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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

The potential of plant sterols to induce remyelination in multiple sclerosis and other demyelinating diseases

Promotor :
dr. Tim VANMIERLO
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Hafidat Lmalem

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Acknowledgements

From November 2014, till June 2015, my senior internship took place at the biomedical research institute of the university of Hasselt. The field I was submerged in during this time-period was neuro-immunology. To my own surprise, this subject interested me even more than I previously thought it would. Not only have I acquired a lot of knowledge, I have also gained a lot of experience during this period of time. Because of this, and many more reasons, I would like to thank everybody who helped and encouraged me during this practical training.

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Hafida Lmalem

June 9, 2015

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List of Abbreviations

ABCA1 = ATP-Binding Casette Transporter A1
ACTB = Beta-actin
AD = Alzheimer's Disease
ALS = Amyotrphic Lateral Sclerosis
ANOVA = Analysis of Varances
ApoE = Apolipoprotein E
BBB = Blood-Brain Barrier
BME = Basal Medium with Earle's salts
CCL2= C-C motif ligand 2 chemokine
CCR2 = C-C chemokine receptor type 2
CHD = Coronary Heart Disease
CNS = Central Nervous System
Ct = Cycle threshold
CycA = Cyclin A
DAPI = 4',6-diamidino-2-phenylindole
DMEM = Dulbecco's Modified Eagle Medium
(c)DNA = (complement) Deoxyribonucleic Acid
EAE = Experimental Autoimmune Encephalomyelitis
EBV = Epstein Barr Virus
EtOH = Ethanol
FCS = Fetal Calf Serum
GAIT = Gamma Interferon Activated Inhibitor of Translation
GW = LXR synthetic agonist GW3965
GWAS = Genome Wide Association Studies
HDL = High Density Lipoprotein
HEK = Human Embryonic Kidney
HLA = Human Leukocyte Antigen
HMBS = Hydroxymethylbilane Synthase
IFN = Interferon
IL = Interleukin
LDL = Low Density Lipoprotein
LIF = Leukemia Inhibitory Factor
LPC = Lysophosphatidylcholine
LPS = Lipopolysaccharide
LXR = Liver X Receptor
LXRE = Liver X Receptor Response Element
MBP = Myelin Basic Protein
MOG = Myelin Oligodendrocyte Glycoprotein
MS = Multiple Sclerosis
N-CoR = Nuclear receptor corepressor
NED = N-1-Naphthylethylenediamine Dihydrochloride
NF-kB = Nuclear Factor kappa-light-chain-enhancer of activated B cells
NF = Neurofilament
NGF = Neurotrophic Growth Factor
NO = Nitric Oxide
ONGP = O-nitrophenyl-beta-D-galactopyranoside
PBS = Phosphate Buffered Saline
PDGF = Platelet-derived Growth Factor
PFA = Paraformol Aldehyde
PGK1 = Phosphoglycerate Kinase 1
PLP = Proteolipid Protein
PPMS = Primary Progressive Multiple Sclerosis
P/S = Penicillin/Streptomycin
PS = Plant Sterols
ROS = Reactive Oxygen species
RRMS = Relapsing-Remissting Multiple Sclerosis
RNA = Ribonucleic Acid
RPL13A = Ribosomal Protein L13a
RT-qPCR = Real Time Polymerase Chain Reaction
RXR = Retinoid X Receptor
SEM = Standard Error Mean
SPMS = Secondary Progressive Multiple Sclerosis
SREBP-1c = Sterol regulatory Element-binding Protein 1c
T09 = Synthetic LXR agonist T0901317
TBP = TATA – binding protein
TGF-β = Transforming Growth Factor beta
Th17 = T helper 17 cells
TNFα = Tumor Necrosis Factor alpha
YWHAZ = Tyrosine 3 monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Abstract

Introduction: Multiple Sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS). The leading characteristic of MS is damage of the myelin sheaths covering the axons of the nerve cells, causing a decrease in saltatory conduction. In this study, we investigate the effects of plant sterols (PS), plant analogues of mammalian cholesterol, on remyelination. PS are natural agonists of liver X receptors (LXRs), which control cholesterol homeostasis, important for (re)myelination. PS can cross the blood-brain barrier (BBB) and they have been described to have anti-inflammatory and sterol regulating properties. Therefore, we hypothesize that PS positively influence remyelination by activating LXRs in microglia.

Materials & methods: First, the effects of PS on microglial expression levels of cytokines, chemokines, growth factors, LXR response genes were investigated. Second, to investigate indirect mechanisms of remyelination, PS-conditioned microglial medium was transferred to oligodendrocytes. Third, we used an organotypic cerebellar slice model to elucidate the potential of PS in stimulating remyelination in a multicellular environment.

Results: We found that PS do not activate LXRs in microglia. In addition, PS are only limitedly involved in directing the inflammatory profile of microglia. In line herewith, myelination in oligodendrocytes was only modestly influenced by a PS-conditioned medium transfer. Also, PS do not seem to stimulate remyelination in a multicellular environment.

Discussion & conclusion: The role of PS on microlgia in remyelination seems to be limited. On a multicellular level, remyelination could not be stimulated by the addition of PS. Moreover, in contrast to previous studies, we could not confirm LXR activation by PS.

Keywords: Multiple Sclerosis, plant sterols, liver X receptor, remyelination

Samenvatting

Introductie: Multiple Sclerosis (MS) is een chronische, inflammatoire, auto-immune aandoening van het centraal zenuwstelsel (CZS). De hoofdeigenschap van MS is schade aan de myeline schede rondom de axonen van de zenuwcellen, resulterend in een verlaging van de snelheid van de zenuwimpuls geleiding. In deze studie bestuderen wij het effect van plantensterolen (PS), de plant analogen van zoogdierlijk cholesterol, op remyelinisatie. PS zijn natuurlijke agonisten van lever X receptoren (LXRs), die de cholesterol homeostase controleren, hetgeen belangrijk is voor remyelinisatie. PS kunnen doorheen de bloed-brein barrière (BBB) migreren, en eerder werd al beschreven dat zij anti-inflammatoire en sterol regulerende eigenschappen hebben. Omwille van deze redenen, stellen wij als hypothese dat PS remyelinisatie positief beïnvloeden door LXRs te activeren in microglia.

Materialen & Methode: In een eerste instantie werden de effecten van PS op de expressie niveaus van cytokines, chemokines, groeifactoren en LXR respons genen in microglia onderzocht. Om daarna de indirecte mechanismes van remyelinisatie te onderzoeken, werd PS-geconditioneerd medium van de microglia overgebracht naar de oligodendrocyten. Als laatste maakten we gebruik van een organotypisch cerebellair model om het remyelinisatie stimulerend vermogen van PS in een multicellulaire omgeving op te helderen.

Resultaten: In onze resultaten konden wij geen LXR activatie door PS in microglia onderscheiden. Bovendien waren PS slechts matig betrokken bij het inflammatoir profiel van microglia. Bovendien werd ook de myelinisatie in oligodendrocyten slechts miniem beïnvloed door een PS-geconditioneerd medium transfer. Ook in een multicellulaire omgeving stimuleerden de PS de remyelinisatie niet.

Discussie & conclusie: De rol van PS op microglia in remyelinisatie lijkt beperkt te zijn. Op een multicellulair niveau kon remyelinisatie niet gestimuleerd worden door toevoeging van PS. In tegenstelling tot eerdere studies konden wij dus geen LXR activatie door PS bevestigen.

Sleutelwoorden: Multiple Sclerosis, plantensterolen, lever X receptor, remyelinisatie

1 Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory, auto-immune and neurodegenerative disease of the central nervous system (CNS). It is characterized by demyelination, focal T cell and macrophage infiltrates, oligodendrocyte loss and axonal damage (3, 4).

MS can be divided into three phenotypes. The first is primary progressive MS (PPMS), which has the lowest inflammatory and highest neurodegenerative impact. Relapses do not occur often, but a chronic progressive accumulation of disability is experienced (5). The most common form of MS is relapsing-remitting MS (RRMS). Periods of complete or partial stability are followed by episodes of attacks, causing disability. At onset, about 85% of MS patients have RRMS. More than half of these patients may develop secondary progressive MS (SPMS) in time. During SPMS, no complete relapses and remissions occur, leading to gradual worsening of the disability (6).

Even though the disease affects approximately 2.3 million people worldwide, the etiology of MS is, to this day, still not completely understood. However, evidence does suggest a complex relationship between environmental and genetic factors, causing an aberrant immune response to environmental triggers in people who are genetically predisposed (3, 6). For example, the risk for MS can be increased by specific alleles of cytokines and their receptors (7). The first allele identified was the human leukocyte antigen (HLA) class II haplotype HLA-DRB 1501. Today, large genome wide association studies (GWAS) have been performed which described numerous alleles associated with the disease, including genes encoding receptors for interleukin (IL)-2, IL-7 IL-12A and IL-12β (8). Environmental factors, such as vitamin D_3 deficiency, Epstein Barr virus (EBV), systemic infections, and smoking have also been related to MS. Typically, people suffering from MS, predominantly women (twice as many women as men) (9), develop first symptoms in the third decade of life. Symptoms include motor, visual, and sensory disturbances, limb weakness, gait problems, and bladder and bowel symptoms (6).

More investigation is necessary to establish a correlation between types of nutrition and recovery in MS. Research by Hadgkiss *et al.* (10) shows a higher likelihood of a better quality of life and a lower level of disability amongst MS patients consuming a higher intake of fruit and vegetables, healthy fat,

and no meat or dairy. Swank *et al.* (11) performed a study in 1990 (a time before randomized controlled trials) in which a better health was observed in MS participants who were on a very low fat diet supplemented with cod liver or vegetable oils, during 34 years, compared to subjects who did not maintain the diet. Another, more recent study, performed by Weinstock-Guttman *et al.* (12) demonstrated a relapse rate reduction and physical and mental health improvements in subjects maintaining a low fat diet supplemented with fish oil. Further, fruits and vegetables obtain intrinsic compounds which can affect inflammatory and oxidative pathways, by for example phytosterols, polyphenols and caretenoids. Dietary phytosterols, found in plants, legumes, nuts and seeds, are thought to have cholesterol-lowering and immunomodulatory effects (10, 13). These findings stimulate an increase fruit and vegetable intake amongst MS patients.

1.2 Pathophysiology of MS

MS is pathophysiologically featured by blood-brain barrier (BBB) leakage, destruction of myelin sheaths, oligodendrocyte damage, cell death, axonal damage and loss, glial scar formation and the presence of inflammatory infiltrates. The inflammatory infiltrates are mainly autoreactive T cells, macrophages, and mast cells (14). During viral infections or inflammatory stimulation, lymphocytes, mostly myelin autoreactive T cells, migrate through the BBB after autoreactive activation in the periphery (7, 15). The secretion of C-X-C and C-C chemokines by activated microglia contribute to the recruitment of macrophages and T lymphocytes from the periphery, as well as to the migration of microglia to sites of CNS inflammation (16). In the CNS, the autoreactive T cells, such as Th17 cells, are reactivated and release pro-inflammatory cytokines, such as IL-6 and Tumor Necrosis Factor alpha (TNFα). They then initiate and propagate an auto-immune response against the CNS (7, 15). Myelin proteins, such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and proteolipid protein (PLP), lipids, including sulfatide, sphingomyelin and oxidized lipids, and glycans are identified as possible autoantigens (7).

Demyelination of axons is a main characteristic of MS (17), partially because the pathophysiology of MS involves an attack of the host immune system on oligodendrocytes. These cells are important for synthesis and maintenance of the myelin sheaths surrounding the axons of the neurons within the CNS (3). The main function of these sheaths is insulation of the axons to guarantee fast saltatory conduction of action potentials (17).

To this day, there is no cure available for MS. However, treatments, aiming to reduce the immune response do exist. The first treatment for MS was interferon-β1b (IFNβ1b) and got FDA approved in

1993. Since then, other treatments have been introduced with different modes of action and side effects. These medications include different beta-interferons, glatiramer acetate, Tysabri, Gilenya, and mitoxantrone. Also, a large number of clinical trials on different medications are being conducted. Developing therapies targeting the neuro-axonal pathology, stays a challenge. New therapies focussing on repair processes (such as remyelination) therefore hold promise. At the moment, a lot of effort is focused on the development of safer and orally available medications (18).

1.3 The importance of the glial cells in (re)myelination

Besides neurons, the most prevalent cells in the CNS are the glial cells, being astrocytes, microglia and oligodendrocytes (figure 1) (2).

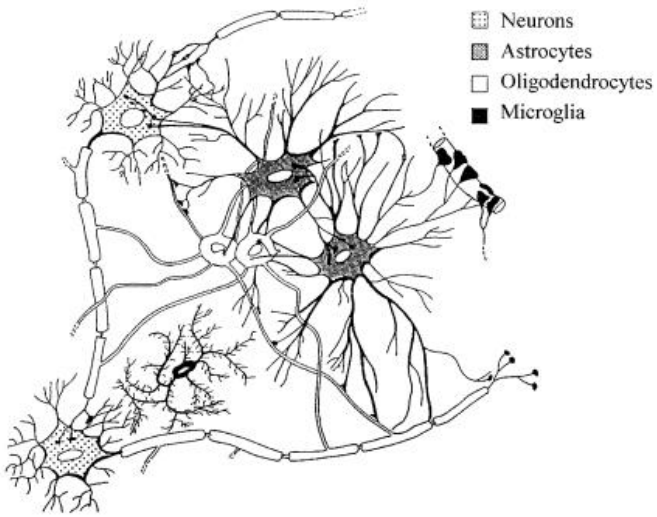

Figure 1: Representation of the different cells in the CNS. Neurons (black dots) are represented with myelinated axons. The glial cells, being astrocytes (grey), oligodendrocytes (white) and microglia (black) can also be observed with interactions between the different cell types and between the same cell types (2).

Astrocytes are star shaped, stellate cells with processes which are in contact with multiple cells and cell types in the CNS (2). Astrocytes are present in white as well as in grey matter and their end-feet are in close relation with cerebral endothelial cells, lining the BBB. In a healthy CNS, these glial cells support neurons and perform immune-related actions by secreting multiple factors, such as cytokines and chemokines. In MS, astocytes stimulate as well as restrain neuroinflammation and tissue damage (19). Astrocytes have previously been shown to promote oligodendrocyte survival *in vitro* through secretion of growth factors such as platelet-derived growth factor (PDGF) and leukemia inhibitory factor (LIF). Furthermore, these cells can stimulate remyelination, by supporting/directing oligodendrocyte precursor cell (OPC) migration, proliferation, and differentiation, via excretion of trophic and chemotactic factors (figure 2) (1, 2, 20). In contrast, astrocytes are also involved in degeneration and demyelination, by promoting inflammation and damaging oligodendrocytes and axons, subsequently leading to glial scarring. Astrocytes can achieve these effects in multiple ways, for example by secreting more pro-inflammatory (such as IL-1 and TNF-α) or anti-inflammatory (such as IL-10 and IL-11) cytokines,

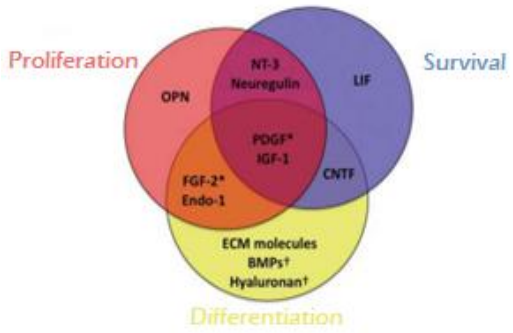

Figure 2: The influence of factors secreted by astrocytes on OPC proliferation, differentiation and/or survival (1).

† negative regulators of OPC differentiation

* positive and negative influence on oligodendrogenesis

creating more or less inflammatory damage respectively. Secretion of chemokines, such as CCL2, help attract other inflammatory cells to the damaged site. Other ways are by limiting the availability of cholesterol to aid in remyelination, or by excreting less neurotrophic factors, responsible for the survival, the growth and the maintenance of neurons (20).

The role of microglia in MS has also been shown to be double. On one hand, activated microglia can be toxic to neuronal and oligodendrocyte precursor cells, release proteases, inflammatory cytokines and free radicals, and recruit and reactivate T lymphocytes in the CNS. On the other hand, microglia are known to stimulate axon regeneration, assist in promoting remyelination, clear myelin debris, and release growth- and neurotrophic factors (21). This is in line with studies demonstrating a switch from pro-inflammatory M1 microglia to a more anti-inflammatory M2 phenotype at onset of remyelination. The classically activated or M1 phenotypes, activated by lipopolysaccharide (LPS), TNF-α or IFNγ, are associated with release of pro-inflammatory cytokines, reactive oxygen species (ROS), and nitrogen species. The alternative or M2-polarized phenotypes, activated by transforming growth factor beta (TGF-β), IL-4 or IL-13, secrete anti-inflammatory cytokines (22, 23). Recent work by Franklin *et al.* (22) has demonstrated that M2 cell depletion inhibits oligodendrocyte differentiation. The chronic stages of MS are featured by a block in oligodendrocyte differentiation. An enhancement of oligodendrocyte differentiation was seen *in vitro*, when using M2 cell-conditioned media, and an impairment was seen *in vivo* after an intralesional M2 cell depletion (22). Together, these results illustrate the importance of myelin clearance by regenerative microglia and macrophages.

Oligodendrocytes exist in different maturation stages. First, they originate from mitotic oligodendrocyte precursor cells (OPCs) that differentiate into pre-oligodendrocytes progenitors, immature oligodendrocytes, non-myelinating mature oligodendrocytes and eventually myelinating mature oligodendrocytes (figure 3). Every maturation stage can be distinguished by the expression of developmental markers, a panel of cell specific antibodies, such as A2B5, O4 and MBP (2).

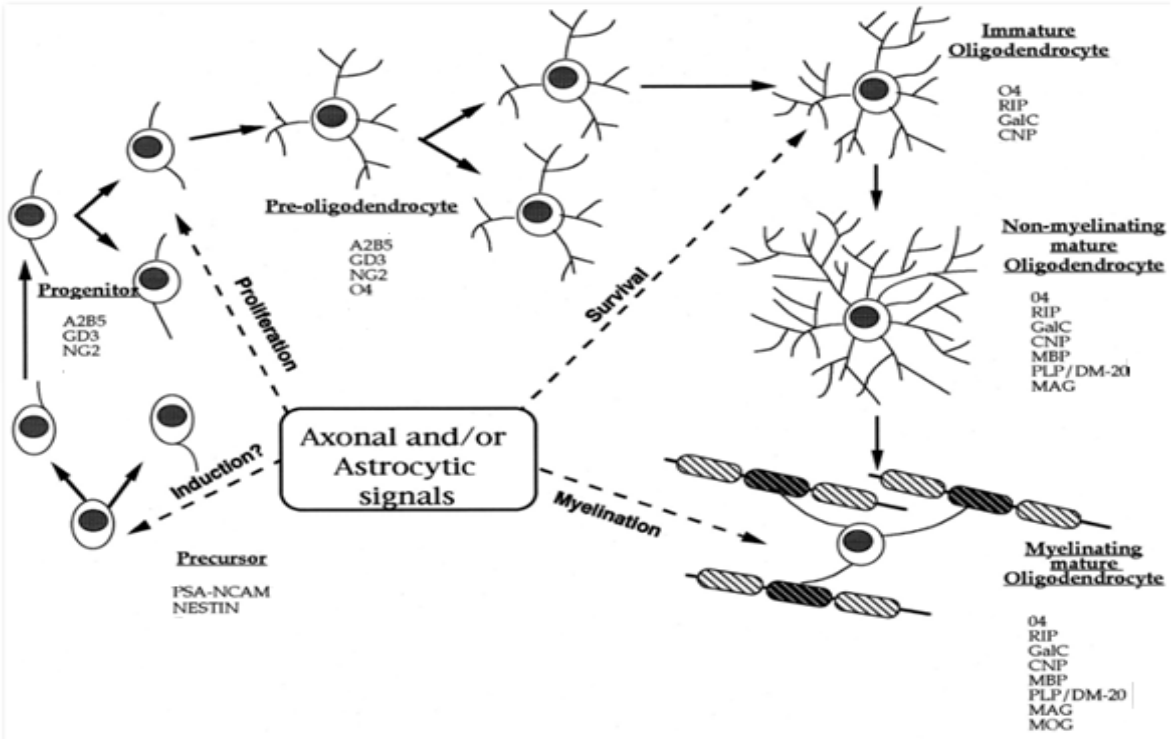

Figure 3: The maturation stages of oligodendrocytes. The dotted lines indicate the timing of axonal and/or astrocytic signaling. The different stage-specific markers are summed up underneath the developmental stage name (2).

1.4 Cerebral cholesterol homeostasis, a role for Liver X receptors

Myelin mainly consists of proteins (30%) and lipids (70%), of which 25% is cholesterol. The brain is the most cholesterol-rich organ in the body, originating from *in situ* synthesis by developing neurons and glial cells. (17)

Deficits in cholesterol homeostasis, result in severe neurological disability. Moreover, disturbances in the cholesterol metabolism are involved in CNS disorders, such as Alzheimer's disease (AD), MS, and amyotrophic lateral sclerosis (ALS) (24). A reduction in cholesterol synthesis by oligodendrocytes leads to impaired myelination, which is associated with MS and other demyelinating diseases (25).

Modulation of the liver X receptors (LXRs), which are nuclear receptors crucially involved in directing the cellular cholesterol homeostasis and regulation of inflammation, is thought to be involved in controlling diverse neurodegenerative diseases. Two receptor isoforms are described: LXRα (NR1H3) and LXRβ (NR1H2). These receptor isoforms belong to the nuclear receptor superfamily of ligand activated transcription factors. The human LXRα gene is located on chromosome 11p11.2 and the

LXRβ gene is located on chromosome 19q13.3. LXRα has a higher expression in metabolically active tissues (liver, small intestine, kidney, macrophages and adipose tissue), while LXRβ is more ubiquitously expressed, with high levels found in the developing brain (26). When comparing their DNA-binding domain and ligand-binding domain, the receptors show 80% similar amino acid identity. In addition, the genes have been shown to be highly conserved in mammals (27, 28). Preceding binding to LXR response element (LXRE) of its target genes, consisting of a direct repeat of 5'-AGGTCA-3', LXR activation requires heterodimerization with the retinoid X receptor (RXR) (29). Activation of specific RXR subtypes was recently shown to be critically involved in CNS remyelination. (17, 30). LXRs regulate multiple genes involved in lipid metabolism, such as those participating in reversed sterol transport (cholesterol efflux) and fatty acid biosynthesis (27). The activated genes include the ATP-binding cassette transporters ABCA1 and ABCG1, lipoprotein lipase, sterol regulatory element-binding protein 1c (SREBP-1c) and apolipoprotein E (ApoE) (31). LXRs act as cholesterol sensors. When the concentration of intracellular cholesterol increases, LXRs induce the transcription of genes that protect cells from a cholesterol overload (27, 28). When a cell is loaded with cholesterol, resulting in an increased production of oxysterols, LXRs get stimulated and upregulate the expression of ABCA1 and ABCG1 sterol transporters. Subsequently, cholesterol efflux is enhanced from the plasma membrane to lipid-poor ApoA-I and high density lipoprotein (HDL), respectively. This way, a regulatory feedback mechanism is provided, maintaining a constant cholesterol content (32). Even though cholesterol itself is not a ligand for LXRs, its metabolites, such as the oxysterols 22(R)-hydroxyxholesterol, 24(S),25 epoxycholesterol and 27-hydroxycholesterol can activate LXRs (33).

Alternatively, LXRs are involved in transrepression of pro-inflammatory genes. After ligand binding and SUMO-modification, an interaction of LXR with a subunit of the transcriptional nuclear receptor corepressor (N-CoR) complex is promoted (34). Consequently, no dissociation of the N-CoR complex from nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) occurs, and the transcription of pro-inflammatory genes is blocked (29, 35).

As LXRs are master regulators of cholesterol homeostasis, tightly regulate the inflammatory response, and have the potential to induce remyelination via the permissive LXR/RXR heterodemerization, these nuclear receptors are very interesting therapeutic targets.

1.5 Plant sterols

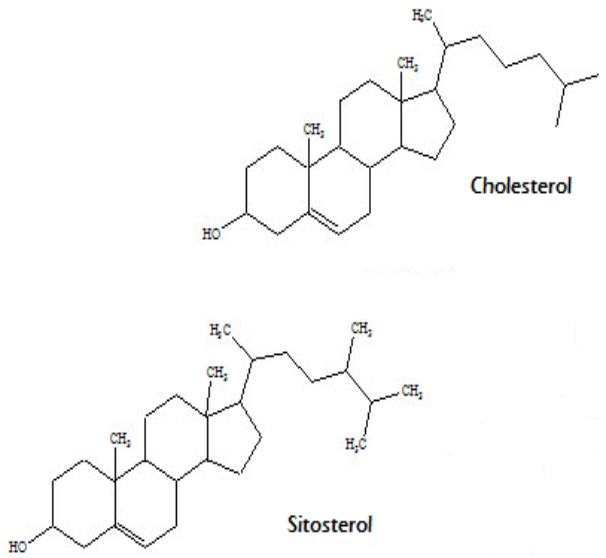

Figure 4: Structures of animal sterols and plant sterols. Cholesterol (the mammalian sterol) and sitosterol (a plant sterol) show similar structures, as is the case with many other plant sterols.

Plant sterols (PS) resemble mammalian cholesterol both structurally and functionally. The main plant sterols, such as sitosterol and campesterol, differ from cholesterol in their side chain by the addition of an ethyl or methyl group at C24 and/or a double bond at C22 (figure 3) (36). The only source of plant sterols is dietary intake, through for example fruits, vegetables, cereals, and nuts (37). Depending on the diet, the most prevalent PS are sitosterol (65%), campesterol (32%) and stigmasterol (3%). The average intake of cholesterol and PS (+/- 300 mg/day) is comparable in a Western diet. However, the absorption difference of both sterols, 30-70% of the available cholesterol is absorbed in contrast to 5% of the dietary available plant sterols, leads to low PS concentrations in the plasma (36, 38).

Accumulation of cholesterol in plasma is a risk factor for development of atherosclerosis and coronary heart disease (CHD) (39). High levels of PS supplementation have been shown to reduce plaque formation in studies concerning atherosclerosis (40). For decades, PS enriched dairy products have been marketed as functional food to lower plasma cholesterol concentrations (37). Indeed, research has shown that plant sterol esters are able to dose dependently decrease serum low density lipoprotein (LDL) cholesterol concentrations (41). The effect may be caused by a reduction in cholesterol absorption into the circulation from the intestinal lumen, through a competition between plant sterols and cholesterol for incorporation into mixed micelles. The intracellular cholesterol levels can also be secreted into the intestinal lumen by the activation of the nuclear LXR target genes, ABCG5 and ABCG8. Since PS are easily taken into enterocytes but are poorly absorbed to the plasma, hypertriglyceridemia, occurring when using synthetic LXR agonists such as T0901317 (T09) and GW3965 (GW), is not present (42). However, this is not the case in patients with sitosterolemia, an autosomal recessive disorder characterized by defective ABCG5 and/or ABCG8, which are normally only significantly expressed in hepatocytes and enterocytes (43). PS are, in contrast to cholesterol,

the preferred substrates of these transporters. In these patients, increased serum PS concentrations can be observed, since a defect in one or both of the transporters leads to a reduced biliary excretion, also shown in mice with an inactivated ABCG5 and/or ABCG8. The result of this defect could be a development of premature CHD and accelerated atherothrombosis (43, 44).

In contrast to cholesterol, PS are able to cross the BBB, enabling them to affect CNS specific cell types (45). Indeed, PS have been shown to be beneficial in animal studies concerning MS (13). However, the conducted research was mainly based on their immunomodulatory potential and behavioural changes upon intake. PS can for example protect against the development of experimental autoimmune encephalomyelitis (EAE), an animal model for MS, by reducing infiltration and inflammation of immune cells into the CNS, leading to a decreased demyelination. An up-regulation of 10% of the anti-inflammatory IL-10 was shown, while pro-inflammatory CCL2 was inhibited by 50%. Furthermore, PS seemed to delay disease onset and to reduce the severity of EAE (13).

Overall, PS could stimulate remyelination by activating LXRs in astrocytes and microglia, causing a cholesterol efflux, which could then be taken up by oligodendrocytes via their LDL receptor and be used to (re)myelinate axons. PS could also have an anti-inflammatory effect (because of chemokines, cytokines or inflammatory mediators). The results of these effects may cause PS to be important for the recovery of MS.

1.6 Aims

PS are known to be beneficial in studies concerning MS. Previous research has been performed on immunized EAE mice. Experiments on this model organism has led to more information about the effects of PS on inflammation. The effects of PS in MS, and especially on (re)myelination of the neuronal axons, is a relatively new topic, limiting the amount of information available about this subject. PS seem to have multiple benefits, causing them to be of interest in studies concerning MS and other diseases, such as Azheimer's disease. Not only are they able to cross the BBB, and have the potential to induce myelin-associated genes, such as MBP, they are also LXR agonists, important for cholesterol metabolism, and have anti-inflammatory effects, all very important for recovery in MS.

In this study, we will investigate whether LXR activation occurs in microglia treated with PS, and whether LXR activation in these cells is beneficial for remyelination. The hypothesis underlying this research is that PS both activate LXRs in microglia, leading to cholesterol efflux, taken up by oligodendrocytes, and lead to beneficial circumstances (such as excretion of more anti-inflammatory

cytokines) for remyelination of the CNS to occur. To (dis)prove this hypothesis, the potential of PS to enhance remyelination via LXRs within a multicellular environment will be determined by using an *ex vivo* organotypic cerebellar slice model. Next, the effect of PS on microglia will be studied *in vitro*, using both primary cells and cell lines. Lastly, the effects of LXR activation in microglia on oligodendrocytes will be studied *in vitro* by medium-transfer experiments.

To our knowledge, no such studies have been published, and more information concerning the molecular mechanisms is necessary. This study could eventually lead to more understanding of the remyelination process and it could also lead to the discovery of new targets to help develop new therapies.

2 Materials and methods

2.1 Animals

Six female and two male C57BL/6 mice were purchased from Harlan Netherlands B.V. (Horst, The Netherlands). Pups from these mice were used for the experiments in this research project. The animals were housed in the animal facility of the Biomedical Research Institute of the University of Hasselt in Diepenbeek. The institutional guidelines were respected during the conduction of the experiments, which were approved by the ethical committee of animal experiments of Hasselt University.

2.2 Cell lines and primary mouse cells

2.2.1 Cell culture

The BV-2 microglial cell line (a gift from prof. Frank Stassen, Maastricht University) was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S). The cells were incubated at 37°C and 5% CO_2.

The Oli-neu (oligodendrocyte) cell line (a gift from prof. Jack van Horssen VUmc) was cultured in SATO medium with 2% FCS. Cells were harvested and seeded in 24-well plates and were subsequently incubated at 37°C at 8.5% CO_2.

Primary mouse cells were isolated from mouse brains (P1-P2) after mechanical dissociation, and the homogenate was cultured in T125 culture flasks in DMEM containing 10% FCS and 1% P/S. Two-thirds of the medium was refreshed on day 4, 7, 11 and 14. Starting from day 7, 5µg/ml Bovine insulin (Sigma) was added. A shake-off was conducted to collect the different glial cell types. After a preshake (180 rpm, 1h), microglia were collected. Next, OPCs were isolated from the astrocyte monolayer by an overnight shake-off (240 rpm, 18h).

2.3 Direct effects on expression levels in microglia and macrophages

Prior to investigation of the expression levels of different cytokines, chemokines, neurotrophic factors and LXR (target) genes in the BV-2 cell line, cells were treated for 24 hours with ethanol (EtOH), 25 µM plant sterol mixture (Ingmar Wester, Raisio) containing sitosterol (45%), campesterol (15%) and stigmasterol (30%), 2 µM LXR agonist GW3965 (a gift from prof. Knut Steffensen) or 25 µM cholesterol. The BV-2 cells were incubated at a density of 1.10^5-1.10^6 cells/ml in a 24-well plate (100 µl). After 18 hours of incubation, IFNɣ and IL-1β (Sigma-Aldrich, 100 ng/ml) were added to stimulate the cells. Next, a time-dependent experiment was performed, incubating BV-2 microglia overnight in a 24-well plate (500 µl) at a density of $2{,}5.10^5$ cells/ml, giving the cells time to first properly attach. Subsequently, conditions were added for 24 hours. In half of the wells IFNɣ and IL-1β were added for 6, 12 or 23 hours, after which RT-qPCR was performed.

2.3.1 Indirect effect on oligodendrocyte expression levels

Primary macrophages or BV-2 cells were cultured in a 24-well plate (100 µl) at a density of 1.10^5 – 1.10^6 cells/ml in DMEM containing 10% FCS and 1% P/S. The cells were exposed to different concentrations of PS mixture or cholesterol (0 µM, 10 µM, 25 µM and 50 µM) for 72 hours. Next, the medium was removed and replaced by SATO medium after washing the cells once with phosphate buffered saline (PBS). After 24 hours, the medium was transferred into a new 24-well plate containing attached oli-neu cells (4.10^4 – 4.10^5 cells/ml) for 24 hours. Next, RT-qPCR was used to measure expression levels of myelin basic protein (MBP) and proteolipid protein (PLP).

2.4 Real-time qPCR

Total RNA was extracted using the Qiagen RNeasy mini kit, according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop spectophotometer (Isogen Life science). cDNA synthesis was performed using Qscript cDNA SuperMix (Quanta Biosciences) according to the manufacturer's instructions. In brief, 500ng RNA was dissolved in a mixture of RNase free water and QScript. After PCR (protocol: first 5 minutes at 25°C, then 30 minutes at 42°C, afterwards 5 minutes at 85°C and the incubation was held at 4°C), RNase free water was added, to achieve an end concentration of 5 ng/µl RNA.

A 96-well plate was prepared, containing 25% cDNA and 75% mastermix (66.67% µl SYBR Green, 4% 1/10th diluted forward primer and 1/10th diluted reverse primer and 25.33% RNase free water). Results were analyzed using the relative quantification ($\Delta\Delta C_T$) method. Normalization of gene expression was achieved by determining the most stable reference genes. For this, expression of various reference gene candidates were assessed by RT-qPCR and analyzed with GeNorm (46). Primers were chosen according to literature or designed using Primer-Express (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (table 1). The reference genes tested were YWHAZ, TBP, ACTB, HMBS, CYCA, PGK1, RPL13A and 18S.

Table 1: Forward and reverse primers of the genes used

Name experimental gene	Forward primer	Reverse primer
CCL2	GGCTCAGCCACATGCAGTTAA	AGCCTACTCATTGGGATCATCTT
CCR2	CAGGTGACAGAGACTCTTGGAATG	GAACTTCTCTCCAACAAAGGCATAA
TNFα	CCAGACCCTCACACTCAG	CACTTGGTGGTTTGCTACGAC
IL-6	TGTCTATACCACTTCACAAGTCGGAG	GCACAACTCTTTTCTCATTTCCAC
IL-1β	ACCCTGCAGCTGGAGAGTGT	TTGACTTCTATCTTGTTGAAGACAAACC
IL-10	AATAACTGCACCCACTTCCCA	CAGCTGGTCCTTTGTTTGAAAG
LXRα	TGTTGCAGCCTCTCTACTTGGA	TCTGCAGACCGGCCCAACGTG
LXRβ	AAGGACTTCACCTACAGCAAGGA	GAACTCGAAGATGGGATTGATGA
ABCA1	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
ABCG1	CAAGACCCTTTTGAAAGGGATCTC	GCCAGAATATTCATGAGTGTGGAC
SREBP1c	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGCCCAG
ApoE	CCTGAACCGCTTCTGGGATT	GCTCTTCCTGGACCTGGTCA
TGFβ	GGGCTACCATGCCAACTTCTG	GAGGGCAAGGACCTTGCTGTA
LIF	CGCCTAACATGACAGACTTCCCAT	AGGCCCCTCATGACGTCTATAGTA
NGF	CAGACCCGGAACATCACTGTA	CCATGGGCCTGGAAGTCTAG
PLP	TTGTTTGGGAAAATGGCTAGGA	GCAGATGGACAGAAGGTTCGA
MBP	TCACAGAAGAGACCCTCACAGC	GAGTCAAGGATGCCCGTGTC
YWHAZ	GCAACGATGTACTGTCTCTTTTGG	GTCCACAATTCCTTTCTTGTCATC
RPL13a	GGATCCCTCCACCCTATGACA	CTGGTACTTCCACCCGACCTC
CYCA	GCGTCTCCTTCGAGCTGTT	AAGTCACCACCCTGGCA
PGK1	GAAGGGAAGGGAAAAAGATGC	GCTATGGGCTCGGTGTGC

2.5 Nitric oxide assay

Nitric Oxide (NO) levels were measured using the Griess Reagent System (Promega). 50 µl experimental medium from each well was added to a 96 well plate. An equal amount of sulfanilamide solution was added to each well and incubated for 10 minutes at room temperature, protected from light. Next, 50 µl of N-1-naphthylethylenediamine dihydrochloride (NED) was added

to each well and incubated for 10 minutes at room temperature, again protected from light. Absorbance was measured within 30 minutes, at 550 nm using a microplate reader (BIORAD).

2.6 Immunocytochemistry

Antibodies used include: The early oligodendrocyte marker A2B5 (1/1000, Millipore), the mature oligodendrocyte marker MBP (1/500, Millipore), the oligodendrocyte marker Olig 2 (1/500, Millipore), Alexa fluor 555 goat anti-mouse IgM for A2B5 (1/600, Invitrogen), Alexa fluor 488 donkey anti-rabbit for Olig 2 and Alexa fluor 647 gooat anti-rat for MBP.

In short, the cells were first fixated in 4% paraformaldehyde (PFA) during 20 min at room temperature. Next, they were washed using 1x PBS, after which a blocking took place for 30 min using 1x PBS with 0.5% Triton-X-100 + 10% goat/donkey serum (Millipore). After this time-period the primary antibodies were added. The incubation took place at room temperature during 2 hours. Next, the secondary antibodies were added. After a 45 min incubation period at room temperature, the cells stained with 4',6-diamidino-2-phenylindole (DAPI) during 10 minutes. Finally, they were mounted with anti-fade mounting medium before examination (Leica fluorescence microscope).

2.7 Luciferase reporter assay

Bacterial transformation necessary for the luciferase reporter assay was previously performed on DH5α *E. coli* cells by other members of our research group. Plasmids were isolated from these pellets according to the Plasmid Midi kit (Qiagen) protocol. Afterwards, the luciferase reporter assay was initiated. In a first step, $18.3.10^4$ cells/ml growth medium (DMEM containing 10% FCS and 1% P/S) were seeded in a 60 mm cell culture dish (BD Falcon 353004) and were incubated (37°C, 5% CO_2) until cells were attached to the culture dishes. Since human embryonic kidney (HEK) cells are known to be easily transfected, these cells were tested alongside the cells of interest, the BV-2 cells. After attachment, the growth medium was replaced by new growth medium with plasmid mix, and the dishes were incubated overnight at 37°C and 5% CO_2. The plasmid mix contained 50% plasmid cocktail (100 ng/µl pG5-TK-GL3 (GAL5) 50 ng/µl pCMV-Bgal, 20 ng/µl nucleotide receptor plasmid and NaCl 0.9%) and 50% transfection mix (6% JetPEI (Polyplus 101-10) and 94% NaCl 0.9%). The next day, the cells were first trypsinised to detach them from the culture dishes, after which growth medium without FCS was added and this medium was pipetted into a 96-well plate. The plate was then incubated at 37°C for 5-6 hours. When the cells were attached properly, they were treated with

EtOH (control), and the LXR agonists, 2 µM GW and T09. The plates were then incubated at 37°C and 5% CO_2 for 18 hours, after which the cells were washed with PBS and subsequently lysed. The lysis buffer (1 plate) contained 25% Glycyl-Glycine (Sigma, G-3028, 100 mM, pH 7.8), 25% Triton 4x (Sigma, X100), 15% $MgSO_4$ (Sigma, M-2643, 100 mM), 1.6% EGTA (Sigma, E-3889, 250 mM) and 33.4% MilliQ.

For the luciferase assay, lysate and One-Glo (Promega, E6120) were added in a white 96-well plate (Greiner BIO, G55075). Luminescence was measured in a luminometer (FLUOstar OPTIMA, BMG Labtech). β-galactosidase was used as an internal control. For this assay lysate and β-gal buffer were pipette into every well of non-ELISA 96 well plates (non-sticky, Greiner BIO 655101). The β-gal buffer contained 80% buffer Z (10% Na_2HPO_4 (VWR prolabo) 1 M, 1% ml KCl (VWR prolabo) 1 M, 0.1% $MgSO_4$ (Merck) 1 M and 88,9% MilliQ), 20% O-nitrophenyl-beta-D-galactopyranoside (ONPG) (1 g ONGP (Sigma, N-1127), 8.2% Na_2HPO_4 (VWR prolabo) 1 M, 1.8% NaH_2PO_4 (Merck) 1 M and 90% MilliQ) and 3.4 µl β-mercaptoethanol (Fluka, BioChemica)) per ml Buffer Z. When a yellow color became visible, the optical densities of the samples were determined spectrophotometrically at 410 nm.

2.8 Neonatal brain slice experiments

P7 mouse pups were decapacitated, and the brains with cerebellum were submerged in filtered dissection medium containing 100 g/l glucose in 200 ml 1X PBS. After separating the cerebellum from the brain stem, and removing the meninges, the cerebellum was cut with a McIlwain tissue chopper 350 µm thick. The slices were then separated in dissection medium, after which they were placed in a 24-well plate with Millicell inserts (Millipore) in which culture medium was previously incubated overnight. The dissection medium contained 50% Basal Medium with Earle's salts (BME, Life Technologies), 2.5% Hanks' Balanced Salts Solution (HBSS, Life Techologies), 9% D-(+)-Glucose solution (Sigma), 1% GlutaMAX 200 mM (Life Technologies), 11.5% H_2O, 25% heat inactivated horse serum (Life Technologies) and 1% Penicillin-Streptomycin (5,000 U/ml, Life Technologies). At the end of dissection the culture medium was changed after heating it to 37°C (400 µl/well). This medium was changed every 2 or 3 days by replacing half the medium.

After two days, lysophosphatidylcholine (LPC), dissolved in chloroform:methanol (50:50), was added to the medium to demyelinate the brain slices. After 16 hours, the medium was completely removed, and new medium was added in a different plate. The same day, EtOH, PS mixture (25 µM), cholesterol (25 µM) or the positive control, a PDE 4-inhibitor was added. Medium was changed every

2 or 3 days by replacing half the medium with culture medium containing one of the conditions for 1 and 2 weeks. To examine remyelination regarding to the complete axonal network, immunolabeling was performed using MBP and neurofilament (NF) antibodies (Millipore). To quantify remyelination, lines were drawn on the pictures resulting from fluorescence microscopy, and the ratio of non-myelinated to myelinated axons was made.

2.9 Statistics

Data were analyzed using Graph Pad Prism 6 software and are expressed as as mean values ± standard error (SEM) for 'n' experiments.. Normality was tested using the D'Agostino and Pearson omnibus normality test. When the data were normally distributed, an analysis of variances (ANOVA) was used. If not normally distributed, data were first transformed using the statistical software R. The Kruskal-Wallis test was used when data could not be normally distributed. *P<0.05, **P<0.01 and ***P<0.001.

3 Results

The main objective of this study was to identify which mechanism underlies the effects of PS, observed previously by Jo Mailleux *et al.* (unpublished results), on MBP and PLP expression. Experiments performed on oligodendrocytes by our research group showed that unlike a synthetic LXR agonist, PS significantly upregulated the MBP gene and the myelin PLP gene. This indicated that PS did not directly affect oligodendrocytes via LXRs. (Jo Mailleux *et al*., unpublished results). An indirect effect however could not be excluded. The indirect effects of PS on oligodendrocytes via astrocytes were previously examined by our research group, but no significant effects could be distinguished. In this study, microglia (cell line and primary cells) were the main cell types investigated in regards to indirect effects on remyelination. However, since remyelination was the endpoint we were interested in, experiments in a multicellular environment were also performed.

3.1 Direct effects of PS on microglia

3.1.1 No direct effects of PS on BV-2 cell expression levels of cytokines, chemokines and neurotrophic factors

Multiple factors could have an influence on the remyelination properties of oligodendrocytes. Cytokines, chemokines, neurotrophic factors and LXR (target) genes were investigated.

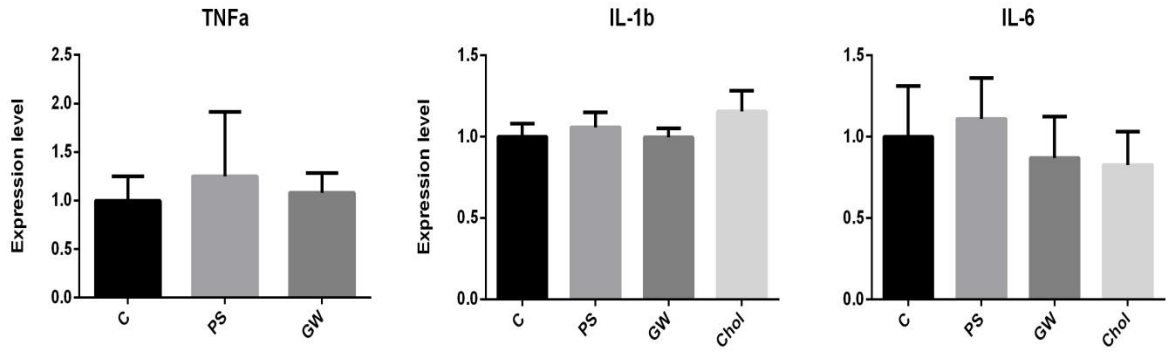

Figure 5: Expression level of the pro-inflammatory cytokines in the BV-2 microglial cell line. Cells were treated with EtOH (vehicle), 25 µM sitosterol-β, 2 µM GW 3965 or 25 µM cholesterol for 24 hours, and were subsequently stimulated with IFNɣ and IL-1β (100 ng/ml) after 18 hours of treatment. The expression level of TNFα (n=4 per group), IL-1β (n=6 per group) and IL-6 (n=6 per group) was taken into account, to establish the inflammatory features of the microglial cell line after stimulation. Data are presented as ± SEM.

In a first step, the expression level of three pro-inflammatory cytokines, TNFα, IL-1β and IL-6 was measured, after treatment with β-sitosterol (25 µM), the synthetic LXR agonist, GW3965 (2 µM) or cholesterol (25 µM). The cells were stimulated using IFNɣ and IL-1β (100 ng/ ml) after 18 hours of exposure to the different conditions. No significant lower expression of these cytokines could be observed (figure 5).

Next, the expression level of LIF, NGF and TGFβ was examined. The synthetic and natural LXR agonists did not significantly influence the expression levels. LIF and NGF were significantly upregulated by cholesterol, when compared to the control condition (figure 6).

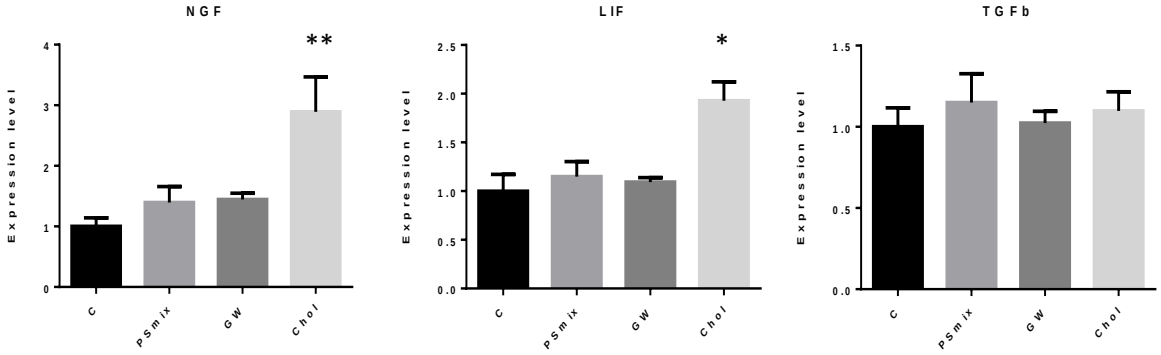

Figure 6: BV-2 expression level of NGF, LIF and TGF-β. The BV-2 cell line was treated for 24 hours with EtOH (vehicle), 25 µM PS mixture (containing 60% sitosterol, 25% campesterol and 15% stigmasterol), 2 µM GW3965 or 25 µM cholesterol. The last six hours, the cells were activated with IFNɣ and IL-1β (100 ng/ml). The synthetic and natural LXR did not significantly influence expression levels (n=6). Cholesterol (n=4) however did significantly influence NGF (**p<0.01) and LIF (*p<0.05) expression when compared to the control condition (n=6). Data are presented as ± SEM.

Apart from the cytokines, the neurotrophic factor and the growth factor, chemokines were also investigated. Chemokines are important for the attraction of monocytes and T lymphocytes. The expression levels of the chemokine CCL2 and the chemokine receptor CCR2 were measured after 24 hours exposure to one of the four conditions and six hours of TNFɣ and IL-1β stimulation. However, when comparing the expression of these factors, no significant difference could be distinguished between the different conditions (figure 7).

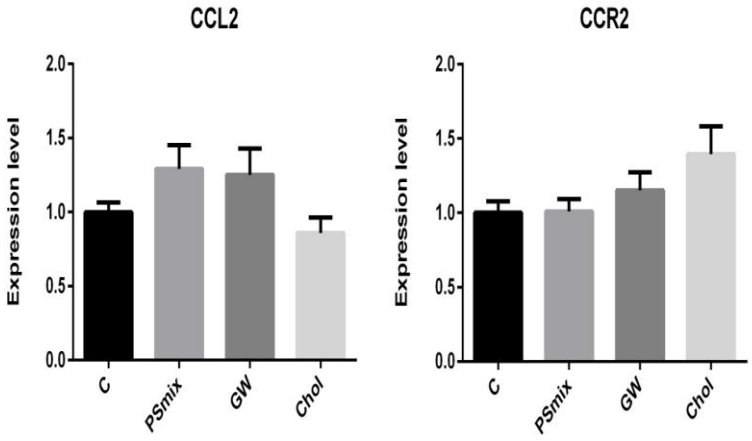

Figure 7: BV-2 cell line expression level of the chemokine CCL2 and the chemokine receptor CCR2. Expression levels of CCL2 and CCR2 are measured after 24 hours of treatment with EtOH (vehicle), 25 µM PS mix, 2 µM GW3965 or 25 µM cholesterol and stimulation by IFNɣ and IL-1β (100 ng/ml) during the last six hours of treatment. No significant differences could be achieved (n=8). Data are presented as ± SEM.

3.1.2 An LXR mediated effect of PS on BV-2 cells could not be distinguished

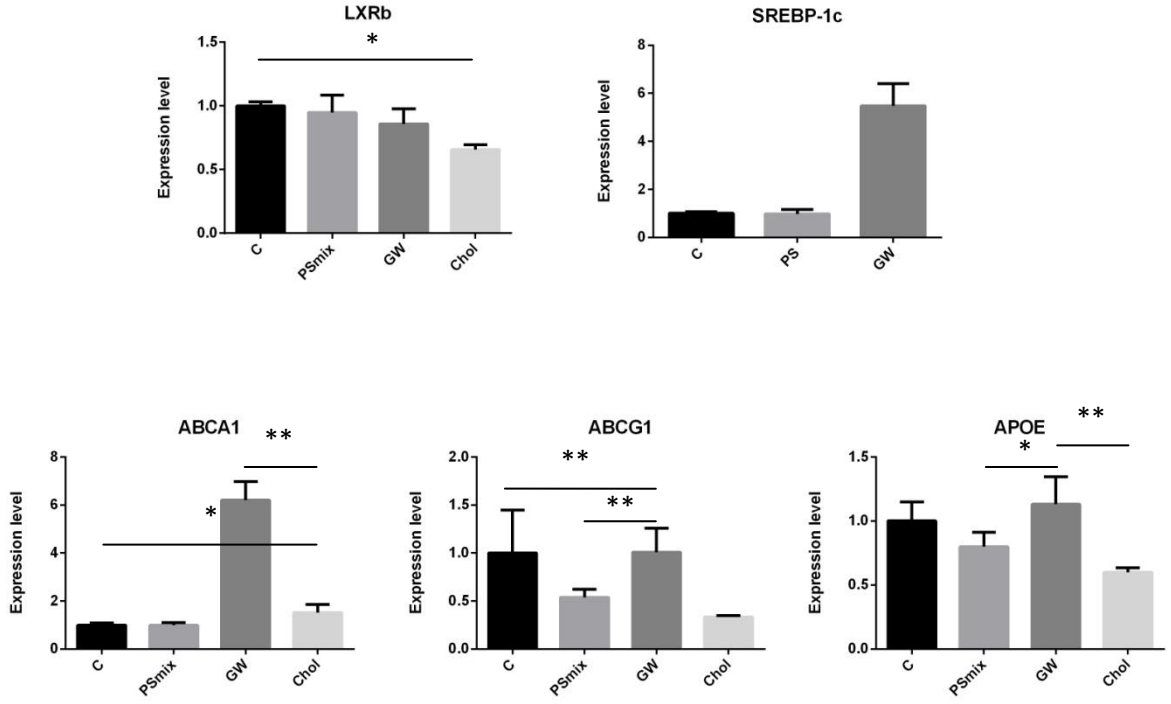

Figure 8: Expression level of LXRβ and different LXR target genes in BV-2 microglia. Cells were treated for 24 hours with EtOH (vehicle), 25 µM PS mix, 2 µM GW3965 or 25 µM cholesterol, and they were stimulated with IFNɣ and IL-1β (100 ng/ml)during the last 6 hours of treatment. GW3965 causes the highest LXR target gene expression (n=6), however, not the highest LXRβ expression (n=6). Data are presented as ± SEM (**p<0.01 and *p<0.05).

An important feature of the hypothesis underlying this research was that the effects that PS show, are at least partially caused by their agonistic properties towards the LXRs. In this regard, we were also interested in the expression levels of the LXR genes and their target genes. The synthetic LXR

agonist, GW3965, activated, as expected, all LXR target genes (figure 8). However, PS and cholesterol did not. Remarkably, GW did not significantly influence the expression level of LXRβ.

3.1.3 Effects of stimulation duration on different targets in BV-2 cells

To investigate the effect of IL-1β and IFNɣ stimulation on BV2 microglia, a time-dependent experiment was set up. EtOH, 25 µM PS mix, 2 µM GW and 25 µM cholesterol were added for a time period of 24 hours. However, this time, the BV-2 cell line was exposed to 100 ng/ml IFNɣ and IL-1β for different time periods (0, 6, 12 and 23 hours) (figure 9).

As expected, ABCA1 showed the highest expression level when the synthetic agonist was added for all the different stimulation periods. The highest level however, was observed when no stimulation was added. IFNɣ and IL-1β did not seem to contribute much in the other conditions, showing similar expression levels at every time period. The highest expression of the anti-inflammatory gene, Arginase, could be observed after six hours of stimulation, while the lowest expression is observed after twelve hours, for all the conditions. The reverse is true for the pro-inflammatory gene TNFα, showing the highest expression level at twelve hours, while six hours of stimulation does not upregulate the expression of this gene. The chemokine receptor CCR2, did not show much difference at the different time points of expression level measurement. However, an upregulation was observed after 23 hours of stimulation in the control condition. The best stimulation period for NGF was around 23 hours, or employing no stimulation at all. However, at 23 hours, all the conditions seemed to have a downregulating impact on gene expression when compared to the control condition.

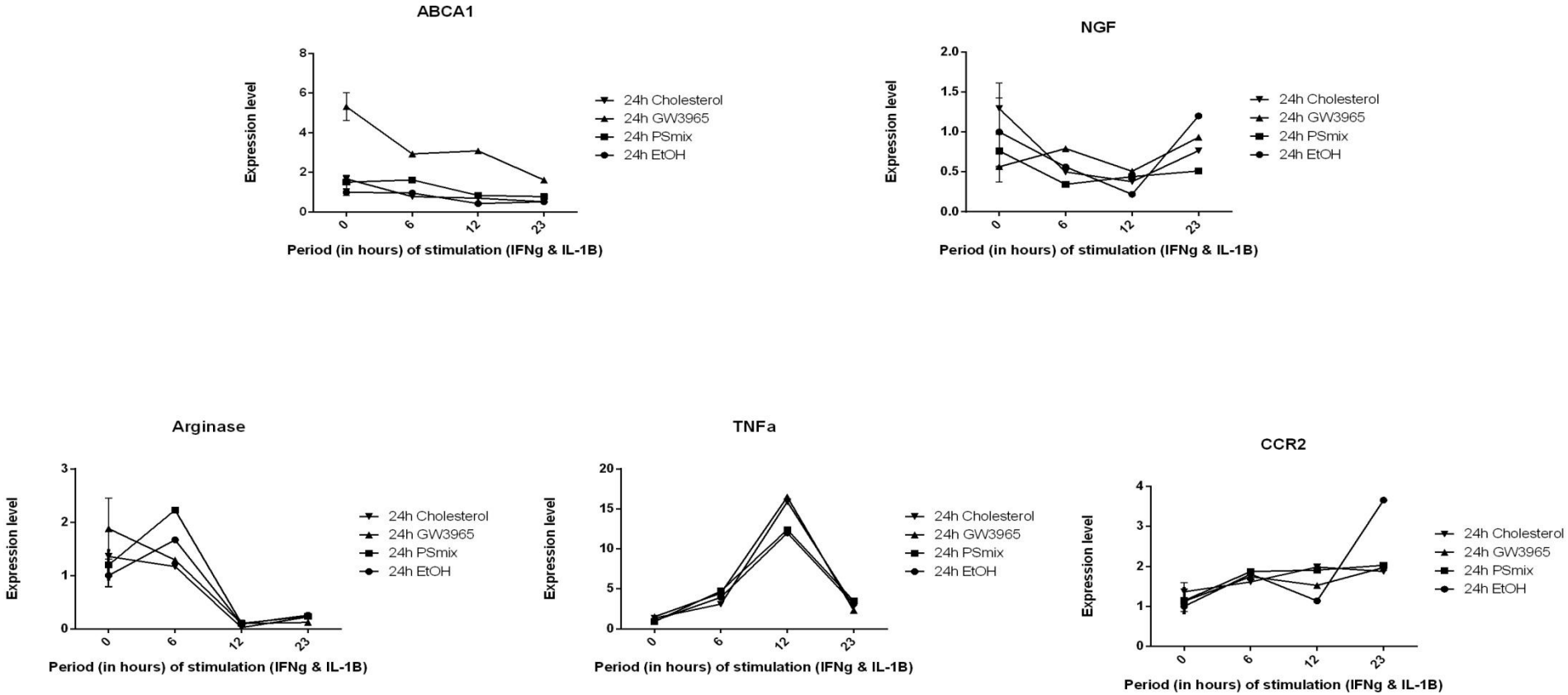

Figure 9: Time-dependent experiment of IFNɣ and IL-1β stimulation on BV-2 cells. BV-2 cells were exposed to medium containing EtOH (vehicle), GW (2 µM), PSmix (25 µM) or cholesterol (25 µM) for a time period of 24 hours. During this time, cells were stimulated with IFNɣ or IL-1β for 0 (n=3), 6 (n=1), 12 (n=1) or 23 (n=1) hours. The highest expression level of LXR target gene ABCA1 is observed when GW was added, for all the time periods. The highest expression of the anti-inflammatory gene Arginase is observed when stimulating the cells for six hours. An expression level close to zero is achieved for all the conditions when stimulation for 12 and 23 hours. The pro-inflammatory cytokine, TNFα, has the highest expression when stimulated for 12 hours for all the conditions. The chemokine receptor CCR2 does not seem to be much influenced by addition of IFNɣ and IL-1β, except after 23 hours of stimulation in the control condition. The growth factor NGF, seems to have the highest expression level at zero or 23 hours of stimulation. Data are presented as ± SEM.

3.2 Indirect effects of PS on oligodendrocytes

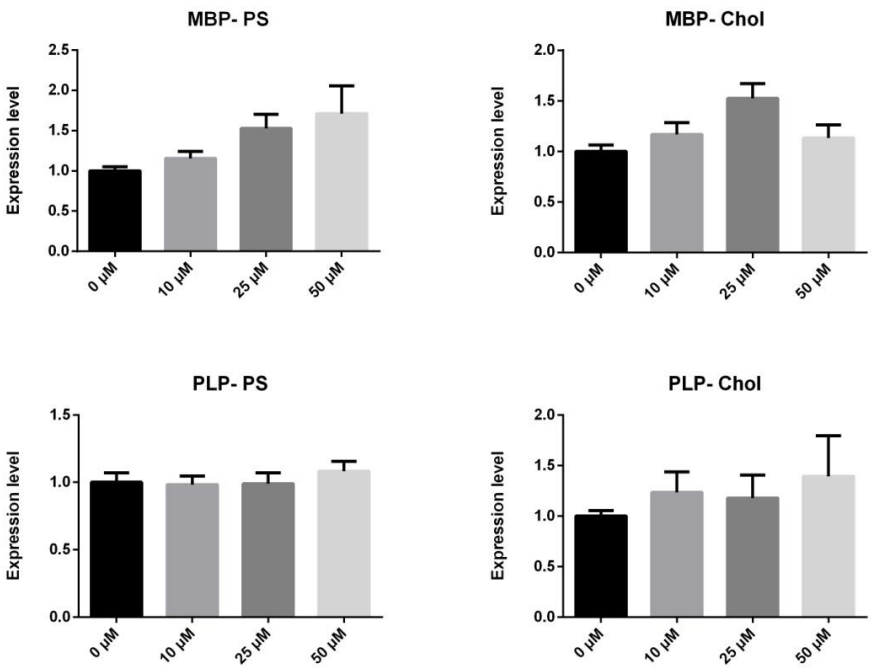

Figure 10: The indirect effect of BV-2 microglia exposed to PSmix and cholesterol on MBP and PLP expression level in oli-neu cells. The microglial cell line was exposed to DMEM with 10% FCS and 1% P/S containing different concentrations of PSmix (n=13) or cholesterol (n=7) (0 µM, 10 µM, 25 µM and 50 µM) for 72 hours. Next, this medium was replaced by SATO medium, to which the cells were exposed to for 24 hours. This medium was then transferred onto the oli-neu cell line to investigate the effect of factors excreted by microglia on the MBP and PLP expression of the oli-neu cell line. However, no significant difference could be achieved. Data are presented as ± SEM.

The expression level of MBP and PLP in oli-neu cells was investigated after being exposed to PS or cholesterol conditioned medium, transferred from BV-2 cells (figure 10) or primary macrophages (figure 11). If PS had an effect on remyelination, it would be expected to observe an upregulation of the myeline genes, preferably with a rising concentration of PS. However, the resulting gene expression levels after qPCR did not differ significantly between the different PS or cholesterol concentrations.

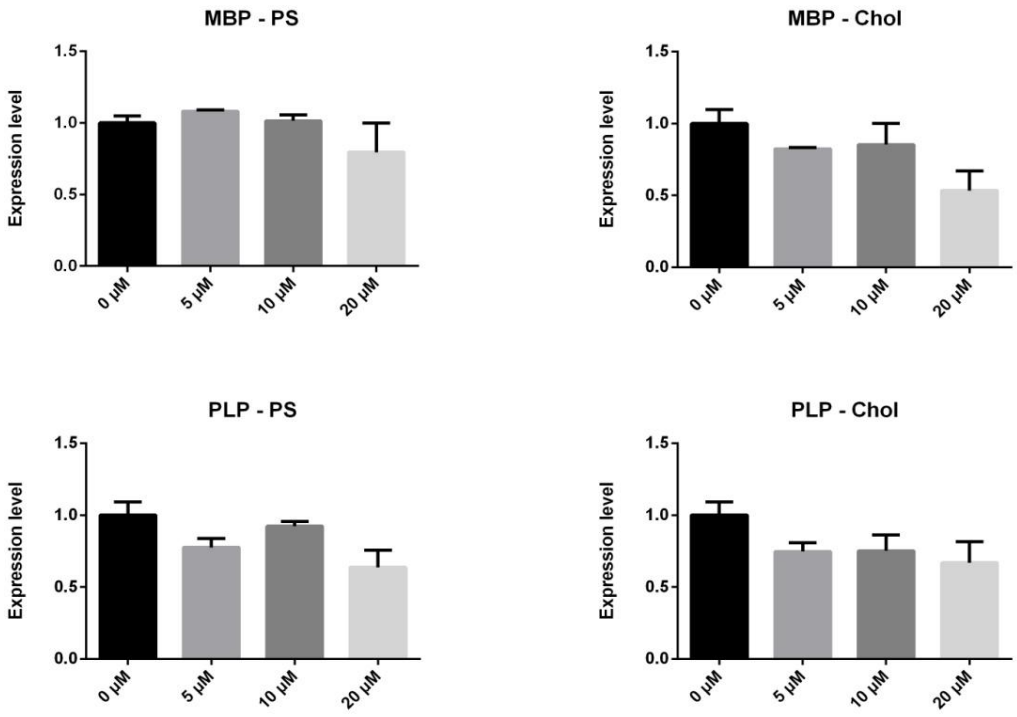

Figure 11: The indirect effect of primary macrophages exposed to PSmix and cholesterol on MBP and PLP expression level in oli-neu cells. The primary macrophages were exposed to DMEM with 10% FCS and 1% P/S containing PSmix or cholesterol in different concentrations (0 µM, 5 µM, 10 µM and 20 µM) for 72 hours. This medium was then replaced by SATO medium, to which the cells were exposed to for 24 hours. Next, medium was transferred onto the oli-neu cell line to investigate the effect of factors excreted by the macrophages on the MBP and PLP expression of the oli-neu cell line. However, no significant difference could be achieved. Data are presented as ± SEM, with n=2.

3.3 PS do not stimulate remyelination in in an organotypic cerebellar slice culture

Demyelination of the axons was obtained by adding LPC to the brain slices. Naturally occurring remyelination was then stimulated by the addition of a PDE 4-inhibitor (0.5 µM). EtOH, PSmix (25 µM) and Cholesterol (25 µM) were also added to obtain more information on their ability to stimulate remyelination. After one week (figure 12), and after two weeks (figure 13), the amount of remyelination was measured. PS did not stimulate remyelination in the cerebellar brain slice culture after one week as well as after two weeks, compared to EtOH. The PDE 4-inhibitor was the only compound which seemed to stimulate remyelination better than EtOH. A higher remyelination percentage was achieved after one week than after twee weeks of exposure to every condition. Since we used only one slice per condition, no significant results could be achieved.

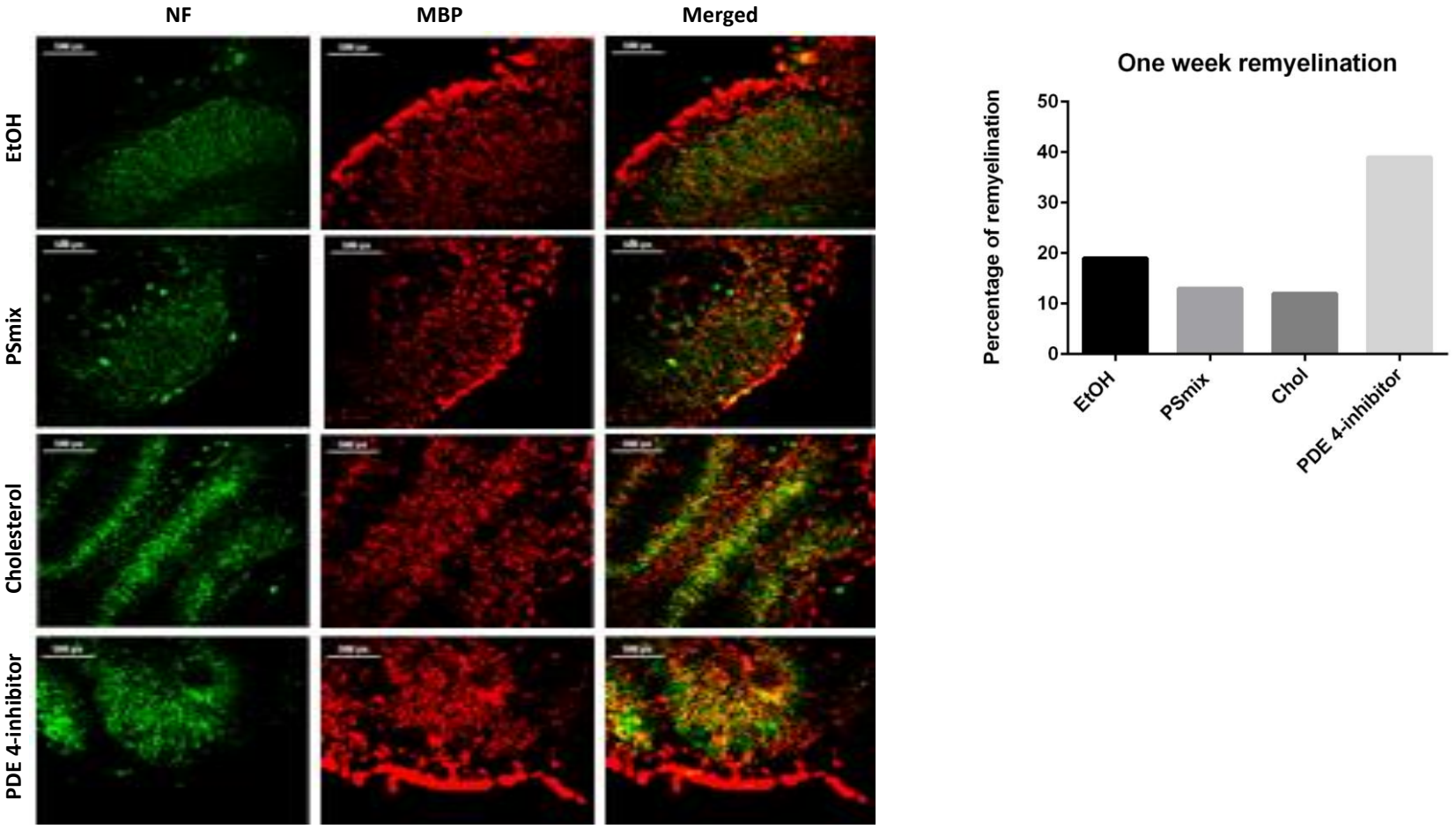

Figure 12: Remyelination of the brain slice culture, one week after demyelination. Cerebellar brain slices were demyelinated using LPC during 16 hours, after which remyelination was stimulated by the addition of EtOH (vehicle), 25 µM PSmix, 25 µM cholesterol or 0.5 µM PDE 4 inhibitor. After one week the slices were colored with MBP (red) and NF (green). Naturally occurring remyelination was already stimulated by the PDE 4 inhibitor (n=1) after one week. In all the conditions (n=1), OPCs could be distinguished, suggesting differentiation and migration towards the axons.

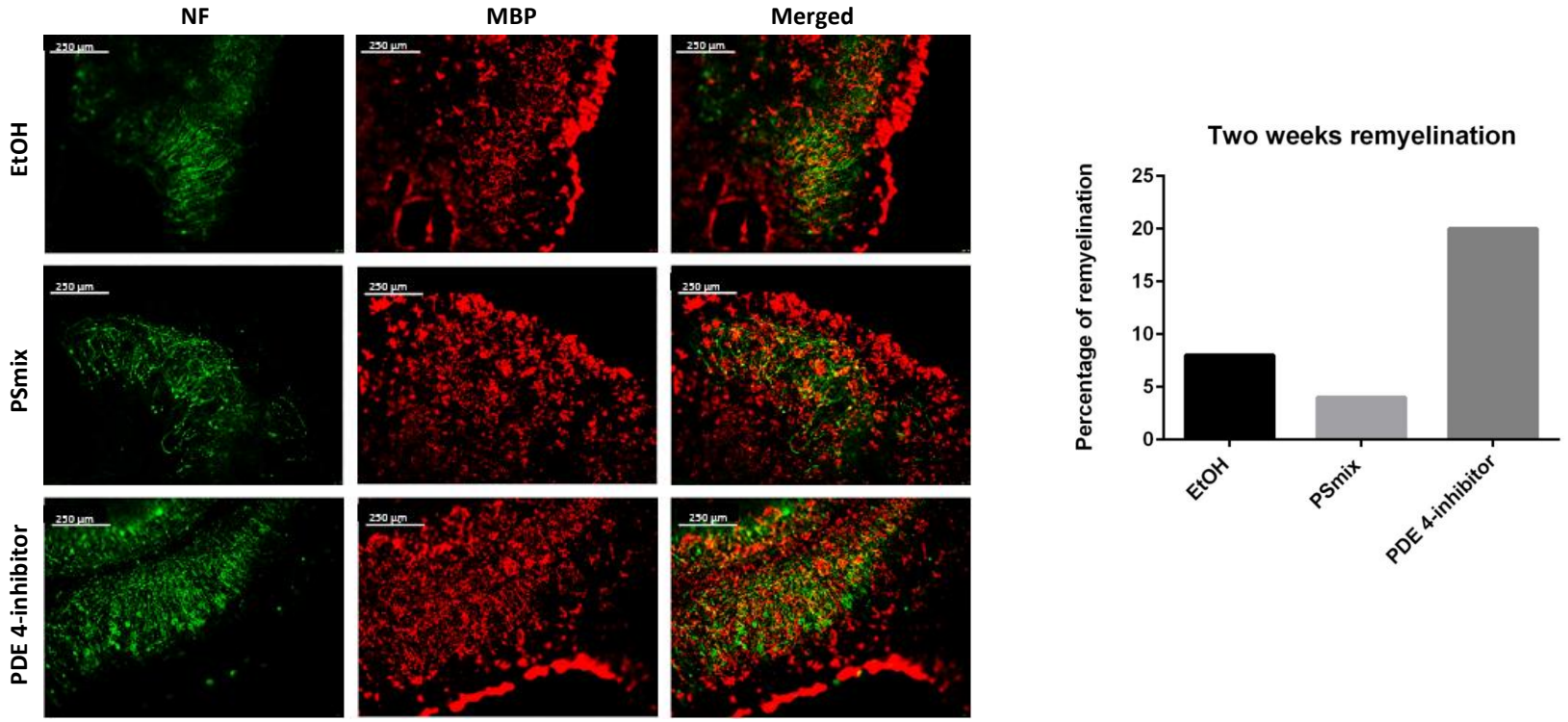

Figure 13: Remyelination of the brain slice culture, one week after demyelination. Cerebellar brain slices were demyelinated using LPC during 16 hours, after which remyelination was stimulated by the addition of EtOH (vehicle), 25 µM PSmix, 25 µM cholesterol or 0.5 µM PDE 4 inhibitor. After twee weeks, the slices were colored with MBP (red) and NF (green). The amount of remyelination was evaluated by calculating the ratio of myelinated axons (yellow)/nude axons (green). The PDE 4 inhibitor (n=1) stimulated remyelination the strongest. PSmix did not aid in the remyelination process (n=1).

Discussion

4 Discussion

The hypothesis underlying this research was that PS activate LXRs in microglia and lead to excretion of beneficial factors, such as anti-inflammatory cytokines, creating a favourable environment for remyelination of the CNS to occur. Since the discovery that PS are able to cross the BBB and accumulate in the brain (47), the amount of research considering their possible role in the CNS has been rising, leading to findings which suggest both beneficial as well as detrimental effects of PS in the CNS (48). Despite the multiple studies claiming activation of LXRs by several different PS (42), we did not observe any activation of LXRs. Several experiments, including on EAE mice (13), show anti-inflammatory effects of PS. However, these findings could not be supported by our study. The inflammatory properties of microglia and oligodendrocytes did not seem to be influenced, respectively directly nor indirectly, after addition of PS.

The first experiment was conducted to achieve more information on the direct effects of a mixture of the most abundant unsaturated PS in nature on LXR target genes, in a microglial cell line. PS never seemed to cause up- or downregulation of any of the target genes. In studies investigating the intestinal absorption of cholesterol, similar findings on LXR target genes were achieved. For example, a study conducted by Calpe-Berdiel *et al.*(49) demonstrated that the phytosterol-mediated inhibition of intestinal cholesterol absorption was independent of the ABCA1 transporter. Also, a report by Plösch *et al.* (50) stated that the observed plant sterol and – stanol-induced reduction in cholesterol absorption was not influenced by ABCG5 deficiency in mice.

The inflammatory related genes were also not altered after addition of PS, questioning the immune modulatory effects of PS. However, a study conducted by Nashed *et al.* (51) showed that Phytosterol-enriched diets were not only strongly associated with reduced plasma cholesterol concentrations and atherosclerosis, but the diets also caused a higher anti-inflammatory (IL-10) and lower pro-inflammatory (IL-6 and TNF-α) cytokine production. Calpe-Berdiel *et al.* (52) contradicted these results by concluding that phytosterols modulated the T-helper immune response, but no apparent anti-inflammatory effects could be distinguished. Both studies used apoE−/− C57BL/6J mice. Aspects explaining the different results could be the type of phytosterol mixture fed to the mice (58% β-sitosterol, 19% campesterol, 13% dihydrobrassicasterol, and 10% stigmasterol vs. 20% campesterol,

22% stigmasterol, and 41% β-sitosterol) and the immunization method (ovoalbumin vs. a single turpentine injection). Even though these results were achieved after *in vivo* experiments, and we conducted *in vitro* experiments, these results explain that multiple factors, such as the amount and type of PS, can influence the results.

Neurotrophic factors and growth factors were also not affected by the addition of PS. A synthetic LXR agonist was always tested along with a mixture of PS, to have an indication on whether possible trends or significances were LXR mediated. Cholesterol was also always tested alongside PSmix to distinguish a sterol effect from a PS effect. A significant higher expression of NGF and LIF was achieved after addition of cholesterol. Since the focus of the effects of PS are predominantly placed on cholesterol and inflammatory modulating properties, less attention is dedicated to neurotrophic - and growth factors.

To have an indication on the possible effects of IFNɣ and IL-1β on the expression levels of different genes, the experiment was repeated, taking different time-periods of stimulation (0, 6, 12 and 23 hours) into account. In the previous experiment, we observed a significant influence of cholesterol on NGF after six hours of stimulation (the conventional amount of time) but we could not distinguish this significance any longer in the time-dependent experiment, partially due to a small sample size. Our results did indicate different optimal stimulation periods are required depending on the gene of interest. However, these results still need to be confirmed in bigger experimental setups.

Since the main cell type used for most of the experiments was the immortalized murine microglial BV-2 cell line, we researched whether these cells were an appropriate choice for our experiments. The cell type is frequently used as a substitute for primary microglia. In a study conducted by Henn *et al.* (53) the strengths and weaknesses of these cells were re-evaluated, eventually supporting the use of the BV-2 cell line in many experimental settings. One of their main findings, was that the average upregulation of genes was less pronounced in BV-2 cells, after stimulation with LPS. However, BV-2 cells did show a normal regulation of NO production and a functional response to IFNɣ. We also performed an NO assay, to measure the effect of different substances added to the BV-2 cells on NO production. However, we could not detect the production of this free radical. To exclude possible deficits being present in the kit, the assay was used on other cell types. With these cells, NO production could be measured. The reason of failure in our experiment could have been due to a too low cell density (1.10^4 – 1.10^5 BV-2 cells) for any real detection to be observed. A last finding was that the BV-2 cells were able to stimulate other glial cells, primarily cortical astrocytes. In contrast, we

investigated the possibility of BV-2 cells to stimulate MBP and PLP production after a medium-transfer in the oli-neu oligodendrocyte cell line. The first time this experiment was conducted, significant results were achieved, with the expression level of MBP and PLP correlating with PS concentration. However, cholesterol was not included, so we could not conclude that the resulting effect was PS mediated, instead of sterol mediated. We then repeated this experiment, but we could not confirm the previous results.

Since cell renewal in the adult CNS is limited, and even blocked in inflammatory conditions, Butovsky *et al.* (54) researched the contribution of microglia in neurogenesis and oligodendrogenesis. In this experiment IL-4-activated microglia affected oligodendrogenesis more, whereas the IFNɣ activated microglia affected neurogenesis more. These findings were the result of a co-culture experiment, instead of a medium-transfer experiment. However, these findings do suggest stimulatory effects of microglia. In a study conducted by Pang *et al.* (55), LPS-activated microglia were deleterious to OPCs (co-culture as well as medium-transfer), reducing the production of MBP. In both these studies, an inflammatory-like environment was created by the addition of IL-4 and IFNɣ on the one hand and LPS on the other hand. We did not add any stimulation in our experiment. Our experimental setup, similar to the one by Pang *et al.*, without the activation seemed to work in a first trial of the experiment. In the next several trials, no influence of the BV-2 cells on the oli-neu cell line could be distinguished. In the future, if this experiment should be repeated with PS or other stimulations, both samples with as without activation by IFNɣ should be taken into account.

To be sure that the right cell type was used, the qualification of the oligodedroglial cell line was researched. The oli-neu cell type was previously characterized as qualified to substitute OPCs. In a study conducted by Pereira et al. (56) a comparison was made between the oli-neu and the N20.1 oligodendroglial cell line. These two cell lines appear to be at slightly different maturation stages, with oli-neu cells being slightly further maturated. However, according to this study, both cell lines are likely representative of oligodendrocytes at an immature stage, causing them to be useful for investigation of the regulation of myelin gene expression at slightly different stages.

In a next experiment, we examined the fluorescent intensity of OPC maturation markers A2B5, O4 and MBP in a slightly different experimental setup. In this setup we exposed the microglia to PSmix or cholesterol (only 0 and 25 µM) during 24 hours instead of 72 hours, after which the cells were incubated in SATO medium for 72 hours instead of 24 hours before medium transfer. MBP staining could not be detected with the secondary antibody, Alexa fluor 647. Furthermore, the amount of

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Olig2^{+} oligodendrocytes was low compared to the amount of type II astrocytes perceived by nonspecific binding of Alexa fluor 488, the secondary antibody of Olig 2, and as A2B5 positive cells (See addendum, Figure 1).

Multiple protocols have been described in regards to isolation of mouse OPCs. According to a study performed by Vitry S *et al.* in 1999 (57), the creation of oligospheres from transgenic mice is one of the best methods to isolate OPCs. They concluded that this ability could help define the molecular and cellular mechanisms of myelination and remyelination of the CNS. In a more recent study by Media-Rodriguez *et al.* (58) a method, similar to the one we used, was applied to isolate sufficient OPCs from postnatal (P0,P15) and adult (P60, P180) C57/BL6 mouse brains, and from human neurosurgical samples. The OPCs they obtained were capable of migrating and differentiating into myelin forming cells *in vitro*. With additions and adaptations from this last study, we might obtain a higher amount and purity of OPCs (Differences between our study and the study by Media-Rodriguez *et al.* , see addendum table 1).

Because of the high yield of cells, the main origin of OPC cultures had been from rat cortices and optic nerve. Methods include for example the traditional shake-off method or a modified OPC culture approach as described by Jianqin Niu *et al.* (59). On top of changes in the isolation procedure, such as replacement of the Mixed cell-medium by modified OPC growth-medium and detachment of the OPCs from the underlying astrocytes with gentle aspirating, an extra step was added in this approach to minimize the astrocyte contamination. After three days, but still prior to purification, medium was again replaced by modified OPC isolation-medium. Subsequently, gentle shaking (50-60 rpm) was performed on a horizontal orbital shaker. The resulting cells were then the purified OPCs. Implementing these steps in the isolation of mouse OPCs could help diminish the amount of astrocyte contamination in our experiment, taking into account that primary cells obtained from mice need different substances and factors in their medium to survive than primary rat cells do. Especially the trophic requirements seem to differ (60).

To have an indication on whether plant sterols had an effect on remyelination, we used a cerebellar brain slice model to observe this effect on. The *ex vivo* slice culture systel was previously characterized, and concluded to be a promising way of studying the biology of remyelination by Zhang *et al.* (61). The OPCs in our brain slice system seemed to respond to the demyelination by differentiating, proliferating and migrating towards the demyelinated axons to remyelinate them. Remyelination is very similar to myelination, but the axons that need to be remyelinated may have

been changed, and are in the presence of inflammatory debris and tissue injury. Cerebellar slice cultures have the advantage that most myelinated axons are Purkinje cell axons, instead of a mixture of fibre types and diameters (61). One week after demyelination, we could already distinguish some differentiating OPCs, preparing to remyelinate the nude axons. However, this time point could be regarded as a mid-term evaluation of the brain slices, as it was still too early for much remyelination to occur (61). After one more week, remyelination was again examined. The difference between the two time points was apparent, however not the way we expected. Remyelination seemed to be better established one week after demyelination than after two weeks for every condition. A difference in the amount of myelin formation could be observed between the different conditions. As expected, the highest percentage of remyelination could be observed when our positive control, a PDE 4 inhibitor, which elevates cyclic adenosine monophosphate and thereby enhances OPC differentiation by activation of mitogen-activated protein kinase signaling (62), was added to the cerebellar brain slices. PSmix did not seem to stimulate remyelination, as the percentage after both one and two weeks was lower than the control condition. Unfortunately, we could not evaluate the effect caused by cholesterol after two weeks, since this brain slice disintegrated during incubation.

In summary, MS is a demyelinating disease of the CNS, only affecting humans. Because it does not occur in other species, it makes it difficult to study the disease at a molecular level and to establish a cure. Activation of LXRs and LXR target genes could lead to stimulation of remyelination (63). In our case, we investigated the agonistic effect of PS on LXRs. However, the LXR target genes (SREBP-1c, ABCG1, ABCA1 and APOE) were only significantly upregulated when they were exposed to the synthetic LXR agonist GW3965, indicating that the PSmix used does not activate LXRs or LXR target genes. To confirm this, we conducted a luciferase assay to test for LXR activation, however the transfection was not optimal. On account of time deficit, we could not optimize the assay. The genes important in inflammation, were also not significantly up- or downregulated after PSmix addition, nor after LXR activation by the synthetic agonist. In a cerebellar slice culture environment, the PSmix did not seem to stimulate remyelination. Overall, in contrast to previous studies, we could not confirm LXR activation nor influences in the inflammatory aspect of MS. Moreover, we could not observe any stimulation of remyelination by the PSmix used in our study.

Discussion

5 References

1. Moore CS, Abdullah SL, Brown A, Arulpragasam A, Crocker SJ. How factors secreted from astrocytes impact myelin repair. J Neurosci Res. 2011;89(1):13-21.
2. Baumann N, Pham-Dinh D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev. 2001;81(2):871-927.
3. Markowitz CE. Multiple sclerosis update. Am J Manag Care. 2013;19(16 Suppl):s294-300.
4. Ciccarelli O, Barkhof F, Bodini B, De Stefano N, Golay X, Nicolay K, et al. Pathogenesis of multiple sclerosis: insights from molecular and metabolic imaging. Lancet Neurol. 2014;13(8):807-22.
5. Stellmann JP, Neuhaus A, Lederer C, Daumer M, Heesen C. Validating predictors of disease progression in a large cohort of primary-progressive multiple sclerosis based on a systematic literature review. PLoS One. 2014;9(3):e92761.
6. National Clinical Guideline C. National Institute for Health and Clinical Excellence: Guidance. Multiple Sclerosis: Management of Multiple Sclerosis in Primary and Secondary Care. London: National Institute for Health and Care Excellence (UK)

Copyright (c) National Clinical Guideline Centre, 2014.; 2014.
7. Miljkovic D, Spasojevic I. Multiple sclerosis: molecular mechanisms and therapeutic opportunities. Antioxid Redox Signal. 2013;19(18):2286-334.
8. Hoglund RA, Maghazachi AA. Multiple sclerosis and the role of immune cells. World J Exp Med. 2014;4(3):27-37.
9. Compston A, Coles A. Multiple sclerosis. Lancet. 2008;372(9648):1502-17.
10. Hadgkiss EJ, Jelinek GA, Weiland TJ, Pereira NG, Marck CH, van der Meer DM. The association of diet with quality of life, disability, and relapse rate in an international sample of people with multiple sclerosis. Nutr Neurosci. 2015;18(3):125-36.
11. Swank RL, Dugan BB. Effect of low saturated fat diet in early and late cases of multiple sclerosis. Lancet. 1990;336(8706):37-9.
12. Weinstock-Guttman B, Baier M, Park Y, Feichter J, Lee-Kwen P, Gallagher E, et al. Low fat dietary intervention with omega-3 fatty acid supplementation in multiple sclerosis patients. Prostaglandins Leukot Essent Fatty Acids. 2005;73(5):397-404.
13. 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. Inflamm Res. 2011;60(5):457-65.
14. Hernandez-Pedro NY, Espinosa-Ramirez G, de la Cruz VP, Pineda B, Sotelo J. Initial immunopathogenesis of multiple sclerosis: innate immune response. Clin Dev Immunol. 2013;2013:413465.
15. Cheng W, Chen G. Chemokines and chemokine receptors in multiple sclerosis. Mediators Inflamm. 2014;2014:659206.
16. Zhang-Gandhi CX, Drew PD. Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes. J Neuroimmunol. 2007;183(1-2):50-9.
17. Nelissen K, Mulder M, Smets I, Timmermans S, Smeets K, Ameloot M, et al. Liver X receptors regulate cholesterol homeostasis in oligodendrocytes. J Neurosci Res. 2012;90(1):60-71.
18. Minagar A. Current and future therapies for multiple sclerosis. Scientifica (Cairo). 2013;2013:249101.
19. Miljkovic D, Timotijevic G, Mostarica Stojkovic M. Astrocytes in the tempest of multiple sclerosis. FEBS Lett. 2011;585(23):3781-8.
20. Williams A, Piaton G, Lubetzki C. Astrocytes--friends or foes in multiple sclerosis? Glia. 2007;55(13):1300-12.

21. Rawji KS, Yong VW. The benefits and detriments of macrophages/microglia in models of multiple sclerosis. Clin Dev Immunol. 2013;2013:948976.
22. 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.
23. 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. 2006;129(Pt 2):517-26.
24. Vanmierlo T, Bogie JJ, Mailleux J, Vanmol J, Lutjohann D, Mulder M, et al. Plant sterols: Friend or foe in CNS disorders? Progress in lipid research. 2015.
25. Saher G, Brügger B, Lappe-Siefke C, Möbius W, Tozawa R, Wehr MC, et al. High cholesterol level is essential for myelin membrane growth. Nat Neurosci. 2005;8(4):468-75.
26. 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.
27. Yang C, McDonald JG, Patel A, Zhang Y, Umetani M, Xu F, et al. Sterol intermediates from cholesterol biosynthetic pathway as liver X receptor ligands. J Biol Chem. 2006;281(38):27816-26.
28. Zhao C, Dahlman-Wright K. Liver X receptor in cholesterol metabolism. J Endocrinol. 2010;204(3):233-40.
29. Gabbi C, Warner M, Gustafsson JA. Action mechanisms of Liver X Receptors. Biochem Biophys Res Commun. 2014;446(3):647-50.
30. Huang JK, Jarjour AA, Nait Oumesmar B, Kerninon C, Williams A, Krezel W, et al. Retinoid X receptor gamma signaling accelerates CNS remyelination. Nat Neurosci. 2011;14(1):45-53.
31. Beyea MM, Heslop CL, Sawyez CG, Edwards JY, Markle JG, Hegele RA, et al. Selective up-regulation of LXR-regulated genes ABCA1, ABCG1, and APOE in macrophages through increased endogenous synthesis of 24(S),25-epoxycholesterol. J Biol Chem. 2007;282(8):5207-16.
32. Cavelier C, Lorenzi I, Rohrer L, von Eckardstein A. Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1. Biochim Biophys Acta. 2006;1761(7):655-66.
33. Huang C. Natural modulators of liver X receptors. J Integr Med. 2014;12(2):76-85.
34. 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.
35. Im SS, Osborne TF. Liver x receptors in atherosclerosis and inflammation. Circ Res. 2011;108(8):996-1001.
36. Vanmierlo T, Husche C, Schott HF, Pettersson H, Lutjohann D. Plant sterol oxidation products--analogs to cholesterol oxidation products from plant origin? Biochimie. 2013;95(3):464-72.
37. 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.
38. Salen G, Ahrens EH, Grundy SM. Metabolism of beta-sitosterol in man. J Clin Invest. 1970;49(5):952-67.
39. Toth PP, Grabner M, Punekar RS, Quimbo RA, Cziraky MJ, Jacobson TA. Cardiovascular risk in patients achieving low-density lipoprotein cholesterol and particle targets. Atherosclerosis. 2014;235(2):585-91.
40. Sehayek E, Breslow JL. Plasma plant sterol levels: another coronary heart disease risk factor? Arterioscler Thromb Vasc Biol. 25. United States2005. p. 5-6.
41. Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin Proc. 2003;78(8):965-78.
42. Plat J, Nichols JA, Mensink RP. Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. J Lipid Res. 2005;46(11):2468-76.

43. 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. Proc Natl Acad Sci U S A. 2002;99(25):16237-42.
44. Klett EL, Lu K, Kosters A, Vink E, Lee MH, Altenburg M, et al. A mouse model of sitosterolemia: absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol. BMC Med. 2004;2:5.
45. 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.
46. 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 biology. 2002;3(7):RESEARCH0034.
47. Jansen PJ, Lütjohann D, Abildayeva K, Vanmierlo T, Plösch T, Plat J, et al. Dietary plant sterols accumulate in the brain. Biochim Biophys Acta. 2006;1761(4):445-53.
48. Vanmierlo T, Bogie JJ, Mailleux J, Vanmol J, Lütjohann D, Mulder M, et al. Plant sterols: Friend or foe in CNS disorders? Prog Lipid Res. 2015.
49. Calpe-Berdiel L, Escola-Gil JC, Blanco-Vaca F. Phytosterol-mediated inhibition of intestinal cholesterol absorption is independent of ATP-binding cassette transporter A1. Br J Nutr. 2006;95(3):618-22.
50. Plösch T, Kruit JK, Bloks VW, Huijkman NC, Havinga R, Duchateau GS, et al. Reduction of cholesterol absorption by dietary plant sterols and stanols in mice is independent of the Abcg5/8 transporter. J Nutr. 2006;136(8):2135-40.
51. Nashed B, Yeganeh B, HayGlass KT, Moghadasian MH. Antiatherogenic effects of dietary plant sterols are associated with inhibition of proinflammatory cytokine production in Apo E-KO mice. J Nutr. 2005;135(10):2438-44.
52. 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 Sci. 2007;80(21):1951-6.
53. Henn A, Lund S, Hedtjärn M, Schrattenholz A, Pörzgen P, Leist M. The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. ALTEX. 2009;26(2):83-94.
54. 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.
55. Pang Y, Campbell L, Zheng B, Fan L, Cai Z, Rhodes P. Lipopolysaccharide-activated microglia induce death of oligodendrocyte progenitor cells and impede their development. Neuroscience. 2010;166(2):464-75.
56. Pereira GB, Dobretsova A, Hamdan H, Wight PA. Expression of myelin genes: comparative analysis of Oli-neu and N20.1 oligodendroglial cell lines. J Neurosci Res. 2011;89(7):1070-8.
57. Vitry S, Avellana-Adalid V, Hardy R, Lachapelle F, Baron-Van Evercooren A. Mouse oligospheres: from pre-progenitors to functional oligodendrocytes. J Neurosci Res. 1999;58(6):735-51.
58. Medina-Rodríguez EM, Arenzana FJ, Bribián A, de Castro F. Protocol to isolate a large amount of functional oligodendrocyte precursor cells from the cerebral cortex of adult mice and humans. PLoS One. 2013;8(11):e81620.
59. Niu J, Wang L, Liu S, Li C, Kong J, Shen HY, et al. An efficient and economical culture approach for the enrichment of purified oligodendrocyte progenitor cells. J Neurosci Methods. 2012;209(1):241-9.
60. Horiuchi M, Lindsten T, Pleasure D, Itoh T. Differing in vitro survival dependency of mouse and rat NG2+ oligodendroglial progenitor cells. J Neurosci Res. 2010;88(5):957-70.
61. Zhang H, Jarjour AA, Boyd A, Williams A. Central nervous system remyelination in culture--a tool for multiple sclerosis research. Exp Neurol. 2011;230(1):138-48.

62. Syed YA, Baer A, Hofer MP, Gonzalez GA, Rundle J, Myrta S, et al. Inhibition of phosphodiesterase-4 promotes oligodendrocyte precursor cell differentiation and enhances CNS remyelination. EMBO Mol Med. 2013;5(12):1918-34.
63. 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.

6 Addendum

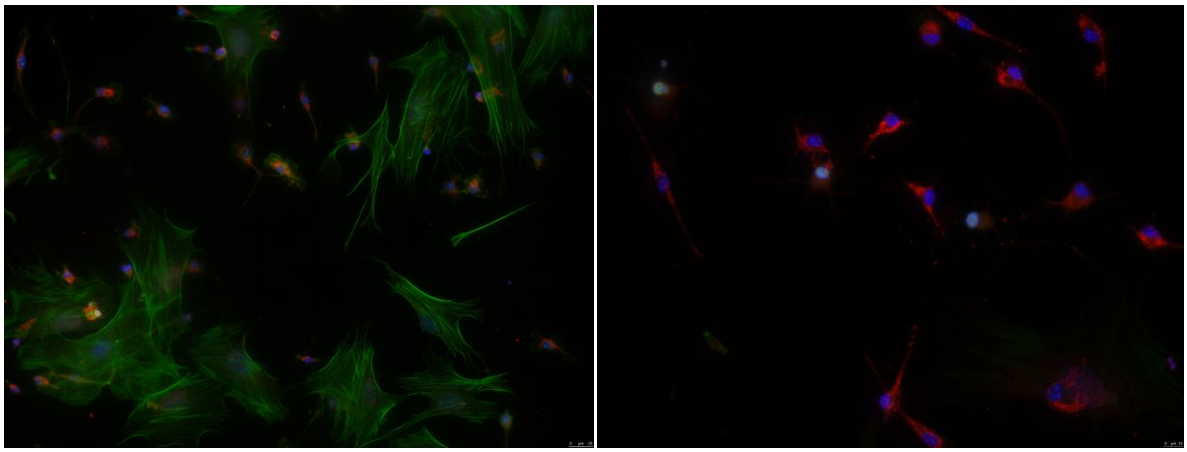

Figure 1: Olig 2, A2B5 and DAPI staining of OPCs. The amount of Olig 2 (green) specific positive cells (OPCs) was much smaller than the amount of olig 2 nonspecific positive cells (type II astrocytes). A2B5 (red) is a marker for progenitor oligodendrocytes, as well as for type II astrocytes. So, only when an overlap of Olig2 and DAPI (blue) is observed, then, the presence of OPCs is a fact.

Table 1: Comparison in OPC isolation methods

Our study	Study by Media-Rodriguez *et al.* (50)
Use HBSS or DMEM.	Use ice-cold HBSS (without Ca^{2+} and Mg^{2+}), favoring papain digestion.
Add papaine as a way of digestion.	Add papaine as a way of digestion.
/	Add DNAse solution to stop papaine digestion.
Resuspend and vortex at 1200 rpm for 5 minutes.	/
Vortex shortly , then place falcon tube 20 min in a hot water bath at 37°C.	Incubate at 37°C for 5 minutes.
Add cold DMEM + 10% FCS + 1% P/S (resuspend) and centrifuge for 5 min at 1200 rpm.	Centrifuge at 900 rpm for 10 minutes.
/	Pass cell suspension (10 ml) through a 100 µm nylon mesh strainer.
Seed resuspended cells in flasks previously coated with PLL.	Seed recovered cells in Poly-L Ornithin coated flasks and incubate at 5% CO_2 and 37°C for one day
Replace medium after a couple of hours (we waited 5.5 hours).	Replace medium with OPC medium supplemented with platelet derived growth factor (PDGF) –AA (Not necessary for P0). No longer then 10-15 days.
Shake-off one hour at 180 rpm (microglia), then, add new medium containing 5 µg/ml insulin and shake—off overnight at 225 rpm (OPCs).	Shake-off overnight (18-20 hours) in an orbital shaker at 250 rpm and 37°C.
/	Collect medium and pass through a 40 µm nylon mesh strainer. Centrifuge flow-through at 900 rpm for 10 minutes.
Collect medium in Petri-dishes and incubate during 20 min at 8.5% CO_2 and 37°C.	Plate resuspended cells in Petri dish at 37°C for 45 min (allowing microglial cells to attach).
/	Collect unattached cells and repeat the process for 30 minutes.
Centrifuge medium at 1200 rpm for 10 min.	Centrifuge OPC-enriched supernatant at 900 rpm for 10 min.

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The potential of plant sterols to induce remyelination in multiple sclerosis and other demyelinating diseases

Richting: **master in de biomedische wetenschappen-milieu en gezondheid**
Jaar: **2015**

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Datum: **9/06/2015**