruuls SR, Weber KS, Lodder IJ, Dopp EA, Dijkstra CD. Macrophage phagocytosis of myelin in vitro determined by flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor necrosis factor-alpha and nitric oxide. J Neuroimmunol. 1996;70(2):145-52.
99. Mosley K, Cuzner ML. Receptor-mediated phagocytosis of myelin by macrophages and microglia: effect of opsonization and receptor blocking agents. Neurochem Res. 1996;21(4):481-7.
100. Reichert F, Rotshenker S. Complement-receptor-3 and scavenger-receptor-AI/II mediated myelin phagocytosis in microglia and macrophages. Neurobiol Dis. 2003;12(1):65-72.
101. da Costa CC, van der Laan LJ, Dijkstra CD, Bruck W. The role of the mouse macrophage scavenger receptor in myelin phagocytosis. The European journal of neuroscience. 1997;9(12):2650-7.
102. Lalive PH. Autoantibodies in inflammatory demyelinating diseases of the central nervous system. Swiss Med Wkly. 2008;138(47-48):692-707.
103. Kerlero de Rosbo N, Honegger P, Lassmann H, Matthieu JM. Demyelination induced in aggregating brain cell cultures by a monoclonal antibody against myelin/oligodendrocyte glycoprotein. J Neurochem. 1990;55(2):583-7.
104. Schluesener HJ, Sobel RA, Linington C, Weiner HL. A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. J Immunol. 1987;139(12):4016-21.
105. Breij EC, Brink BP, Veerhuis R, van den Berg C, Vloet R, Yan R, et al. Homogeneity of active demyelinating lesions in established multiple sclerosis. Ann Neurol. 2008;63(1):16-25.
106. Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. Ann Neurol. 2004;55(4):458-68.
107. Piddlesden SJ, Storch MK, Hibbs M, Freeman AM, Lassmann H, Morgan BP. Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis. J Immunol. 1994;152(11):5477-84.
108. Gaultier A, Wu X, Le Moan N, Takimoto S, Mukandala G, Akassoglou K, et al. Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. J Cell Sci. 2009;122(Pt 8):1155-62.
109. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, et al. Microglial and macrophage polarization-new prospects for brain repair. Nature reviews Neurology. 2015;11(1):56-64.
110. Huitinga I, van Rooijen N, de Groot CJ, Uitdehaag BM, Dijkstra CD. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J Exp Med. 1990;172(4):1025-33.
111. Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. Nat Neurosci. 2011;14(9):1142-9.

112. Mildner A, Schlevogt B, Kierdorf K, Bottcher C, Erny D, Kummer MP, et al. Distinct and non-redundant roles of microglia and myeloid subsets in mouse models of Alzheimer's disease. J Neurosci. 2011;31(31):11159-71.
113. Saederup N, Cardona AE, Croft K, Mizutani M, Cotleur AC, Tsou CL, et al. Selective chemokine receptor usage by central nervous system myeloid cells in CCR2-red fluorescent protein knock-in mice. PLoS One. 2010;5(10):e13693.
114. Ruckh JM, Zhao JW, Shadrach JL, van Wijngaarden P, Rao TN, Wagers AJ, et al. Rejuvenation of regeneration in the aging central nervous system. Cell Stem Cell. 2012;10(1):96-103.
115. Kotter MR, Li WW, Zhao C, Franklin RJ. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. J Neurosci. 2006;26(1):328-32.
116. GrandPre T, Nakamura F, Vartanian T, Strittmatter SM. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature. 2000;403(6768):439-44.
117. Prinz M, Tay TL, Wolf Y, Jung S. Microglia: unique and common features with other tissue macrophages. Acta Neuropathol. 2014;128(3):319-31.
118. Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguero EG, et al. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat Neurosci. 2013;16(3):273-80.
119. Henderson AP, Barnett MH, Parratt JD, Prineas JW. Multiple sclerosis: distribution of inflammatory cells in newly forming lesions. Ann Neurol. 2009;66(6):739-53.
120. Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, et al. Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Mol Cell Neurosci. 2006;31(1):149-60.
121. Huizinga R, van der Star BJ, Kipp M, Jong R, Gerritsen W, Clarner T, et al. Phagocytosis of neuronal debris by microglia is associated with neuronal damage in multiple sclerosis. Glia. 2012;60(3):422-31.
122. Nikic I, Merkler D, Sorbara C, Brinkoetter M, Kreutzfeldt M, Bareyre FM, et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat Med. 2011;17(4):495-9.
123. Bhasin JM, Chakrabarti E, Peng DQ, Kulkarni A, Chen X, Smith JD. Sex specific gene regulation and expression QTLs in mouse macrophages from a strain intercross. PloS one. 2008;3(1):e1435.
124. Goldmann T, Wieghofer P, Muller PF, Wolf Y, Varol D, Yona S, et al. A new type of microglia gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. Nat Neurosci. 2013;16(11):1618-26.
125. Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hovelmeyer N, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. Nat Med. 2005;11(2):146-52.
126. van Horssen J, Singh S, van der Pol S, Kipp M, Lim JL, Peferoen L, et al. Clusters of activated microglia in normal-appearing white matter show signs of innate immune activation. Journal of neuroinflammation. 2012;9:156.
127. van Noort JM, van den Elsen PJ, van Horssen J, Geurts JJ, van der Valk P, Amor S. Preactive multiple sclerosis lesions offer novel clues for neuroprotective therapeutic strategies. CNS Neurol Disord Drug Targets. 2011;10(1):68-81.
128. Liu Y, Hao W, Letiembre M, Walter S, Kulanga M, Neumann H, et al. Suppression of microglial inflammatory activity by myelin phagocytosis: role of p47-PHOX-mediated generation of reactive oxygen species. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006;26(50):12904-13.
129. Butovsky O, Landa G, Kunis G, Ziv Y, Avidan H, Greenberg N, et al. Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis. The Journal of clinical investigation. 2006;116(4):905-15.
130. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci. 2013;16(9):1211-8.
131. Goldmann T, Wieghofer P, Jordao MJ, Prutek F, Hagemeyer N, Frenzel K, et al. Origin, fate and dynamics of macrophages at central nervous system interfaces. Nature immunology. 2016;17(7):797-805.

132. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol. 2013;13(10):709-21.
133. Bauer J, Huitinga I, Zhao W, Lassmann H, Hickey WF, Dijkstra CD. The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis. Glia. 1995;15(4):437-46.
134. Fabriek BO, Van Haastert ES, Galea I, Polfliet MM, Dopp ED, Van Den Heuvel MM, et al. CD163-positive perivascular macrophages in the human CNS express molecules for antigen recognition and presentation. Glia. 2005;51(4):297-305.
135. Polfliet MM, van de Veerdonk F, Dopp EA, van Kesteren-Hendrikx EM, van Rooijen N, Dijkstra CD, et al. The role of perivascular and meningeal macrophages in experimental allergic encephalomyelitis. Journal of neuroimmunology. 2002;122(1-2):1-8.
136. Greter M, Heppner FL, Lemos MP, Odermatt BM, Goebels N, Laufer T, et al. Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nat Med. 2005;11(3):328-34.
137. Stadelmann C, Kerschensteiner M, Misgeld T, Bruck W, Hohlfeld R, Lassmann H. BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? Brain : a journal of neurology. 2002;125(Pt 1):75-85.
138. Vanderlocht J, Hellings N, Hendriks JJ, Vandenabeele F, Moreels M, Buntinx M, et al. Leukemia inhibitory factor is produced by myelin-reactive T cells from multiple sclerosis patients and protects against tumor necrosis factor-alpha-induced oligodendrocyte apoptosis. Journal of neuroscience research. 2006;83(5):763-74.
139. Bogie JF, Stinissen P, Hendriks JJ. Macrophage subsets and microglia in multiple sclerosis. Acta Neuropathol. 2014;128(2):191-213.
140. Vercellino M, Votta B, Condello C, Piacentino C, Romagnolo A, Merola A, et al. Involvement of the choroid plexus in multiple sclerosis autoimmune inflammation: a neuropathological study. Journal of neuroimmunology. 2008;199(1-2):133-41.
141. Engelhardt B, Wolburg-Buchholz K, Wolburg H. Involvement of the choroid plexus in central nervous system inflammation. Microsc Res Tech. 2001;52(1):112-29.
142. Brown DA, Sawchenko PE. Time course and distribution of inflammatory and neurodegenerative events suggest structural bases for the pathogenesis of experimental autoimmune encephalomyelitis. J Comp Neurol. 2007;502(2):236-60.
143. Axtell RC, Steinman L. Gaining entry to an uninflamed brain. Nature immunology. 2009;10(5):453-5.
144. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, et al. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nature immunology. 2009;10(5):514-23.
145. Shechter R, Miller O, Yovel G, Rosenzweig N, London A, Ruckh J, et al. Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus. Immunity. 2013;38(3):555-69.
146. Szmydynger-Chodobska J, Strazielle N, Gandy JR, Keefe TH, Zink BJ, Ghersi-Egea JF, et al. Posttraumatic invasion of monocytes across the blood-cerebrospinal fluid barrier. J Cereb Blood Flow Metab. 2012;32(1):93-104.
147. Dragunow M. Meningeal and choroid plexus cells--novel drug targets for CNS disorders. Brain research. 2013;1501:32-55.
148. Kivisakk P, Imitola J, Rasmussen S, Elyaman W, Zhu B, Ransohoff RM, et al. Localizing central nervous system immune surveillance: meningeal antigen-presenting cells activate T cells during experimental autoimmune encephalomyelitis. Annals of neurology. 2009;65(4):457-69.
149. Ransohoff RM, Cardona AE. The myeloid cells of the central nervous system parenchyma. Nature. 2010;468(7321):253-62.
150. Garabedian BV, Lemaigre-Dubreuil Y, Mariani J. Central origin of IL-1beta produced during peripheral inflammation: role of meninges. Brain research Molecular brain research. 2000;75(2):259-63.
151. Wu Z, Zhang J, Nakanishi H. Leptomeningeal cells activate microglia and astrocytes to induce IL-10 production by releasing pro-inflammatory cytokines during systemic inflammation. Journal of neuroimmunology. 2005;167(1-2):90-8.
152. Johansson EM, Sanabra C, Cortes R, Vilaro MT, Mengod G. Lipopolysaccharide administration in vivo induces differential expression of cAMP-specific

phosphodiesterase 4B mRNA splice variants in the mouse brain. Journal of neuroscience research. 2011;89(11):1761-72.
153. Reuter U, Bolay H, Jansen-Olesen I, Chiarugi A, Sanchez del Rio M, Letourneau R, et al. Delayed inflammation in rat meninges: implications for migraine pathophysiology. Brain : a journal of neurology. 2001;124(Pt 12):2490-502.
154. Lucchinetti CF, Popescu BF, Bunyan RF, Moll NM, Roemer SF, Lassmann H, et al. Inflammatory cortical demyelination in early multiple sclerosis. The New England journal of medicine. 2011;365(23):2188-97.
155. Wu Z, Hayashi Y, Zhang J, Nakanishi H. Involvement of prostaglandin E2 released from leptomeningeal cells in increased expression of transforming growth factor-beta in glial cells and cortical neurons during systemic inflammation. Journal of neuroscience research. 2007;85(1):184-92.
156. Olson RE. Discovery of the lipoproteins, their role in fat transport and their significance as risk factors. The Journal of nutrition. 1998;128(2 Suppl):439S-43S.
157. Hanukoglu I. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. The Journal of steroid biochemistry and molecular biology. 1992;43(8):779-804.
158. Berg JM. The Biosynthesis of Membrane Lipids and Steroids. Biochemistry 5th edition Chapter 26. 2002:New York: W.H. Freeman and Company.
159. Nieweg K, Schaller H, Pfrieger FW. Marked differences in cholesterol synthesis between neurons and glial cells from postnatal rats. J Neurochem. 2009;109(1):125-34.
160. Berg JM. Biochemistry. 2002(5th ed.).
161. Feingold KR, Grunfeld C. Introduction to Lipids and Lipoproteins. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.
162. Annema W, Tietge UJ. Regulation of reverse cholesterol transport - a comprehensive appraisal of available animal studies. Nutr Metab (Lond). 2012;9(1):25.
163. Wang X, Collins HL, Ranalletta M, Fuki IV, Billheimer JT, Rothblat GH, et al. Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo. The Journal of clinical investigation. 2007;117(8):2216-24.
164. Vitali C, Wellington CL, Calabresi L. HDL and cholesterol handling in the brain. Cardiovasc Res. 2014;103(3):405-13.
165. Koch S, Donarski N, Goetze K, Kreckel M, Stuerenburg HJ, Buhmann C, et al. Characterization of four lipoprotein classes in human cerebrospinal fluid. J Lipid Res. 2001;42(7):1143-51.
166. Camargo N, Goudriaan A, van Deijk AF, Otte WM, Brouwers JF, Lodder H, et al. Oligodendroglial myelination requires astrocyte-derived lipids. PLoS Biol. 2017;15(5):e1002605.
167. Kim WS, Weickert CS, Garner B. Role of ATP-binding cassette transporters in brain lipid transport and neurological disease. J Neurochem. 2008;104(5):1145-66.
168. Zhao S, Hu X, Park J, Zhu Y, Zhu Q, Li H, et al. Selective expression of LDLR and VLDLR in myelinating oligodendrocytes. Dev Dyn. 2007;236(9):2708-12.
169. Eisenberg S. High density lipoprotein metabolism. Journal of lipid research. 1984;25(10):1017-58.
170. Arsenault BJ, Lemieux I, Despres JP, Wareham NJ, Stroes ES, Kastelein JJ, et al. Comparison between gradient gel electrophoresis and nuclear magnetic resonance spectroscopy in estimating coronary heart disease risk associated with LDL and HDL particle size. Clinical chemistry. 2010;56(5):789-98.
171. Castro GR, Fielding CJ. Early incorporation of cell-derived cholesterol into pre-beta-migrating high-density lipoprotein. Biochemistry. 1988;27(1):25-9.
172. Rigotti A, Trigatti B, Babitt J, Penman M, Xu S, Krieger M. Scavenger receptor BI--a cell surface receptor for high density lipoprotein. Current opinion in lipidology. 1997;8(3):181-8.
173. Williamson R, Lee D, Hagaman J, Maeda N. Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(15):7134-8.
174. Schmitz G, Robenek H, Lohmann U, Assmann G. Interaction of high density lipoproteins with cholesteryl ester-laden macrophages: biochemical and morphological

characterization of cell surface receptor binding, endocytosis and resecretion of high density lipoproteins by macrophages. The EMBO journal. 1985;4(3):613-22.
175. Choi HY, Karten B, Chan T, Vance JE, Greer WL, Heidenreich RA, et al. Impaired ABCA1-dependent lipid efflux and hypoalphalipoproteinemia in human Niemann-Pick type C disease. The Journal of biological chemistry. 2003;278(35):32569-77.
176. Jauhiainen M, Dolphin PJ. Human plasma lecithin-cholesterol acyltransferase. An elucidation of the catalytic mechanism. The Journal of biological chemistry. 1986;261(15):7032-43.
177. Francone OL, Haghpassand M, Bennett JA, Royer L, McNeish J. Expression of human lecithin:cholesterol acyltransferase in transgenic mice: effects on cholesterol efflux, esterification, and transport. Journal of lipid research. 1997;38(4):813-22.
178. Fielding CJ. The origin and properties of free cholesterol potential gradients in plasma, and their relation to atherogenesis. Journal of lipid research. 1984;25(13):1624-8.
179. Brown MS, Goldstein JL. Receptor-mediated control of cholesterol metabolism. Science. 1976;191(4223):150-4.
180. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. The New England journal of medicine. 1989;320(14):915-24.
181. Lusis AJ. Atherosclerosis. Nature. 2000;407(6801):233-41.
182. Chapman MJ, Guerin M, Bruckert E. Atherogenic, dense low-density lipoproteins. Pathophysiology and new therapeutic approaches. European heart journal. 1998;19 Suppl A:A24-30.
183. McNamara JR, Small DM, Li Z, Schaefer EJ. Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B. Journal of lipid research. 1996;37(9):1924-35.
184. Jorissen W, Wouters E, Bogie JF, Vanmierlo T, Noben JP, Sviridov D, et al. Relapsing-remitting multiple sclerosis patients display an altered lipoprotein profile with dysfunctional HDL. Sci Rep. 2017;7:43410.
185. de Graaf J, Hak-Lemmers HL, Hectors MP, Demacker PN, Hendriks JC, Stalenhoef AF. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. Arterioscler Thromb. 1991;11(2):298-306.
186. Chancharme L, Therond P, Nigon F, Lepage S, Couturier M, Chapman MJ. Cholesteryl ester hydroperoxide lability is a key feature of the oxidative susceptibility of small, dense LDL. Arterioscler Thromb Vasc Biol. 1999;19(3):810-20.
187. Berneis KK, Krauss RM. Metabolic origins and clinical significance of LDL heterogeneity. Journal of lipid research. 2002;43(9):1363-79.
188. Goedeke L, Fernandez-Hernando C. Regulation of cholesterol homeostasis. Cell Mol Life Sci. 2012;69(6):915-30.
189. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell. 1997;89(3):331-40.
190. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature. 1990;343(6257):425-30.
191. Sakakura Y, Shimano H, Sone H, Takahashi A, Inoue N, Toyoshima H, et al. Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis. Biochemical and biophysical research communications. 2001;286(1):176-83.
192. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science. 1986;232(4746):34-47.
193. Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat. 1992;1(6):445-66.
194. Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. Nat Immunol. 2013;14(8):812-20.
195. Triantafilou M, Miyake K, Golenbock DT, Triantafilou K. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci. 2002;115(Pt 12):2603-11.
196. Swirski FK, Nahrendorf M. Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure. Science. 2013;339(6116):161-6.

197. Tolani S, Pagler TA, Murphy AJ, Bochem AE, Abramowicz S, Welch C, et al. Hypercholesterolemia and reduced HDL-C promote hematopoietic stem cell proliferation and monocytosis: studies in mice and FH children. Atherosclerosis. 2013;229(1):79-85.
198. Feng Y, Schouteden S, Geenens R, Van Duppen V, Herijgers P, Holvoet P, et al. Hematopoietic stem/progenitor cell proliferation and differentiation is differentially regulated by high-density and low-density lipoproteins in mice. PLoS One. 2012;7(11):e47286.
199. Moore KJ, Rayner KJ, Suarez Y, Fernandez-Hernando C. microRNAs and cholesterol metabolism. Trends Endocrinol Metab. 2010;21(12):699-706.
200. Gent J, Braakman I. Low-density lipoprotein receptor structure and folding. Cell Mol Life Sci. 2004;61(19-20):2461-70.
201. Ho YK, Brown MS, Goldstein JL. Hydrolysis and excretion of cytoplasmic cholesteryl esters by macrophages: stimulation by high density lipoprotein and other agents. Journal of lipid research. 1980;21(4):391-8.
202. Aviram M. Interaction of oxidized low density lipoprotein with macrophages in atherosclerosis, and the antiatherogenicity of antioxidants. Eur J Clin Chem Clin Biochem. 1996;34(8):599-608.
203. Bogie JF, Timmermans S, Huynh-Thu VA, Irrthum A, Smeets HJ, Gustafsson JA, et al. Myelin-derived lipids modulate macrophage activity by liver x receptor activation. PLoS One. 2012;7(9):e44998.
204. Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes & development. 2000;14(22):2819-30.
205. van de Bovenkamp P, Kosmeijer-Schuil TG, Katan MB. Quantification of oxysterols in Dutch foods: egg products and mixed diets. Lipids. 1988;23(11):1079-85.
206. Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. The Journal of clinical investigation. 1991;88(6):1785-92.
207. Valenzuela A, Sanhueza J, Nieto S. Cholesterol oxidation: health hazard and the role of antioxidants in prevention. Biol Res. 2003;36(3-4):291-302.
208. Sevanian A, Hodis HN, Hwang J, McLeod LL, Peterson H. Characterization of endothelial cell injury by cholesterol oxidation products found in oxidized LDL. Journal of lipid research. 1995;36(9):1971-86.
209. Brown AJ, Dean RT, Jessup W. Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. Journal of lipid research. 1996;37(2):320-35.
210. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, et al. Role of LXRs in control of lipogenesis. Genes & development. 2000;14(22):2831-8.
211. Collins JL, Fivush AM, Watson MA, Galardi CM, Lewis MC, Moore LB, et al. Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. Journal of medicinal chemistry. 2002;45(10):1963-6.
212. Loren J, Huang Z, Laffitte BA, Molteni V. Liver X receptor modulators: a review of recently patented compounds (2009 - 2012). Expert Opin Ther Pat. 2013;23(10):1317-35.
213. Tontonoz P, Mangelsdorf DJ. Liver X receptor signaling pathways in cardiovascular disease. Molecular endocrinology. 2003;17(6):985-93.
214. Attie AD, Kastelein JP, Hayden MR. Pivotal role of ABCA1 in reverse cholesterol transport influencing HDL levels and susceptibility to atherosclerosis. Journal of lipid research. 2001;42(11):1717-26.
215. Yu L, Hammer RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC, et al. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(25):16237-42.
216. Ghisletti S, Huang W, Ogawa S, Pascual G, Lin ME, Willson TM, et al. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. Mol Cell. 2007;25(1):57-70.
217. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat Med. 2003;9(2):213-9.

218. Huang W, Ghisletti S, Saijo K, Gandhi M, Aouadi M, Tesz GJ, et al. Coronin 2A mediates actin-dependent de-repression of inflammatory response genes. Nature. 2011;470(7334):414-8.
219. Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, et al. Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice. The Journal of clinical investigation. 2007;117(12):3900-8.
220. Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, Lamkanfi M, Han S, et al. Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. Circulation. 2008;118(18):1837-47.
221. Murphy AJ, Akhtari M, Tolani S, Pagler T, Bijl N, Kuo CL, et al. ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. The Journal of clinical investigation. 2011;121(10):4138-49.
222. Li P, Spann NJ, Kaikkonen MU, Lu M, Oh DY, Fox JN, et al. NCoR repression of LXRs restricts macrophage biosynthesis of insulin-sensitizing omega 3 fatty acids. Cell. 2013;155(1):200-14.
223. Rong X, Albert CJ, Hong C, Duerr MA, Chamberlain BT, Tarling EJ, et al. LXRs regulate ER stress and inflammation through dynamic modulation of membrane phospholipid composition. Cell Metab. 2013;18(5):685-97.
224. Gerbod-Giannone MC, Li Y, Holleboom A, Han S, Hsu LC, Tabas I, et al. TNFalpha induces ABCA1 through NF-kappaB in macrophages and in phagocytes ingesting apoptotic cells. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(9):3112-7.
225. N AG, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. Immunity. 2009;31(2):245-58.
226. Bjorkhem I, Meaney S. Brain cholesterol: long secret life behind a barrier. Arterioscler Thromb Vasc Biol. 2004;24(5):806-15.
227. Curtiss LK, Boisvert WA. Apolipoprotein E and atherosclerosis. Current opinion in lipidology. 2000;11(3):243-51.
228. Kockx M, Jessup W, Kritharides L. Regulation of endogenous apolipoprotein E secretion by macrophages. Arterioscler Thromb Vasc Biol. 2008;28(6):1060-7.
229. Tenger C, Zhou X. Apolipoprotein E modulates immune activation by acting on the antigen-presenting cell. Immunology. 2003;109(3):392-7.
230. Baitsch D, Bock HH, Engel T, Telgmann R, Muller-Tidow C, Varga G, et al. Apolipoprotein E induces antiinflammatory phenotype in macrophages. Arterioscler Thromb Vasc Biol. 2011;31(5):1160-8.
231. Grainger DJ, Reckless J, McKilligin E. Apolipoprotein E modulates clearance of apoptotic bodies in vitro and in vivo, resulting in a systemic proinflammatory state in apolipoprotein E-deficient mice. J Immunol. 2004;173(10):6366-75.
232. Ali K, Middleton M, Pure E, Rader DJ. Apolipoprotein E suppresses the type I inflammatory response in vivo. Circ Res. 2005;97(9):922-7.
233. Zhong N, Weisgraber KH. Understanding the association of apolipoprotein E4 with Alzheimer disease: clues from its structure. The Journal of biological chemistry. 2009;284(10):6027-31.
234. Hatters DM, Peters-Libeu CA, Weisgraber KH. Apolipoprotein E structure: insights into function. Trends Biochem Sci. 2006;31(8):445-54.
235. Zhong N, Ramaswamy G, Weisgraber KH. Apolipoprotein E4 domain interaction induces endoplasmic reticulum stress and impairs astrocyte function. The Journal of biological chemistry. 2009;284(40):27273-80.
236. Liu CC, Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nature reviews Neurology. 2013;9(2):106-18.
237. Williams DL, Dawson PA, Newman TC, Rudel LL. Apolipoprotein E synthesis in peripheral tissues of nonhuman primates. The Journal of biological chemistry. 1985;260(4):2444-51.
238. Zechner R, Moser R, Newman TC, Fried SK, Breslow JL. Apolipoprotein E gene expression in mouse 3T3-L1 adipocytes and human adipose tissue and its regulation

by differentiation and lipid content. The Journal of biological chemistry. 1991;266(16):10583-8.
239. Werb Z, Chin JR, Takemura R, Oropeza RL, Bainton DF, Stenberg P, et al. The cell and molecular biology of apolipoprotein E synthesis by macrophages. Ciba Found Symp. 1986;118:155-71.
240. Gafencu AV, Robciuc MR, Fuior E, Zannis VI, Kardassis D, Simionescu M. Inflammatory signaling pathways regulating ApoE gene expression in macrophages. The Journal of biological chemistry. 2007;282(30):21776-85.
241. Galetto R, Albajar M, Polanco JI, Zakin MM, Rodriguez-Rey JC. Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region. The Biochemical journal. 2001;357(Pt 2):521-7.
242. Laffitte BA, Repa JJ, Joseph SB, Wilpitz DC, Kast HR, Mangelsdorf DJ, et al. LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(2):507-12.
243. Huwait EA, Greenow KR, Singh NN, Ramji DP. A novel role for c-Jun N-terminal kinase and phosphoinositide 3-kinase in the liver X receptor-mediated induction of macrophage gene expression. Cell Signal. 2011;23(3):542-9.
244. Kang J, Rivest S. Lipid metabolism and neuroinflammation in Alzheimer's disease: a role for liver X receptors. Endocr Rev. 2012;33(5):715-46.
245. Yvan-Charvet L, Wang N, Tall AR. Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. Arterioscler Thromb Vasc Biol. 2010;30(2):139-43.
246. Vance JE, Hayashi H. Formation and function of apolipoprotein E-containing lipoproteins in the nervous system. Biochim Biophys Acta. 2010;1801(8):806-18.
247. Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, et al. CNS synaptogenesis promoted by glia-derived cholesterol. Science. 2001;294(5545):1354-7.
248. Tabernero A, Velasco A, Granda B, Lavado EM, Medina JM. Transcytosis of albumin in astrocytes activates the sterol regulatory element-binding protein-1, which promotes the synthesis of the neurotrophic factor oleic acid. J Biol Chem. 2002;277(6):4240-6.
249. Pfrieger FW, Ungerer N. Cholesterol metabolism in neurons and astrocytes. Prog Lipid Res. 2011;50(4):357-71.
250. Bales KR. Brain lipid metabolism, apolipoprotein E and the pathophysiology of Alzheimer's disease. Neuropharmacology. 2010;59(4-5):295-302.
251. Whitney KD, Watson MA, Collins JL, Benson WG, Stone TM, Numerick MJ, et al. Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. Molecular endocrinology. 2002;16(6):1378-85.
252. Pfrieger FW. Outsourcing in the brain: do neurons depend on cholesterol delivery by astrocytes? Bioessays. 2003;25(1):72-8.
253. Wang L, Schuster GU, Hultenby K, Zhang Q, Andersson S, Gustafsson JA. Liver X receptors in the central nervous system: from lipid homeostasis to neuronal degeneration. Proc Natl Acad Sci U S A. 2002;99(21):13878-83.
254. van de Kraats C, Killestein J, Popescu V, Rijkers E, Vrenken H, Lutjohann D, et al. Oxysterols and cholesterol precursors correlate to magnetic resonance imaging measures of neurodegeneration in multiple sclerosis. Mult Scler. 2014;20(4):412-7.
255. Zhornitsky S, McKay KA, Metz LM, Teunissen CE, Rangachari M. Cholesterol and markers of cholesterol turnover in multiple sclerosis: relationship with disease outcomes. Mult Scler Relat Disord. 2016;5:53-65.
256. Vuletic S, Kennedy H, Albers JJ, Killestein J, Vrenken H, Lutjohann D, et al. Cerebrospinal fluid apolipoprotein E and phospholipid transfer protein activity are reduced in multiple sclerosis; relationships with the brain MRI and CSF lipid variables. Mult Scler Relat Disord. 2014;3(4):533-41.
257. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. Nat Rev Immunol. 2015;15(2):104-16.
258. Lefterov I, Bookout A, Wang Z, Staufenbiel M, Mangelsdorf D, Koldamova R. Expression profiling in APP23 mouse brain: inhibition of Abeta amyloidosis and inflammation in response to LXR agonist treatment. Mol Neurodegener. 2007;2:20.

259. Hijmans BS, Tiemann CA, Grefhorst A, Boesjes M, van Dijk TH, Tietge UJ, et al. A systems biology approach reveals the physiological origin of hepatic steatosis induced by liver X receptor activation. FASEB J. 2015;29(4):1153-64.
260. Akhisa T, Kokke W, Tamura T. Naturally occurring sterols and related compounds from plants. Nes GWPaWD, editor. Champaign, Illinois: American Oil Chemists' Society.; 1991.
261. Yang C, Yu L, Li W, Xu F, Cohen JC, Hobbs HH. Disruption of cholesterol homeostasis by plant sterols. The Journal of clinical investigation. 2004;114(6):813-22.
262. Chen Z, Liu J, Fu Z, Ye C, Zhang R, Song Y, et al. 24(S)-Saringosterol from edible marine seaweed Sargassum fusiforme is a novel selective LXRbeta agonist. J Agric Food Chem. 2014;62(26):6130-7.
263. Plat J, Nichols JA, Mensink RP. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. Journal of lipid research. 2005;46(11):2468-76.
264. Hoang MH, Jia Y, Jun HJ, Lee JH, Lee BY, Lee SJ. Fucosterol is a selective liver X receptor modulator that regulates the expression of key genes in cholesterol homeostasis in macrophages, hepatocytes, and intestinal cells. Journal of agricultural and food chemistry. 2012;60(46):11567-75.
265. Kaneko E, Matsuda M, Yamada Y, Tachibana Y, Shimomura I, Makishima M. Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. J Biol Chem. 2003;278(38):36091-8.
266. El Kharrassi Y, Samadi M, Lopez T, Nury T, El Kebbaj R, Andreoletti P, et al. Biological activities of Schottenol and Spinasterol, two natural phytosterols present in argan oil and in cactus pear seed oil, on murine miroglial BV2 cells. Biochemical and biophysical research communications. 2014;446(3):798-804.
267. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, et al. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. The Journal of biological chemistry. 2002;277(37):34182-90.
268. Wilund KR, Yu L, Xu F, Hobbs HH, Cohen JC. High-level expression of ABCG5 and ABCG8 attenuates diet-induced hypercholesterolemia and atherosclerosis in Ldlr-/- mice. Journal of lipid research. 2004;45(8):1429-36.
269. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. The Journal of biological chemistry. 2002;277(21):18793-800.
270. McDaniel AL, Alger HM, Sawyer JK, Kelley KL, Kock ND, Brown JM, et al. Phytosterol feeding causes toxicity in ABCG5/G8 knockout mice. The American journal of pathology. 2013;182(4):1131-8.
271. Plat J, Hendrikx T, Bieghs V, Jeurissen ML, Walenbergh SM, van Gorp PJ, et al. Protective role of plant sterol and stanol esters in liver inflammation: insights from mice and humans. PloS one. 2014;9(10):e110758.
272. Klingberg S, Andersson H, Mulligan A, Bhaniani A, Welch A, Bingham S, et al. Food sources of plant sterols in the EPIC Norfolk population. European journal of clinical nutrition. 2008;62(6):695-703.
273. Valsta LM, Lemstrom A, Ovaskainen ML, Lampi AM, Toivo J, Korhonen T, et al. Estimation of plant sterol and cholesterol intake in Finland: quality of new values and their effect on intake. The British journal of nutrition. 2004;92(4):671-8.
274. AbuMweis SS, Vanstone CA, Lichtenstein AH, Jones PJ. Plant sterol consumption frequency affects plasma lipid levels and cholesterol kinetics in humans. European journal of clinical nutrition. 2009;63(6):747-55.
275. Gylling H, Plat J, Turley S, Ginsberg HN, Ellegard L, Jessup W, et al. Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. Atherosclerosis. 2014;232(2):346-60.
276. Park SW. Intestinal and hepatic niemann-pick c1-like 1. Diabetes & metabolism journal. 2013;37(4):240-8.
277. Lin DS, Steiner RD, Merkens LS, Pappu AS, Connor WE. The effects of sterol structure upon sterol esterification. Atherosclerosis. 2010;208(1):155-60.

278. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science. 2000;290(5497):1771-5.
279. Klett EL, Patel S. Genetic defenses against noncholesterol sterols. Current opinion in lipidology. 2003;14(4):341-5.
280. Lee MH, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, et al. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. Nature genetics. 2001;27(1):79-83.
281. Saeed AA, Genove G, Li T, Luetjohann D, Olin M, Mast N, et al. Effects of a disrupted blood-brain barrier on cholesterol homeostasis in the brain. The Journal of biological chemistry. 2014.
282. Vanmierlo T, Weingartner O, van der Pol S, Husche C, Kerksiek A, Friedrichs S, et al. Dietary intake of plant sterols stably increases plant sterol levels in the murine brain. Journal of lipid research. 2012;53(4):726-35.
283. Smiljanic K, Vanmierlo T, Djordjevic AM, Perovic M, Loncarevic-Vasiljkovic N, Tesic V, et al. Aging induces tissue-specific changes in cholesterol metabolism in rat brain and liver. Lipids. 2013;48(11):1069-77.
284. Jansen PJ, Lutjohann D, Abildayeva K, Vanmierlo T, Plosch T, Plat J, et al. Dietary plant sterols accumulate in the brain. Biochim Biophys Acta. 2006.
285. Panzenboeck U, Balazs Z, Sovic A, Hrzenjak A, Levak-Frank S, Wintersperger A, et al. ABCA1 and scavenger receptor class B, type I, are modulators of reverse sterol transport at an in vitro blood-brain barrier constituted of porcine brain capillary endothelial cells. The Journal of biological chemistry. 2002;277(45):42781-9.
286. Fransen HP, de Jong N, Wolfs M, Verhagen H, Verschuren WM, Lutjohann D, et al. Customary use of plant sterol and plant stanol enriched margarine is associated with changes in serum plant sterol and stanol concentrations in humans. The Journal of nutrition. 2007;137(5):1301-6.
287. Vanmierlo T, Popp J, Kolsch H, Friedrichs S, Jessen F, Stoffel-Wagner B, et al. The plant sterol brassicasterol as additional CSF biomarker in Alzheimer's disease. Acta psychiatrica Scandinavica. 2011;124(3):184-92.
288. Weingartner O, Lutjohann D, Ji S, Weisshoff N, List F, Sudhop T, et al. Vascular effects of diet supplementation with plant sterols. Journal of the American College of Cardiology. 2008;51(16):1553-61.
289. Shafaati M, Marutle A, Pettersson H, Lovgren-Sandblom A, Olin M, Pikuleva I, et al. Marked accumulation of 27-hydroxycholesterol in the brains of Alzheimer's patients with the Swedish APP 670/671 mutation. Journal of lipid research. 2011;52(5):1004-10.
290. Popp J, Meichsner S, Kolsch H, Lewczuk P, Maier W, Kornhuber J, et al. Cerebral and extracerebral cholesterol metabolism and CSF markers of Alzheimer's disease. Biochemical pharmacology. 2013;86(1):37-42.
291. Simons K, Vaz WL. Model systems, lipid rafts, and cell membranes. Annual review of biophysics and biomolecular structure. 2004;33:269-95.
292. Pike LJ. Lipid rafts: heterogeneity on the high seas. The Biochemical journal. 2004;378(Pt 2):281-92.
293. Ledesma MD, Dotti CG. Amyloid excess in Alzheimer's disease: what is cholesterol to be blamed for? FEBS letters. 2006;580(23):5525-32.
294. Ahmed SN, Brown DA, London E. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. Biochemistry. 1997;36(36):10944-53.
295. Baumgart T, Hess ST, Webb WW. Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension. Nature. 2003;425(6960):821-4.
296. Edidin M. The state of lipid rafts: from model membranes to cells. Annual review of biophysics and biomolecular structure. 2003;32:257-83.
297. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997;387(6633):569-72.
298. Hac-Wydro K, Wydro P, Dynarowicz-Latka P, Paluch M. Cholesterol and phytosterols effect on sphingomyelin/phosphatidylcholine model membranes--thermodynamic

analysis of the interactions in ternary monolayers. Journal of colloid and interface science. 2009;329(2):265-72.
299. Xu X, Bittman R, Duportail G, Heissler D, Vilcheze C, London E. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. The Journal of biological chemistry. 2001;276(36):33540-6.
300. Shi C, Wu F, Xu J. Incorporation of beta-sitosterol into mitochondrial membrane enhances mitochondrial function by promoting inner mitochondrial membrane fluidity. Journal of bioenergetics and biomembranes. 2013;45(3):301-5.
301. Saher G, Brugger B, Lappe-Siefke C, Mobius W, Tozawa R, Wehr MC, et al. High cholesterol level is essential for myelin membrane growth. Nature neuroscience. 2005;8(4):468-75.
302. Xiang Z, Reeves SA. Simvastatin induces cell death in a mouse cerebellar slice culture (CSC) model of developmental myelination. Experimental neurology. 2009;215(1):41-7.
303. Weinstock-Guttman B, Zivadinov R, Mahfooz N, Carl E, Drake A, Schneider J, et al. Serum lipid profiles are associated with disability and MRI outcomes in multiple sclerosis. Journal of neuroinflammation. 2011;8:127.
304. Nelissen K, Mulder M, Smets I, Timmermans S, Smeets K, Ameloot M, et al. Liver X receptors regulate cholesterol homeostasis in oligodendrocytes. Journal of neuroscience research. 2012;90(1):60-71.
305. van de Kraats C, Killestein J, Popescu V, Rijkers E, Vrenken H, Lutjohann D, et al. Oxysterols and cholesterol precursors correlate to magnetic resonance imaging measures of neurodegeneration in multiple sclerosis. Multiple sclerosis. 2014;20(4):412-7.
306. Teunissen CE, Floris S, Sonke M, Dijkstra CD, De Vries HE, Lutjohann D. 24S-hydroxycholesterol in relation to disease manifestations of acute experimental autoimmune encephalomyelitis. Journal of neuroscience research. 2007;85(7):1499-505.
307. Vollmer T, Key L, Durkalski V, Tyor W, Corboy J, Markovic-Plese S, et al. Oral simvastatin treatment in relapsing-remitting multiple sclerosis. Lancet. 2004;363(9421):1607-8.
308. Togha M, Karvigh SA, Nabavi M, Moghadam NB, Harirchian MH, Sahraian MA, et al. Simvastatin treatment in patients with relapsing-remitting multiple sclerosis receiving interferon beta 1a: a double-blind randomized controlled trial. Multiple sclerosis. 2010;16(7):848-54.
309. Chataway J, Schuerer N, Alsanousi A, Chan D, Macmanus D, Hunter K, et al. Effect of high-dose simvastatin on brain atrophy and disability in secondary progressive multiple sclerosis (MS-STAT): a randomised, placebo-controlled, phase 2 trial. Lancet. 2014.
310. Bouic PJ, Lamprecht JH. Plant sterols and sterolins: a review of their immune-modulating properties. Alternative medicine review : a journal of clinical therapeutic. 1999;4(3):170-7.
311. Brull F, Mensink RP, van den Hurk K, Duijvestijn A, Plat J. TLR2 activation is essential to induce a Th1 shift in human peripheral blood mononuclear cells by plant stanols and plant sterols. The Journal of biological chemistry. 2010;285(5):2951-8.
312. Choi JN, Choi YH, Lee JM, Noh IC, Park JW, Choi WS, et al. Anti-inflammatory effects of beta-sitosterol-beta-D-glucoside from Trachelospermum jasminoides (Apocynaceae) in lipopolysaccharide-stimulated RAW 264.7 murine macrophages. Natural product research. 2012;26(24):2340-3.
313. Desai F, Ramanathan M, Fink CS, Wilding GE, Weinstock-Guttman B, Awad AB. Comparison of the immunomodulatory effects of the plant sterol beta-sitosterol to simvastatin in peripheral blood cells from multiple sclerosis patients. International immunopharmacology. 2009;9(1):153-7.
314. Loizou S, Lekakis I, Chrousos GP, Moutsatsou P. Beta-sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. Molecular nutrition & food research. 2010;54(4):551-8.

315. Valerio M, Awad AB. beta-Sitosterol down-regulates some pro-inflammatory signal transduction pathways by increasing the activity of tyrosine phosphatase SHP-1 in J774A.1 murine macrophages. International immunopharmacology. 2011;11(8):1012-7.
316. Valerio MS, Minderman H, Mace T, Awad AB. beta-Sitosterol modulates TLR4 receptor expression and intracellular MyD88-dependent pathway activation in J774A.1 murine macrophages. Cellular immunology. 2013;285(1-2):76-83.
317. Yoo MS, Shin JS, Choi HE, Cho YW, Bang MH, Baek NI, et al. Fucosterol isolated from Undaria pinnatifida inhibits lipopolysaccharide-induced production of nitric oxide and pro-inflammatory cytokines via the inactivation of nuclear factor-kappaB and p38 mitogen-activated protein kinase in RAW264.7 macrophages. Food chemistry. 2012;135(3):967-75.
318. Kim KA, Lee IA, Gu W, Hyam SR, Kim DH. beta-Sitosterol attenuates high-fat diet-induced intestinal inflammation in mice by inhibiting the binding of lipopolysaccharide to toll-like receptor 4 in the NF-kappaB pathway. Molecular nutrition & food research. 2014;58(5):963-72.
319. Awad AB, Toczek J, Fink CS. Phytosterols decrease prostaglandin release in cultured P388D1/MAB macrophages. Prostaglandins, leukotrienes, and essential fatty acids. 2004;70(6):511-20.
320. Moreno JJ. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. Free radical biology & medicine. 2003;35(9):1073-81.
321. Sabeva NS, McPhaul CM, Li X, Cory TJ, Feola DJ, Graf GA. Phytosterols differentially influence ABC transporter expression, cholesterol efflux and inflammatory cytokine secretion in macrophage foam cells. The Journal of nutritional biochemistry. 2011;22(8):777-83.
322. Calpe-Berdiel L, Escola-Gil JC, Benitez S, Bancells C, Gonzalez-Sastre F, Palomer X, et al. Dietary phytosterols modulate T-helper immune response but do not induce apparent anti-inflammatory effects in a mouse model of acute, aseptic inflammation. Life sciences. 2007;80(21):1951-6.
323. Breytenbach U, Clark A, Lamprecht J, Bouic P. Flow cytometric analysis of the Th1-Th2 balance in healthy individuals and patients infected with the human immunodeficiency virus (HIV) receiving a plant sterol/sterolin mixture. Cell biology international. 2001;25(1):43-9.
324. Valerio M, Liu HB, Heffner R, Zivadinov R, Ramanathan M, Weinstock-Guttman B, et al. Phytosterols ameliorate clinical manifestations and inflammation in experimental autoimmune encephalomyelitis. Inflammation research : official journal of the European Histamine Research Society [et al]. 2011;60(5):457-65.
325. Fricke CB, Schroder M, Poulsen M, von Bergmann K, Wester I, Knudsen I, et al. Increased plant sterol and stanol levels in brain of Watanabe rabbits fed rapeseed oil derived plant sterol or stanol esters. The British journal of nutrition. 2007;98(5):890-9.
326. Shi C, Wu F, Zhu XC, Xu J. Incorporation of beta-sitosterol into the membrane increases resistance to oxidative stress and lipid peroxidation via estrogen receptor-mediated PI3K/GSK3beta signaling. Biochim Biophys Acta. 2013;1830(3):2538-44.
327. Adcox C, Boyd L, Oehrl L, Allen J, Fenner G. Comparative effects of phytosterol oxides and cholesterol oxides in cultured macrophage-derived cell lines. Journal of agricultural and food chemistry. 2001;49(4):2090-5.
328. Khabazian I, Bains JS, Williams DE, Cheung J, Wilson JM, Pasqualotto BA, et al. Isolation of various forms of sterol beta-D-glucoside from the seed of Cycas circinalis: neurotoxicity and implications for ALS-parkinsonism dementia complex. Journal of neurochemistry. 2002;82(3):516-28.
329. Tabata RC, Wilson JM, Ly P, Zwiegers P, Kwok D, Van Kampen JM, et al. Chronic exposure to dietary sterol glucosides is neurotoxic to motor neurons and induces an ALS-PDC phenotype. Neuromolecular medicine. 2008;10(1):24-39.
330. Panov A, Kubalik N, Brooks BR, Shaw CA. In vitro effects of cholesterol beta-D-glucoside, cholesterol and cycad phytosterol glucosides on respiration and reactive

oxygen species generation in brain mitochondria. The Journal of membrane biology. 2010;237(2-3):71-7.
331. Lin X, Ma L, Racette SB, Anderson Spearie CL, Ostlund RE, Jr. Phytosterol glycosides reduce cholesterol absorption in humans. American journal of physiology Gastrointestinal and liver physiology. 2009;296(4):G931-5.
332. Lin X, Ma L, Moreau RA, Ostlund RE, Jr. Glycosidic bond cleavage is not required for phytosteryl glycoside-induced reduction of cholesterol absorption in mice. Lipids. 2011;46(8):701-8.
333. Cui G, Qin X, Wu L, Zhang Y, Sheng X, Yu Q, et al. Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation. The Journal of clinical investigation. 2011;121(2):658-70.
334. Hindinger C, Hinton DR, Kirwin SJ, Atkinson RD, Burnett ME, Bergmann CC, et al. Liver X receptor activation decreases the severity of experimental autoimmune encephalomyelitis. Journal of neuroscience research. 2006;84(6):1225-34.
335. Huang JK, Jarjour AA, Nait Oumesmar B, Kerninon C, Williams A, Krezel W, et al. Retinoid X receptor gamma signaling accelerates CNS remyelination. Nature neuroscience. 2011;14(1):45-53.
336. Gutendorf B, Westendorf J. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology. 2001;166(1-2):79-89.
337. Vivancos M, Moreno JJ. beta-Sitosterol modulates antioxidant enzyme response in RAW 264.7 macrophages. Free radical biology & medicine. 2005;39(1):91-7.
338. Gold SM, Voskuhl RR. Estrogen treatment in multiple sclerosis. Journal of the neurological sciences. 2009;286(1-2):99-103.
339. Spence RD, Voskuhl RR. Neuroprotective effects of estrogens and androgens in CNS inflammation and neurodegeneration. Frontiers in neuroendocrinology. 2012;33(1):105-15.
340. Crawford DK, Mangiardi M, Song B, Patel R, Du S, Sofroniew MV, et al. Oestrogen receptor beta ligand: a novel treatment to enhance endogenous functional remyelination. Brain : a journal of neurology. 2010;133(10):2999-3016.
341. Tiwari-Woodruff S, Morales LB, Lee R, Voskuhl RR. Differential neuroprotective and antiinflammatory effects of estrogen receptor (ER)alpha and ERbeta ligand treatment. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(37):14813-8.
342. Emery B. Regulation of oligodendrocyte differentiation and myelination. Science. 2010;330(6005):779-82.
343. Spassky N, Olivier C, Cobos I, LeBras B, Goujet-Zalc C, Martinez S, et al. The early steps of oligodendrogenesis: insights from the study of the plp lineage in the brain of chicks and rodents. Dev Neurosci. 2001;23(4-5):318-26.
344. Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nature neuroscience. 2006;9(2):173-9.
345. Dawson MR, Polito A, Levine JM, Reynolds R. NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Molecular and cellular neurosciences. 2003;24(2):476-88.
346. Fancy SP, Zhao C, Franklin RJ. Increased expression of Nkx2.2 and Olig2 identifies reactive oligodendrocyte progenitor cells responding to demyelination in the adult CNS. Molecular and cellular neurosciences. 2004;27(3):247-54.
347. Chang A, Staugaitis SM, Dutta R, Batt CE, Easley KE, Chomyk AM, et al. Cortical remyelination: a new target for repair therapies in multiple sclerosis. Ann Neurol. 2012;72(6):918-26.
348. de Castro F, Bribian A. The molecular orchestra of the migration of oligodendrocyte precursors during development. Brain research Brain research reviews. 2005;49(2):227-41.
349. Freeman MR, Rowitch DH. Evolving concepts of gliogenesis: a look way back and ahead to the next 25 years. Neuron. 2013;80(3):613-23.
350. Chong SY, Rosenberg SS, Fancy SP, Zhao C, Shen YA, Hahn AT, et al. Neurite outgrowth inhibitor Nogo-A establishes spatial segregation and extent of

oligodendrocyte myelination. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(4):1299-304.
351. Fields RD. Myelination: an overlooked mechanism of synaptic plasticity? Neuroscientist. 2005;11(6):528-31.
352. Franz DN, Iggo A. Conduction failure in myelinated and non-myelinated axons at low temperatures. The Journal of physiology. 1968;199(2):319-45.
353. Hartline DK, Colman DR. Rapid conduction and the evolution of giant axons and myelinated fibers. Current biology : CB. 2007;17(1):R29-35.
354. Prineas JW, Barnard RO, Kwon EE, Sharer LR, Cho ES. Multiple sclerosis: remyelination of nascent lesions. Annals of neurology. 1993;33(2):137-51.
355. Raine CS, Wu E. Multiple sclerosis: remyelination in acute lesions. Journal of neuropathology and experimental neurology. 1993;52(3):199-204.
356. Lassmann H, Bruck W, Lucchinetti C, Rodriguez M. Remyelination in multiple sclerosis. Multiple sclerosis. 1997;3(2):133-6.
357. Patrikios P, Stadelmann C, Kutzelnigg A, Rauschka H, Schmidbauer M, Laursen H, et al. Remyelination is extensive in a subset of multiple sclerosis patients. Brain : a journal of neurology. 2006;129(Pt 12):3165-72.
358. Goldschmidt T, Antel J, Konig FB, Bruck W, Kuhlmann T. Remyelination capacity of the MS brain decreases with disease chronicity. Neurology. 2009;72(22):1914-21.
359. Barkhof F, Bruck W, De Groot CJ, Bergers E, Hulshof S, Geurts J, et al. Remyelinated lesions in multiple sclerosis: magnetic resonance image appearance. Arch Neurol. 2003;60(8):1073-81.
360. Patani R, Balaratnam M, Vora A, Reynolds R. Remyelination can be extensive in multiple sclerosis despite a long disease course. Neuropathol Appl Neurobiol. 2007;33(3):277-87.
361. Nave KA. Myelination and the trophic support of long axons. Nature reviews Neuroscience. 2010;11(4):275-83.
362. Chari DM, Blakemore WF. New insights into remyelination failure in multiple sclerosis: implications for glial cell transplantation. Multiple sclerosis. 2002;8(4):271-7.
363. Franklin RJ, ffrench-Constant C, Edgar JM, Smith KJ. Neuroprotection and repair in multiple sclerosis. Nature reviews Neurology. 2012;8(11):624-34.
364. Chang A, Tourtellotte WW, Rudick R, Trapp BD. Premyelinating oligodendrocytes in chronic lesions of multiple sclerosis. The New England journal of medicine. 2002;346(3):165-73.
365. Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2000;20(17):6404-12.
366. Harlow DE, Macklin WB. Inhibitors of myelination: ECM changes, CSPGs and PTPs. Experimental neurology. 2014;251:39-46.
367. Lau LW, Cua R, Keough MB, Haylock-Jacobs S, Yong VW. Pathophysiology of the brain extracellular matrix: a new target for remyelination. Nature reviews Neuroscience. 2013;14(10):722-9.
368. Mei F, Christin Chong SY, Chan JR. Myelin-based inhibitors of oligodendrocyte myelination: clues from axonal growth and regeneration. Neurosci Bull. 2013;29(2):177-88.
369. Yang Y, Liu Y, Wei P, Peng H, Winger R, Hussain RZ, et al. Silencing Nogo-A promotes functional recovery in demyelinating disease. Annals of neurology. 2010;67(4):498-507.
370. Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, et al. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nature neuroscience. 2004;7(3):221-8.
371. Rodgers JM, Miller SD. Cytokine control of inflammation and repair in the pathology of multiple sclerosis. Yale J Biol Med. 2012;85(4):447-68.
372. Brambilla R, Morton PD, Ashbaugh JJ, Karmally S, Lambertsen KL, Bethea JR. Astrocytes play a key role in EAE pathophysiology by orchestrating in the CNS the inflammatory response of resident and peripheral immune cells and by suppressing remyelination. Glia. 2014;62(3):452-67.

373. Schmitz T, Chew LJ. Cytokines and myelination in the central nervous system. ScientificWorldJournal. 2008;8:1119-47.
374. Harlow DE, Honce JM, Miravalle AA. Remyelination Therapy in Multiple Sclerosis. Front Neurol. 2015;6:257.
375. Norton WT, Poduslo SE. Myelination in rat brain: changes in myelin composition during brain maturation. Journal of neurochemistry. 1973;21(4):759-73.
376. Chrast R, Saher G, Nave KA, Verheijen MH. Lipid metabolism in myelinating glial cells: lessons from human inherited disorders and mouse models. Journal of lipid research. 2011;52(3):419-34.
377. Verrips A, Hoefsloot LH, Steenbergen GC, Theelen JP, Wevers RA, Gabreels FJ, et al. Clinical and molecular genetic characteristics of patients with cerebrotendinous xanthomatosis. Brain : a journal of neurology. 2000;123 (Pt 5):908-19.
378. Verheijen MH, Camargo N, Verdier V, Nadra K, de Preux Charles AS, Medard JJ, et al. SCAP is required for timely and proper myelin membrane synthesis. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(50):21383-8.
379. Karrenbauer VD, Leoni V, Lim ET, Giovannoni G, Ingle GT, Sastre-Garriga J, et al. Plasma cerebrosterol and magnetic resonance imaging measures in multiple sclerosis. Clin Neurol Neurosurg. 2006;108(5):456-60.
380. Teunissen CE, Dijkstra CD, Polman CH, Hoogervorst EL, von Bergmann K, Lutjohann D. Decreased levels of the brain specific 24S-hydroxycholesterol and cholesterol precursors in serum of multiple sclerosis patients. Neuroscience letters. 2003;347(3):159-62.
381. Berghoff SA, Gerndt N, Winchenbach J, Stumpf SK, Hosang L, Odoardi F, et al. Dietary cholesterol promotes repair of demyelinated lesions in the adult brain. Nat Commun. 2017;8:14241.
382. Hansen SW, Ohtani K, Roy N, Wakamiya N. The collectins CL-L1, CL-K1 and CL-P1, and their roles in complement and innate immunity. Immunobiology. 2016;221(10):1058-67.
383. Roy N, Ohtani K, Matsuda Y, Mori K, Hwang I, Suzuki Y, et al. Collectin CL-P1 utilizes C-reactive protein for complement activation. Biochim Biophys Acta. 2016;1860(6):1118-28.
384. Nakamura K, Ohya W, Funakoshi H, Sakaguchi G, Kato A, Takeda M, et al. Possible role of scavenger receptor SRCL in the clearance of amyloid-beta in Alzheimer's disease. J Neurosci Res. 2006;84(4):874-90.
385. Zhao C, Dahlman-Wright K. Liver X receptor in cholesterol metabolism. J Endocrinol. 2010;204(3):233-40.
386. Fryer JD, Demattos RB, McCormick LM, O'Dell MA, Spinner ML, Bales KR, et al. The low density lipoprotein receptor regulates the level of central nervous system human and murine apolipoprotein E but does not modify amyloid plaque pathology in PDAPP mice. The Journal of biological chemistry. 2005;280(27):25754-9.
387. Jurukovska-Nospal M, Arsova V, Levchanska J, Sidovska-Ivanovska B. Effects of statins (atorvastatin) on serum lipoprotein levels in patients with primary hyperlipidemia and coronary heart disease. Prilozi. 2007;28(2):137-48.
388. de Oliveira J, Moreira EL, dos Santos DB, Piermartiri TC, Dutra RC, Pinton S, et al. Increased susceptibility to amyloid-beta-induced neurotoxicity in mice lacking the low-density lipoprotein receptor. Journal of Alzheimer's disease : JAD. 2014;41(1):43-60.
389. Murthy S, Born E, Mathur SN, Field FJ. LXR/RXR activation enhances basolateral efflux of cholesterol in CaCo-2 cells. J Lipid Res. 2002;43(7):1054-64.
390. Huang JK, Jarjour AA, Nait Oumesmar B, Kerninon C, Williams A, Krezel W, et al. Retinoid X receptor gamma signaling accelerates CNS remyelination. Nature neuroscience. 2011;14(1):45-53.
391. Smith ME. Phagocytic properties of microglia in vitro: implications for a role in multiple sclerosis and EAE. Microscopy research and technique. 2001;54(2):81-94.
392. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M. Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. Nature. 1990;343(6258):531-5.

393. Nakamura K, Funakoshi H, Miyamoto K, Tokunaga F, Nakamura T. Molecular cloning and functional characterization of a human scavenger receptor with C-type lectin (SRCL), a novel member of a scavenger receptor family. Biochemical and biophysical research communications. 2001;280(4):1028-35.
394. Nakamura K, Funakoshi H, Tokunaga F, Nakamura T. Molecular cloning of a mouse scavenger receptor with C-type lectin (SRCL)(1), a novel member of the scavenger receptor family. Biochimica et biophysica acta. 2001;1522(1):53-8.
395. Drickamer K, Taylor ME. Recent insights into structures and functions of C-type lectins in the immune system. Current opinion in structural biology. 2015;34:26-34.
396. Ohtani K, Suzuki Y, Eda S, Kawai T, Kase T, Keshi H, et al. The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. The Journal of biological chemistry. 2001;276(47):44222-8.
397. Feinberg H, Taylor ME, Weis WI. Scavenger receptor C-type lectin binds to the leukocyte cell surface glycan Lewis(x) by a novel mechanism. The Journal of biological chemistry. 2007;282(23):17250-8.
398. Coombs PJ, Graham SA, Drickamer K, Taylor ME. Selective binding of the scavenger receptor C-type lectin to Lewisx trisaccharide and related glycan ligands. The Journal of biological chemistry. 2005;280(24):22993-9.
399. Yoshida T, Tsuruta Y, Iwasaki M, Yamane S, Ochi T, Suzuki R. SRCL/CL-P1 recognizes GalNAc and a carcinoma-associated antigen, Tn antigen. Journal of biochemistry. 2003;133(3):271-7.
400. Elola MT, Capurro MI, Barrio MM, Coombs PJ, Taylor ME, Drickamer K, et al. Lewis x antigen mediates adhesion of human breast carcinoma cells to activated endothelium. Possible involvement of the endothelial scavenger receptor C-type lectin. Breast cancer research and treatment. 2007;101(2):161-74.
401. Wouters K, Cudejko C, Gijbels MJ, Fuentes L, Bantubungi K, Vanhoutte J, et al. Bone marrow p16INK4a-deficiency does not modulate obesity, glucose homeostasis or atherosclerosis development. PloS one. 2012;7(3):e32440.
402. Albert ML, Kim JI, Birge RB. alphavbeta5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. Nature cell biology. 2000;2(12):899-905.
403. Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood. 2005;106(7):2543-50.
404. van Horssen J, Bo L, Vos CM, Virtanen I, de Vries HE. Basement membrane proteins in multiple sclerosis-associated inflammatory cuffs: potential role in influx and transport of leukocytes. Journal of neuropathology and experimental neurology. 2005;64(8):722-9.
405. Bogie JF, Jorissen W, Mailleux J, Nijland PG, Zelcer N, Vanmierlo T, et al. Myelin alters the inflammatory phenotype of macrophages by activating PPARs. Acta neuropathologica communications. 2013;1(1):43.
406. Nelissen K, Smeets K, Mulder M, Hendriks JJ, Ameloot M. Selection of reference genes for gene expression studies in rat oligodendrocytes using quantitative real time PCR. JNeurosciMethods. 2010;187(1):78-83.
407. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.
408. Haider L, Fischer MT, Frischer JM, Bauer J, Hoftberger R, Botond G, et al. Oxidative damage in multiple sclerosis lesions. Brain. 2011;134(Pt 7):1914-24.
409. Wallberg M, Bergquist J, Achour A, Breij E, Harris RA. Malondialdehyde modification of myelin oligodendrocyte glycoprotein leads to increased immunogenicity and encephalitogenicity. Eur J Immunol. 2007;37(7):1986-95.
410. Wheeler D, Bandaru VV, Calabresi PA, Nath A, Haughey NJ. A defect of sphingolipid metabolism modifies the properties of normal appearing white matter in multiple sclerosis. Brain. 2008;131(Pt 11):3092-102.
411. Bogie JF, Jorissen W, Mailleux J, Nijland PG, Zelcer N, Vanmierlo T, et al. Myelin alters the inflammatory phenotype of macrophages by activating PPARs. Acta neuropathologica communications. 2013;1(43).

412. Carson MJ, Bilousova TV, Puntambekar SS, Melchior B, Doose JM, Ethell IM. A rose by any other name? The potential consequences of microglial heterogeneity during CNS health and disease. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics. 2007;4(4):571-9.
413. Kuhlmann T, Wendling U, Nolte C, Zipp F, Maruschak B, Stadelmann C, et al. Differential regulation of myelin phagocytosis by macrophages/microglia, involvement of target myelin, Fc receptors and activation by intravenous immunoglobulins. Journal of neuroscience research. 2002;67(2):185-90.
414. Yamasaki R, Lu H, Butovsky O, Ohno N, Rietsch AM, Cialic R, et al. Differential roles of microglia and monocytes in the inflamed central nervous system. J Exp Med. 2014;211(8):1533-49.
415. Joseph SB, Bradley MN, Castrillo A, Bruhn KW, Mak PA, Pei L, et al. LXR-dependent gene expression is important for macrophage survival and the innate immune response. Cell. 2004;119(2):299-309.
416. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell. 1998;93(2):241-52.
417. Mori K, Ohtani K, Jang S, Kim Y, Hwang I, Roy N, et al. Scavenger receptor CL-P1 mainly utilizes a collagen-like domain to uptake microbes and modified LDL. Biochimica et biophysica acta. 2014;1840(12):3345-56.
418. Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, Christie G, et al. Inhibitor of neurite outgrowth in humans. Nature. 2000;403(6768):383-4.
419. Appelmelk BJ, van Die I, van Vliet SJ, Vandenbroucke-Grauls CM, Geijtenbeek TB, van Kooyk Y. Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. Journal of immunology. 2003;170(4):1635-9.
420. Guo Y, Feinberg H, Conroy E, Mitchell DA, Alvarez R, Blixt O, et al. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nature structural & molecular biology. 2004;11(7):591-8.
421. Svajger U, Anderluh M, Jeras M, Obermajer N. C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. Cellular signalling. 2010;22(10):1397-405.
422. Ehrhardt C, Kneuer C, Bakowsky U. Selectins-an emerging target for drug delivery. Advanced drug delivery reviews. 2004;56(4):527-49.
423. de Vos AF, van Meurs M, Brok HP, Boven LA, Hintzen RQ, van der Valk P, et al. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. Journal of immunology. 2002;169(10):5415-23.
424. Fabriek BO, Zwemmer JN, Teunissen CE, Dijkstra CD, Polman CH, Laman JD, et al. In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology. Journal of neuroimmunology. 2005;161(1-2):190-4.
425. van Zwam M, Huizinga R, Melief MJ, Wierenga-Wolf AF, van Meurs M, Voerman JS, et al. Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. Journal of molecular medicine. 2009;87(3):273-86.
426. Nair A, Frederick TJ, Miller SD. Astrocytes in multiple sclerosis: a product of their environment. Cellular and molecular life sciences : CMLS. 2008;65(17):2702-20.
427. Barnett MH, Henderson AP, Prineas JW. The macrophage in MS: just a scavenger after all? Pathology and pathogenesis of the acute MS lesion. Multiple sclerosis. 2006;12(2):121-32.
428. Kotter MR, Zhao C, van Rooijen N, Franklin RJ. Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. Neurobiology of disease. 2005;18(1):166-75.
429. Schwab ME. Nogo and axon regeneration. Current opinion in neurobiology. 2004;14(1):118-24.
430. Morell P QR. Characteristic Composition of Myelin. Basic Neurochemistry: Molecular, Cellular and Medical Aspects 6th edition. Philadelphia: Lippincott-Raven; 1999.

431. Spady DK, Dietschy JM. Sterol synthesis in vivo in 18 tissues of the squirrel monkey, guinea pig, rabbit, hamster, and rat. J Lipid Res. 1983;24(3):303-15.
432. Fan X, Kim HJ, Bouton D, Warner M, Gustafsson JA. Expression of liver X receptor beta is essential for formation of superficial cortical layers and migration of later-born neurons. Proc Natl Acad Sci U S A. 2008;105(36):13445-50.
433. Bogie JF, Stinissen P, Hellings N, Hendriks JJ. Myelin-phagocytosing macrophages modulate autoreactive T cell proliferation. J Neuroinflammation. 2011;8:85.
434. Jansen PJ, Lutjohann D, Abildayeva K, Vanmierlo T, Plosch T, Plat J, et al. Dietary plant sterols accumulate in the brain. Biochimica et biophysica acta. 2006;1761(4):445-53.
435. Lutjohann D, Brzezinka A, Barth E, Abramowski D, Staufenbiel M, von Bergmann K, et al. Profile of cholesterol-related sterols in aged amyloid precursor protein transgenic mouse brain. Journal of lipid research. 2002;43(7):1078-85.
436. Vanmierlo T, Bogie JF, Mailleux J, Vanmol J, Lutjohann D, Mulder M, et al. Plant sterols: Friend or foe in CNS disorders? Prog Lipid Res. 2015;58:26-39.
437. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell. 1995;83(6):841-50.
438. Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, et al. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. Proc Natl Acad Sci U S A. 1999;96(1):266-71.
439. Fu X, Menke JG, Chen Y, Zhou G, MacNaul KL, Wright SD, et al. 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. J Biol Chem. 2001;276(42):38378-87.
440. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature. 1996;383(6602):728-31.
441. Forman BM, Ruan B, Chen J, Schroepfer GJ, Jr., Evans RM. The orphan nuclear receptor LXRalpha is positively and negatively regulated by distinct products of mevalonate metabolism. Proc Natl Acad Sci U S A. 1997;94(20):10588-93.
442. Zhou G, Cummings R, Li Y, Mitra S, Wilkinson HA, Elbrecht A, et al. Nuclear receptors have distinct affinities for coactivators: characterization by fluorescence resonance energy transfer. Mol Endocrinol. 1998;12(10):1594-604.
443. Castrillo A, Joseph SB, Marathe C, Mangelsdorf DJ, Tontonoz P. Liver X receptor-dependent repression of matrix metalloproteinase-9 expression in macrophages. J Biol Chem. 2003;278(12):10443-9.
444. Meffre D, Shackleford G, Hichor M, Gorgievski V, Tzavara ET, Trousson A, et al. Liver X receptors alpha and beta promote myelination and remyelination in the cerebellum. Proc Natl Acad Sci U S A. 2015;112(24):7587-92.
445. Wang Z, Sadovnick AD, Traboulsee AL, Ross JP, Bernales CQ, Encarnacion M, et al. Nuclear Receptor NR1H3 in Familial Multiple Sclerosis. Neuron. 2016;90(5):948-54.
446. Huitinga I, Ruuls SR, Jung S, Van Rooijen N, Hartung HP, Dijkstra CD. Macrophages in T cell line-mediated, demyelinating, and chronic relapsing experimental autoimmune encephalomyelitis in Lewis rats. Clin Exp Immunol. 1995;100(2):344-51.
447. Brosnan CF, Bornstein MB, Bloom BR. The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis. J Immunol. 1981;126(2):614-20.
448. Bogie JF, Mailleux J, Wouters E, Jorissen W, Grajchen E, Vanmol J, et al. Scavenger receptor collectin placenta 1 is a novel receptor involved in the uptake of myelin by phagocytes. Sci Rep. 2017;7:44794.
449. Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Lutjohann D, Hendriks JJ, et al. Active liver X receptor signaling in phagocytes in multiple sclerosis lesions. Mult Scler. 2017:1352458517696595.
450. Bogie JF, Jorissen W, Mailleux J, Nijland PG, Zelcer N, Vanmierlo T, et al. Myelin alters the inflammatory phenotype of macrophages by activating PPARs. Acta Neuropathol Commun. 2013;1:43.
451. Giubilei F, Antonini G, Di Legge S, Sormani MP, Pantano P, Antonini R, et al. Blood cholesterol and MRI activity in first clinical episode suggestive of multiple sclerosis. Acta Neurol Scand. 2002;106(2):109-12.

452. Leoni V, Masterman T, Diczfalusy U, De Luca G, Hillert J, Bjorkhem I. Changes in human plasma levels of the brain specific oxysterol 24S-hydroxycholesterol during progression of multiple sclerosis. Neurosci Lett. 2002;331(3):163-6.
453. Mandoj C, Renna R, Plantone D, Sperduti I, Cigliana G, Conti L, et al. Anti-annexin antibodies, cholesterol levels and disability in multiple sclerosis. Neurosci Lett. 2015;606:156-60.
454. Miyake Y, Tajima S, Funahashi T, Yamamoto A. Analysis of a recycling-impaired mutant of low density lipoprotein receptor in familial hypercholesterolemia. J Biol Chem. 1989;264(28):16584-90.
455. Dimeloe S, Burgener AV, Grahlert J, Hess C. T-cell metabolism governing activation, proliferation and differentiation; a modular view. Immunology. 2017;150(1):35-44.
456. Laskowitz DT, Thekdi AD, Thekdi SD, Han SK, Myers JK, Pizzo SV, et al. Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. Exp Neurol. 2001;167(1):74-85.
457. Christensen DJ, Ohkubo N, Oddo J, Van Kanegan MJ, Neil J, Li F, et al. Apolipoprotein E and peptide mimetics modulate inflammation by binding the SET protein and activating protein phosphatase 2A. J Immunol. 2011;186(4):2535-42.
458. Li FQ, Sempowski GD, McKenna SE, Laskowitz DT, Colton CA, Vitek MP. Apolipoprotein E-derived peptides ameliorate clinical disability and inflammatory infiltrates into the spinal cord in a murine model of multiple sclerosis. The Journal of pharmacology and experimental therapeutics. 2006;318(3):956-65.
459. Karussis D, Michaelson DM, Grigoriadis N, Korezyn AD, Mizrachi-Koll R, Chapman S, et al. Lack of apolipoprotein-E exacerbates experimental allergic encephalomyelitis. Mult Scler. 2003;9(5):476-80.
460. Trigunaite A, Dimo J, Jorgensen TN. Suppressive effects of androgens on the immune system. Cell Immunol. 2015;294(2):87-94.
461. Garcia-Ovejero D, Veiga S, Garcia-Segura LM, Doncarlos LL. Glial expression of estrogen and androgen receptors after rat brain injury. J Comp Neurol. 2002;450(3):256-71.
462. Semenkovich CF, Ostlund RE, Jr. Estrogens induce low-density lipoprotein receptor activity and decrease intracellular cholesterol in human hepatoma cell line Hep G2. Biochemistry. 1987;26(16):4987-92.
463. Smith PM, Cowan A, White BA. The low-density lipoprotein receptor is regulated by estrogen and forms a functional complex with the estrogen-regulated protein ezrin in pituitary GH3 somatolactotropes. Endocrinology. 2004;145(7):3075-83.
464. Napolitano M, Blotta I, Montali A, Bravo E. 17beta-estradiol enhances the flux of cholesterol through the cholesteryl ester cycle in human macrophages. Biosci Rep. 2001;21(5):637-52.
465. Mumford SL, Schisterman EF, Siega-Riz AM, Browne RW, Gaskins AJ, Trevisan M, et al. A longitudinal study of serum lipoproteins in relation to endogenous reproductive hormones during the menstrual cycle: findings from the BioCycle study. The Journal of clinical endocrinology and metabolism. 2010;95(9):E80-5.
466. Barnett JB, Woods MN, Lamon-Fava S, Schaefer EJ, McNamara JR, Spiegelman D, et al. Plasma lipid and lipoprotein levels during the follicular and luteal phases of the menstrual cycle. The Journal of clinical endocrinology and metabolism. 2004;89(2):776-82.
467. Magkos F, Patterson BW, Mittendorfer B. No effect of menstrual cycle phase on basal very-low-density lipoprotein triglyceride and apolipoprotein B-100 kinetics. American journal of physiology Endocrinology and metabolism. 2006;291(6):E1243-9.
468. Perez-Lopez FR, Larrad-Mur L, Kallen A, Chedraui P, Taylor HS. Gender differences in cardiovascular disease: hormonal and biochemical influences. Reprod Sci. 2010;17(6):511-31.
469. Kim AM, Tingen CM, Woodruff TK. Sex bias in trials and treatment must end. Nature. 2010;465(7299):688-9.
470. Segatto M, Trapani L, Marino M, Pallottini V. Age- and sex-related differences in extra-hepatic low-density lipoprotein receptor. J Cell Physiol. 2011;226(10):2610-6.

471. Kuhlmann T, Miron V, Cui Q, Wegner C, Antel J, Bruck W. Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. Brain. 2008;131(Pt 7):1749-58.
472. Skripuletz T, Manzel A, Gropengiesser K, Schafer N, Gudi V, Singh V, et al. Pivotal role of choline metabolites in remyelination. Brain. 2015;138(Pt 2):398-413.
473. O'Meara RW, Ryan SD, Colognato H, Kothary R. Derivation of enriched oligodendrocyte cultures and oligodendrocyte/neuron myelinating co-cultures from post-natal murine tissues. J Vis Exp. 2011(54).
474. Matsushima GK, Morell P. The neurotoxicant, cuprizone, as a model to study demyelination and remyelination in the central nervous system. Brain Pathol. 2001;11(1):107-16.
475. Hibbits N, Pannu R, Wu TJ, Armstrong RC. Cuprizone demyelination of the corpus callosum in mice correlates with altered social interaction and impaired bilateral sensorimotor coordination. ASN neuro. 2009;1(3).
476. Maheshwari A, Janssens K, Bogie J, Van Den Haute C, Struys T, Lambrichts I, et al. Local overexpression of interleukin-11 in the central nervous system limits demyelination and enhances remyelination. Mediators Inflamm. 2013;2013:685317.
477. Woodruff RH, Franklin RJ. The expression of myelin protein mRNAs during remyelination of lysolecithin-induced demyelination. Neuropathol Appl Neurobiol. 1999;25(3):226-35.
478. Franklin RJ. Why does remyelination fail in multiple sclerosis? Nature reviews Neuroscience. 2002;3(9):705-14.
479. Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. A quantitative analysis of oligodendrocytes in multiple sclerosis lesions. A study of 113 cases. Brain. 1999;122 (Pt 12):2279-95.
480. Mierzwa AJ, Zhou YX, Hibbits N, Vana AC, Armstrong RC. FGF2 and FGFR1 signaling regulate functional recovery following cuprizone demyelination. Neurosci Lett. 2013;548:280-5.
481. Liu L, Belkadi A, Darnall L, Hu T, Drescher C, Cotleur AC, et al. CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. Nat Neurosci. 2010;13(3):319-26.
482. Lin CY, Gustafsson JA. Targeting liver X receptors in cancer therapeutics. Nat Rev Cancer. 2015;15(4):216-24.
483. Zhou J, Febbraio M, Wada T, Zhai Y, Kuruba R, He J, et al. Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPARgamma in promoting steatosis. Gastroenterology. 2008;134(2):556-67.
484. Dhaliwal BS, Steinbrecher UP. Scavenger receptors and oxidized low density lipoproteins. Clin Chim Acta. 1999;286(1-2):191-205.
485. Yu XH, Fu YC, Zhang DW, Yin K, Tang CK. Foam cells in atherosclerosis. Clin Chim Acta. 2013;424:245-52.
486. Newcombe J, Li H, Cuzner ML. Low density lipoprotein uptake by macrophages in multiple sclerosis plaques: implications for pathogenesis. Neuropathol Appl Neurobiol. 1994;20(2):152-62.
487. Chou MY, Hartvigsen K, Hansen LF, Fogelstrand L, Shaw PX, Boullier A, et al. Oxidation-specific epitopes are important targets of innate immunity. J Intern Med. 2008;263(5):479-88.
488. Licona-Limon I, Garay-Canales CA, Munoz-Paleta O, Ortega E. CD13 mediates phagocytosis in human monocytic cells. J Leukoc Biol. 2015;98(1):85-98.
489. Rowe AH, Argmann CA, Edwards JY, Sawyez CG, Morand OH, Hegele RA, et al. Enhanced synthesis of the oxysterol 24(S),25-epoxycholesterol in macrophages by inhibitors of 2,3-oxidosqualene:lanosterol cyclase: a novel mechanism for the attenuation of foam cell formation. Circ Res. 2003;93(8):717-25.
490. Ali Z, Heverin M, Olin M, Acimovic J, Lovgren-Sandblom A, Shafaati M, et al. On the regulatory role of side-chain hydroxylated oxysterols in the brain. Lessons from CYP27A1 transgenic and Cyp27a1(-/-) mice. J Lipid Res. 2013;54(4):1033-43.
491. Beltowski J. Liver X receptors (LXR) as therapeutic targets in dyslipidemia. Cardiovasc Ther. 2008;26(4):297-316.

492. Nelson JA, Steckbeck SR, Spencer TA. Biosynthesis of 24,25-epoxycholesterol from squalene 2,3;22,23-dioxide. J Biol Chem. 1981;256(3):1067-8.
493. Lange Y, Ye J, Strebel F. Movement of 25-hydroxycholesterol from the plasma membrane to the rough endoplasmic reticulum in cultured hepatoma cells. J Lipid Res. 1995;36(5):1092-7.
494. Meaney S, Bodin K, Diczfalusy U, Bjorkhem I. On the rate of translocation in vitro and kinetics in vivo of the major oxysterols in human circulation: critical importance of the position of the oxygen function. J Lipid Res. 2002;43(12):2130-5.
495. Bjorkhem I, Andersson O, Diczfalusy U, Sevastik B, Xiu RJ, Duan C, et al. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. Proc Natl Acad Sci U S A. 1994;91(18):8592-6.
496. Lund E, Andersson O, Zhang J, Babiker A, Ahlborg G, Diczfalusy U, et al. Importance of a novel oxidative mechanism for elimination of intracellular cholesterol in humans. Arterioscler Thromb Vasc Biol. 1996;16(2):208-12.
497. Diczfalusy U, Lund E, Lutjohann D, Bjorkhem I. Novel pathways for elimination of cholesterol by extrahepatic formation of side-chain oxidized oxysterols. Scand J Clin Lab Invest Suppl. 1996;226:9-17.
498. Heverin M, Ali Z, Olin M, Tillander V, Joibari MM, Makoveichuk E, et al. On the regulatory importance of 27-hydroxycholesterol in mouse liver. J Steroid Biochem Mol Biol. 2017;169:10-21.
499. Kannenberg F, Gorzelniak K, Jager K, Fobker M, Rust S, Repa J, et al. Characterization of cholesterol homeostasis in telomerase-immortalized Tangier disease fibroblasts reveals marked phenotype variability. J Biol Chem. 2013;288(52):36936-47.
500. Xu J, Wagoner G, Douglas JC, Drew PD. Liver X receptor agonist regulation of Th17 lymphocyte function in autoimmunity. J Leukoc Biol. 2009;86(2):401-9.
501. Chen J, Zacharek A, Cui X, Shehadah A, Jiang H, Roberts C, et al. Treatment of stroke with a synthetic liver X receptor agonist, TO901317, promotes synaptic plasticity and axonal regeneration in mice. J Cereb Blood Flow Metab. 2010;30(1):102-9.
502. Sacchetti P, Sousa KM, Hall AC, Liste I, Steffensen KR, Theofilopoulos S, et al. Liver X receptors and oxysterols promote ventral midbrain neurogenesis in vivo and in human embryonic stem cells. Cell Stem Cell. 2009;5(4):409-19.
503. Dai YB, Tan XJ, Wu WF, Warner M, Gustafsson JA. Liver X receptor beta protects dopaminergic neurons in a mouse model of Parkinson disease. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(32):13112-7.
504. Jansen D, Janssen CI, Vanmierlo T, Dederen PJ, van Rooij D, Zinnhardt B, et al. Cholesterol and synaptic compensatory mechanisms in Alzheimer's disease mice brain during aging. Journal of Alzheimer's disease : JAD. 2012;31(4):813-26.
505. Jiang Q, Lee CYD, Mandrekar S, Wilkinson B, Cramer P, Zelcer N, et al. ApoE Promotes the Proteolytic Degradation of Aβ. Neuron. 2008;58(5):681-93.
506. Riddell DR, Zhou H, Comery TA, Kouranova E, Lo CF, Warwick HK, et al. The LXR agonist TO901317 selectively lowers hippocampal Aβ42 and improves memory in the Tg2576 mouse model of Alzheimer's disease. Molecular and Cellular Neuroscience. 2007;34(4):621-8.
507. Vanmierlo T, Rutten K, van Vark-van der Zee LC, Friedrichs S, Bloks VW, Blokland A, et al. Cerebral accumulation of dietary derivable plant sterols does not interfere with memory and anxiety related behavior in Abcg5-/- mice. Plant foods for human nutrition. 2011;66(2):149-56.
508. Zelcer N, Khanlou N, Clare R, Jiang Q, Reed-Geaghan EG, Landreth GE, et al. Attenuation of neuroinflammation and Alzheimer's disease pathology by liver x receptors. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(25):10601-6.
509. Vanmierlo T, Rutten K, Dederen J, Bloks VW, van Vark-van der Zee LC, Kuipers F, et al. Liver X receptor activation restores memory in aged AD mice without reducing amyloid. Neurobiology of aging. 2011;32(7):1262-72.
510. Saher G, Quintes S, Nave KA. Cholesterol: a novel regulatory role in myelin formation. Neuroscientist. 2011;17(1):79-93.

511. Trousson A, Bernard S, Petit PX, Liere P, Pianos A, El Hadri K, et al. 25-hydroxycholesterol provokes oligodendrocyte cell line apoptosis and stimulates the secreted phospholipase A2 type IIA via LXR beta and PXR. Journal of neurochemistry. 2009;109(4):945-58.
512. Ito J, Nagayasu Y, Miura Y, Yokoyama S, Michikawa M. Astrocytes endogenous apoE generates HDL-like lipoproteins using previously synthesized cholesterol through interaction with ABCA1. Brain research. 2014;1570:1-12.
513. Carlin C, Murray L, Graham D, Doyle D, Nicoll J. Involvement of apolipoprotein E in multiple sclerosis: absence of remyelination associated with possession of the APOE epsilon2 allele. Journal of neuropathology and experimental neurology. 2000;59(5):361-7.
514. Genden EM, Watanabe O, Mackinnon SE, Hunter DA, Strasberg SR. Peripheral nerve regeneration in the apolipoprotein-E-deficient mouse. J Reconstr Microsurg. 2002;18(6):495-502.
515. Dayger CA, Rosenberg JS, Winkler C, Foster S, Witkowski E, Benice TS, et al. Paradoxical effects of apolipoprotein E on cognitive function and clinical progression in mice with experimental autoimmune encephalomyelitis. Pharmacol Biochem Behav. 2013;103(4):860-8.
516. Savettieri G, Messina D, Andreoli V, Bonavita S, Caltagirone C, Cittadella R, et al. Gender-related effect of clinical and genetic variables on the cognitive impairment in multiple sclerosis. Journal of neurology. 2004;251(10):1208-14.
517. Parmenter BA, Shucard JL, Benedict RH, Shucard DW. Working memory deficits in multiple sclerosis: comparison between the n-back task and the Paced Auditory Serial Addition Test. J Int Neuropsychol Soc. 2006;12(5):677-87.
518. Pinholt M, Frederiksen JL, Christiansen M. The association between apolipoprotein E and multiple sclerosis. Eur J Neurol. 2006;13(6):573-80.
519. Lill CM, Liu T, Schjeide BM, Roehr JT, Akkad DA, Damotte V, et al. Closing the case of APOE in multiple sclerosis: no association with disease risk in over 29 000 subjects. J Med Genet. 2012;49(9):558-62.
520. Kantarci OH, Hebrink DD, Achenbach SJ, Pittock SJ, Altintas A, Schaefer-Klein JL, et al. Association of APOE polymorphisms with disease severity in MS is limited to women. Neurology. 2004;62(5):811-4.
521. Zwemmer J, Uitdehaag B, van Kamp G, Barkhof F, Polman C. Association of APOE polymorphisms with disease severity in MS is limited to women. Neurology. 2004;63(6):1139; author reply
522. Hui DY, Harmony JA, Innerarity TL, Mahley RW. Immunoregulatory plasma lipoproteins. Role of apoprotein E and apoprotein B. J Biol Chem. 1980;255(24):11775-81.
523. Mistry MJ, Clay MA, Kelly ME, Steiner MA, Harmony JA. Apolipoprotein E restricts interleukin-dependent T lymphocyte proliferation at the G1A/G1B boundary. Cell Immunol. 1995;160(1):14-23.
524. Bocksch L, Stephens T, Lucas A, Singh B. Apolipoprotein E: possible therapeutic target for atherosclerosis. Curr Drug Targets Cardiovasc Haematol Disord. 2001;1(2):93-106.
525. Getz GS, Reardon CA. Apoprotein E as a lipid transport and signaling protein in the blood, liver, and artery wall. J Lipid Res. 2009;50 Suppl:S156-61.
526. Maezawa I, Nivison M, Montine KS, Maeda N, Montine TJ. Neurotoxicity from innate immune response is greatest with targeted replacement of E4 allele of apolipoprotein E gene and is mediated by microglial p38MAPK. FASEB J. 2006;20(6):797-9.
527. Koch-Henriksen N, Sorensen PS. The changing demographic pattern of multiple sclerosis epidemiology. Lancet neurology. 2010;9(5):520-32.
528. Linton MF, Babaev VR, Gleaves LA, Fazio S. A direct role for the macrophage low density lipoprotein receptor in atherosclerotic lesion formation. The Journal of biological chemistry. 1999;274(27):19204-10.
529. Mahley RW, Ji ZS. Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. Journal of lipid research. 1999;40(1):1-16.
530. Bu G. Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. Nature reviews Neuroscience. 2009;10(5):333-44.

531. Lamsa R, Helisalmi S, Herukka SK, Tapiola T, Pirttila T, Vepsalainen S, et al. Genetic study evaluating LDLR polymorphisms and Alzheimer's disease. Neurobiology of aging. 2008;29(6):848-55.
532. Zou F, Gopalraj RK, Lok J, Zhu H, Ling IF, Simpson JF, et al. Sex-dependent association of a common low-density lipoprotein receptor polymorphism with RNA splicing efficiency in the brain and Alzheimer's disease. Human molecular genetics. 2008;17(7):929-35.
533. Fernandez-Castaneda A, Arandjelovic S, Stiles TL, Schlobach RK, Mowen KA, Gonias SL, et al. Identification of the low density lipoprotein (LDL) receptor-related protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris. J Biol Chem. 2013;288(7):4538-48.
534. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005;123(2):321-34.
535. Lampron A, Larochelle A, Laflamme N, Prefontaine P, Plante MM, Sanchez MG, et al. Inefficient clearance of myelin debris by microglia impairs remyelinating processes. J Exp Med. 2015;212(4):481-95.
536. Lillis AP, Van Duyn LB, Murphy-Ullrich JE, Strickland DK. LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies. Physiol Rev. 2008;88(3):887-918.
537. Gaultier A, Arandjelovic S, Niessen S, Overton CD, Linton MF, Fazio S, et al. Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. Blood. 2008;111(11):5316-25.
538. Chuang TY, Guo Y, Seki SM, Rosen AM, Johanson DM, Mandell JW, et al. LRP1 expression in microglia is protective during CNS autoimmunity. Acta Neuropathol Commun. 2016;4(1):68.
539. Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science. 2009;325(5936):100-4.
540. Chroni A, Nieland TJ, Kypreos KE, Krieger M, Zannis VI. SR-BI mediates cholesterol efflux via its interactions with lipid-bound ApoE. Structural mutations in SR-BI diminish cholesterol efflux. Biochemistry. 2005;44(39):13132-43.
541. Werner HB, Kramer-Albers EM, Strenzke N, Saher G, Tenzer S, Ohno-Iwashita Y, et al. A critical role for the cholesterol-associated proteolipids PLP and M6B in myelination of the central nervous system. Glia. 2013;61(4):567-86.
542. Werner H, Jung M, Klugmann M, Sereda M, Griffiths IR, Nave KA. Mouse models of myelin diseases. Brain Pathol. 1998;8(4):771-93.
543. Jurevics H, Hostettler J, Sammond DW, Nave KA, Toews AD, Morell P. Normal metabolism but different physical properties of myelin from mice deficient in proteolipid protein. Journal of neuroscience research. 2003;71(6):826-34.
544. Plat J, Nichols JA, Mensink RP. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. Journal of lipid research. 2005;46(11):2468-76.
545. Wei J, Zheng M, Liang P, Wei Y, Yin X, Tang Y, et al. Apolipoprotein E and its mimetic peptide suppress Th1 and Th17 responses in experimental autoimmune encephalomyelitis. Neurobiol Dis. 2013;56:59-65.
546. Lynch JR, Tang W, Wang H, Vitek MP, Bennett ER, Sullivan PM, et al. APOE genotype and an ApoE-mimetic peptide modify the systemic and central nervous system inflammatory response. J Biol Chem. 2003;278(49):48529-33.
547. Lynch JR, Wang H, Mace B, Leinenweber S, Warner DS, Bennett ER, et al. A novel therapeutic derived from apolipoprotein E reduces brain inflammation and improves outcome after closed head injury. Exp Neurol. 2005;192(1):109-16.
548. Franklin RJ, Ffrench-Constant C. Remyelination in the CNS: from biology to therapy. Nature reviews Neuroscience. 2008;9(11):839-55.
549. Rodgers JM, Robinson AP, Miller SD. Strategies for protecting oligodendrocytes and enhancing remyelination in multiple sclerosis. Discov Med. 2013;16(86):53-63.

550. Lublin FD, Cofield SS, Cutter GR, Conwit R, Narayana PA, Nelson F, et al. Randomized study combining interferon and glatiramer acetate in multiple sclerosis. Ann Neurol. 2013;73(3):327-40.
551. Pardridge WM. The blood-brain barrier: bottleneck in brain drug development. NeuroRx. 2005;2(1):3-14.

Curriculum Vitae

Jo Mailleux werd geboren op 21 augustus 1986 te Genk. In 2005 behaalde hij zijn diploma Economie – Wiskunde aan het Sint-Jan Berchmanscollege in Genk. Vervolgens startte hij zijn opleiding cel- en gentechnologie aan de Provinciale Hogeschool Limburg. Na voltooiing van een schakelprogramma mocht hij starten aan de masteropleiding Biomedische wetenschappen van de universiteit Hasselt. In 2012 behaalde hij zijn masterdiploma. Zijn eindwerk getiteld "*The immunomodulatory effects of phosphatidylserine containing liposomes in EAE rats*" werd uitgevoerd aan het Biomedisch Onderzoeksinstituut (BIOMED) van de universiteit Hasselt (o.l.v. Prof. dr. Jerome Hendriks). In september 2012 startte hij zijn doctoraat aan BIOMED in het kader van een BOF-beurs o.l.v. promotor Prof. dr. Jerome Hendriks en copromotoren Prof. dr. Jack van Horssen en dr. Tim Vanmierlo. In december 2013 behaalde hij een IWT-beurs, getiteld "*The impact of plant sterols on repair processes in multiple sclerosis*". Tijdens de hieropvolgende jaren participeerde hij actief mee in het onderwijs aan de universiteit Hasselt. In 2014 werd hij verkozen als voorzitter van Biomea, de alumnivereniging van Biomedische wetenschappen aan de universiteit Hasselt, en in 2015 als lid van de Doctoral School M&LS board. In 2017 voltooide hij een postgraduaat bedrijfskunde aan de Universiteit Hasselt.

Bibliography

Publications resulting from this work

Active liver X receptor signaling in phagocytes in multiple sclerosis lesions
Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Lütjohann D, Hendriks JJ, van Horssen J
Multiple Sclerosis Journal 2017 (IF: 4,67)

Scavenger receptor collectin placenta 1 is a novel receptor involved in the uptake of myelin by phagocytes
Bogie JF*, **Mailleux J***, Wouters E, Jorissen W, Grajchen E, Vanmol J, Wouters K, Hellings N, van Horssen J, Vanmierlo T, Hendriks JJ
Scientific reports 2017; 7(22794) (IF: 5.23)

Plant sterols: Friend or foe in CNS disorders?
Vanmierlo T*, Bogie JF*, **Mailleux J**, Vanmol J, Lütjohann D, Mulder M, Hendriks JJ.
Progress in Lipid 2015; 58(26) (IF: 11.24)

Low-density lipoprotein receptor deficiency attenuates neuroinflammation through the induction of apolipoprotein E
Mailleux J, Bogie JF, Vanmol J, Vanmierlo T, van Horssen J, Hendriks JJ
Frontiers in Immunology 2017 (IF: 6.429)

The impact of dietary plant sterols on remyelination.
Mailleux J, Bogie JF, Montes Diaz G, Schepers M, Lambrichts I, Lütjohann D, van Horssen J, Vanmierlo T*, Hendriks JJ*
In preparation

Publications in collaboration with other team members

Myelin alters the inflammatory phenotype of macrophages by activating PPARs
Bogie JF, Jorissen W, **Mailleux J**, Nijland PG, Zelcer N, Vanmierlo T, Van Horssen J, Stinissen P, Hellings N, Hendriks JJ
Acta Neuropathologica Communications 2013; 1(43)

Targeting demyelination via α-secretases promoting sAPPα release to enhance remyelination in central nervous system
Llufriu-Dabén G, Carreté A, Chierto E, **Mailleux J**, Camand E, Simon A, Vanmierlo T, Rose C, Allinquant B, Hendriks JJ, Massaad C, Meffre D, Jafarian-Tehrani M
Neurobiology of Disease 2017; (IF 5.020)

The Phosphodiesterase 4 (PDE4) inhibitor roflumilast improves remyelination in a mouse model for multiple sclerosis
Schepers M*, **Mailleux J***, Bogie J, van Goethem N, Hellings N, Hendriks JJ, Prickaerts J, Vanmierlo T
In preparation

Poster presentations

- Altered cholesterol metabolism in MS
  Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Jorissen W, Hendriks JJ, van Horssen J
  International Society for NeuroImmunoModulation meeting, 25th-27th November 2014, Liege, Belgium

- Altered cholesterol metabolism in MS
  Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Jorissen W, Hendriks JJ, van Horssen J
  MS research days, 28th-29th May 2015, Leiden, The Netherlands

- Liver X receptor activation in multiple sclerosis lesions
  Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Hendriks JJ, van Horssen J

Euroglia, 15th-18th July 2015, Bilbao, Spain

- Altered cholesterol metabolism in MS
  Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Jorissen W, Hendriks JJ, van Horssen J
  FWO-WOG symposium, 25 March 2016, Ghent, Belgium

- Active liver X receptor signaling in phagocytes in multiple sclerosis lesions
  Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Hendriks JJ, van Horssen J
  MS research days, 17th – 18th November 2016, Amsterdam, The Netherlands

- Active liver X receptor signaling in phagocytes in multiple sclerosis lesions
  Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Hendriks JJ, van Horssen J
  Venusberg meeting on Neuroinflammation, 11th – 13th May 2017, Bonn, Germany

Poster presentation award

Active liver X receptor signaling in phagocytes in multiple sclerosis lesions
Mailleux J, Vanmierlo T, Bogie JF, Wouters E, Hendriks JJ, van Horssen J
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"Isn't it funny how day by day nothing changes,
but when you look back everything is different?"
C.S. Lewis