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# Siponimod-loaded extracellular vesicles to stimulate remyelination in the central nervous system

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Director: Professor Anne des Rieux

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À Vianney,

avec toute mon admiration

*"La plus grande gloire n'est pas de ne jamais tomber mais de se relever à chaque chute"*

*Confucius*

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## FOREWORD

Multiple sclerosis is a chronic, inflammatory demyelinating disorder of the central nervous system (CNS) and the most common cause of non-traumatic neurological disability in young adults. Despite significant progress in understanding the disease's pathogenesis and slowing its progression, with currently seventeen approved treatments, none of these therapies can repair the myelin sheath that is damaged during the disease. Myelin regeneration, known as remyelination, is a natural process that occurs following demyelination. However, due to chronic inflammation and the impaired differentiation of oligodendrocyte progenitor cells (OPC) into oligodendrocytes – the CNS-resident cells responsible for myelin production – the efficacy of remyelination decreases as the disease progresses. Therefore, it is crucial to develop therapies that can both reduce inflammation and stimulate remyelination.

We have previously observed that stem cells from human apical papilla (SCAP) demonstrate anti-inflammatory and neuroprotective effects through their secretome, particularly when exposed to a pro-inflammatory environment. This secretome includes both soluble factors and extracellular vesicles (EVs). Based on these findings, we hypothesized that EVs derived from SCAP could recapitulate the therapeutic properties of their parent cells. Furthermore, combining these EVs with a drug possessing pro-remyelination effects, such as siponimod, could potentially resolve inflammation and promote remyelination, two critical aspects of multiple sclerosis.

This thesis manuscript has thus been divided into 4 parts:

In **part I – Introduction**, key concepts essential for understanding this thesis will be outlined. This section will cover the physiopathology of multiple sclerosis, current and emerging therapies, as well as siponimod and its possible involvement in remyelination. Additionally, the biogenesis of extracellular vesicles and their potential use as drug delivery systems will also be discussed.

In **part II – Aims**, the main objectives of the thesis will be described.

In **part III – Results**, the main findings from this thesis will be presented. This result section is divided in two different experimental chapters. The first chapter, 'Influence of a pro-inflammatory stimulus on the miRNA and lipid content of human dental stem cell-derived extracellular vesicles and their impact on microglia activation' explores how EV composition is affected by a pro-inflammatory stimulus and assesses the ability of EVs to reduce pro-inflammatory cytokine expression in both *in vitro* and *ex vivo* models. The second chapter, 'Siponimod-loaded EVs increased oligodendrocyte progenitor cell differentiation', focuses on using EVs as drug delivery

system for siponimod and investigates their effects on myelination and oligodendrocyte progenitor cell differentiation.

In **part IV – General discussion**, the main achievements of this PhD thesis will be presented, followed by a critical discussion regarding this work.

# TABLE OF CONTENTS

# ABBREVIATION LIST

| | |
|---|---|
| **AHSCT** | Autologous hematopoietic stem cell transplant |
| **BACE1** | Beta-site APP cleaving enzyme 1 |
| **BBB** | Blood-brain barrier |
| **BDNF** | Brain-derived neurotrophic factor |
| **CIS** | Clinically isolated syndromes |
| **CNS** | Central nervous system |
| **DAMP** | Damage associated molecular pattern |
| **DMT** | Disease-modifying therapy |
| **DNA** | Deoxyribonucleic acid |
| **DNAse** | Deoxyribonuclease |
| **EAE** | Experimental autoimmune encephalomyelitis |
| **EBV** | Epstein-Barr virus |
| **EMA** | European medicines agency |
| **ESCRT** | Endosomal sorting complex required for transport |
| **EV** | Extracellular vesicle |
| **FDA** | Food and drug administration |
| **FGF** | Fibroblast growth factor |
| **GalC** | Galactoceramide |
| **GFAP** | Glial fibrillary acidic protein |
| **GM-CSF** | Granulocyte-Macrophage Colony Stimulating Factor |
| **HLA** | Human leukocyte antigen |
| **Iba-1** | Ionized calcium-binding adaptor molecule 1 |
| **IDO** | Indoleamine 2,3-dioxygenase |
| **IFN** | Interferon |
| **IGF** | Insulin growth factor |
| **IL** | Interleukine |
| **ILV** | Intraluminal vesicle |
| **IN** | Intranasal |
| **IP** | Intraperitoneal |
| **ISEV** | International society of extracellular vesicles |
| **IV** | Intravenous |
| **LDL** | Low-density lipoprotein |
| **LPA** | Lysophosphatidic acid |
| **LPC** | Lysophosphatidylcholine |
| **MAG** | Myelin-associated glycoprotein |
| **MBP** | Myelin basic protein |
| **MHC** | Major histocompatibility complex |
| **miRNA** | microRNA |
| **MISEV** | Minimal information for studies of extracellular vesicles |
| **MOG** | Myelin oligodendrocyte glycoprotein |
| **mRNA** | messenger RNA |
| **MS** | Multiple sclerosis |
| **MSC** | Mesenchymal stem cell |
| **MVB** | Multivesicular body |
| **NAWM** | Normal-appearing white matter |
| **NF-$\varkappa$B** | Nuclear factor-kappa B |
| **NMDA** | N-methyl-D-aspartate receptor |
| **OPC** | Oligodendrocyte progenitor cell |

| | |
|---|---|
| **PDGF** | Platelet-derived growth factor |
| **PDGFRα** | Platelet-derived growth factor receptor α |
| **PLGA** | Poly(lactic-co-glycolic acid) |
| **PLP** | Myelin proteolipid protein |
| **PPMS** | Primary progressive multiple sclerosis |
| **PTEN** | Phosphatase and TENsin homolog |
| **RNA** | Ribonucleic acid |
| **RRMS** | Relapsing-remitting multiple sclerosis |
| **RT-qPCR** | Real time quantitative polymerase chain reaction |
| **S1P** | Sphingosine-1-phosphate |
| **SEC** | Size exclusion chromatography |
| **siRNA** | small interferent RNA |
| **SLO** | Secondary lymphoid organ |
| **SNARE** | Soluble N-ethylmaleimide-sensitive factor attachment protein receptor |
| **SphK** | Sphingosine kinase |
| **SPMS** | Secondary progressive multiple sclerosis |
| **$T_{CM}$** | Central memory T-cell |
| **TCR** | T-cell receptor |
| **$T_{EM}$** | Effector memory T cell |
| **TFF** | Tangential flow filtration |
| **TfR** | Transferrin receptor |
| **TGF** | Transforming growth factor |
| **Th** | T helper cells |
| **TMEV** | Theiler's encephalomyelitis virus |
| **TNF** | Tumor necrosis factor |

# PART I
# INTRODUCTION

# CHAPTER I: MULTIPLE SCLEROSIS

## I. EPIDEMIOLOGY

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating, and neurodegenerative disorder of the central nervous system (CNS). In 2023, it has been reported that MS affects approximately 2.9 million people worldwide [1, 2]. The global prevalence in 2020 was estimated at 35.9 per 100,000 people with a pooled incidence rate of 2.1 per 100,000 people per year [2]. Women are more at risk of developing MS as they constitute 73% of MS patients [1, 2]. The disease typically manifests between 20 and 40 years old, with an average age of MS diagnosis being 32 years old [2].

A regional disparity in both prevalence and incidence is observed, with Europe having the highest prevalence and incidence rates, followed by the Americas, South East Asia, and Africa (Figure 1).

In both hemispheres, we observed an association between increasing latitude towards the poles and increasing prevalence of MS. This is known as the latitudinal prevalence gradient [2].

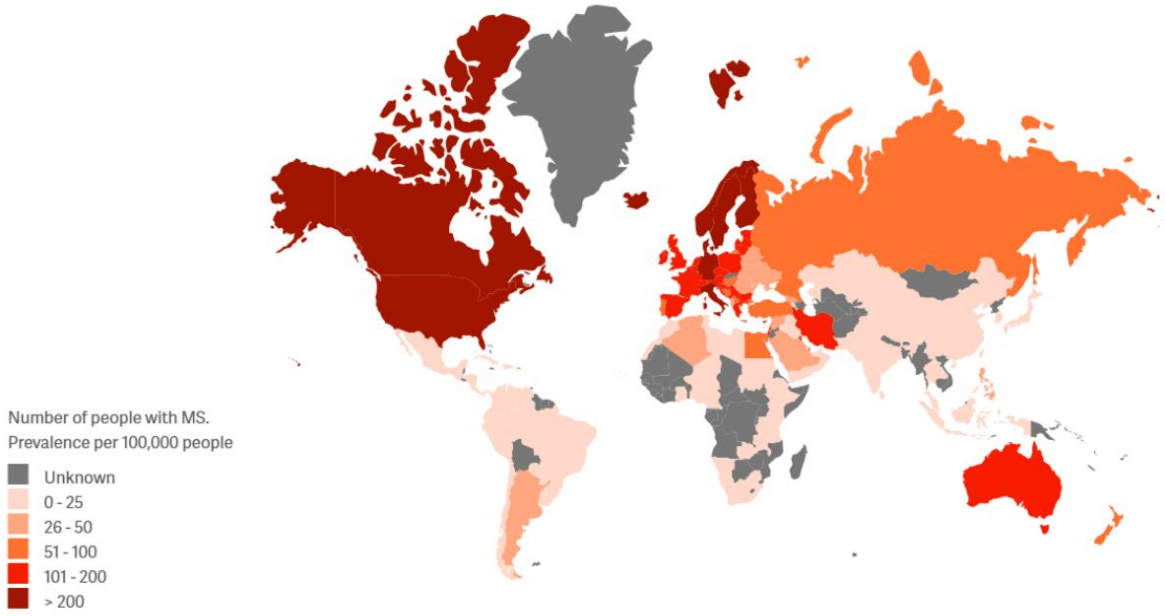



**Figure 1. Map showing geographic variations in MS prevalence per 100,000 people/country [2].**

# II. ETIOLOGY

MS is a complex disease caused by a combination of environmental, genetic, and epigenetic factors [3].

## II.1. ENVIRONMENTAL FACTORS

Several environmental factors such as Epstein-Barr virus infection, smoking, obesity in adolescence, and low vitamin D (associated with a lack of sun exposure) are known to play a part in MS development [4].

### II.1.1. Epstein-Barr virus

For decades, viral infections have been suspected to play an important role in MS onset and Epstein-Barr virus (EBV) is at the top of the list. EBV is a herpes virus, which targets B cells and can stay in a latent state within the cells. A recent study on more than 10 million young adults showed that the risk of MS is 32-fold higher after infection with EBV and established a link between EBV infection and MS. Other viruses such as cytomegalovirus did not increase MS risk [5]. Moreover, a mechanistic link between MS and EBV has been recently demonstrated. Indeed, Lanz et al. highlighted a molecular mimicry between the EBV transcription factor EBV nuclear antigen 1 (EBNA1) and the central nervous system protein glial cell adhesion molecule (GlialCAM). Thus, after EBV infection, the immune system targets also GlialCAM in the myelin sheath [6]. The immortalization of naïve and resting B cells by EBV has also been proposed as a potential mechanism triggering MS, as well as the migration of EBV-infected B cells to the CNS, the deregulation of autoimmune control following EBV infection, and the interaction between EBV and HLA [7].

### II.1.2. Vitamin D and sun exposure

Due to various epidemiological observations, which highlighted a dependence between the latitude and MS incidence, a large number of studies focused on sun exposure and vitamin D levels as an environmental factor for MS. Thus, a case-control study showed that low sun exposure in early life was associated with an increased risk of MS later in age [8]. Later, Munger et al. provided evidence that an increased 25-hydroxyvitamin D level was associated with a decrease in MS activity [9]. This could be explained by the general immunomodulatory role of vitamin D. Indeed, some studies showed that vitamin D supplementation resulted in the stimulation of regulatory T-cells (Tregs) and thus an increase of the production of IL-10, a decrease of the pro-inflammatory T helper (Th)

17 lymphocytes, which led to a decrease of IL-17 production and finally an attenuation of B-cell immunoreactivity [10]. Vitamin D seems also to have direct effects on the CNS as neurons, astrocytes, microglia, and oligodendrocytes express the vitamin D receptor. However, the potential neuroprotector and remyelinating effects of vitamin D remain to be explored [10].

Besides vitamin D level, sun exposure seems also to have benefits for MS-related immune parameters through other mechanisms involving enhanced levels of cis-urocanic acid and melatonin, which both have immunoregulatory effects [11, 12].

### II.1.3. Obesity

Observational studies reported an association between obesity during adolescence and future risk of MS [4]. To confirm these results, Mokry et al. performed a Mendelian randomization study, which highlighted an association between a genetically elevated body mass index and an increased risk of MS [13]. Moreover, a significant interaction between the human leukocyte antigen HLA-DRB1*15 and obesity was observed in two case-control studies [14]. The mechanism behind the obesity-MS relationship is far from clear. However, at least three pathways seem to be involved. First, obesity induces chronic low-grade inflammation due to the secretion of inflammatory mediators by adipose tissue macrophages. Second, levels of circulating 25-hydroxyvitamin D are lower in obese people than in normal-weight people. Third, adipose tissue produces leptin, a promotor of the pro-inflammatory response, proportionally to body fat mass [14]. In a case-control study, it has been shown that leptin was a risk factor for MS among young individuals [15]. This adipokine promotes autoreactive T-cell proliferation and proinflammatory cytokine secretion while inhibiting the proliferation of regulatory T cells [16, 17]).

### II.1.4. Smoking

Several small studies followed by a large case-control study suggested a dose-response relationship between smoking and MS risk [4]. This could be explained by the irritation caused by smoking, which can lead to chronic lung inflammation. This inflammation promotes the activation of the immune system [18]. Furthermore, some studies highlighted a link between smoking and DNA methylation with exposure response relationship [19]. However, in 2020, two Mendelian randomization studies did not confirm any causal relationship between smoking and MS risk [20, 21]. Thus, further investigation still has to be done to elucidate the role of smoking in MS risk.

## II.2. GENETIC AND EPIGENETIC FACTORS

Although MS is not a Mendelian disease and does not have an inheritance pattern, various studies have shown a higher incidence and prevalence in family members of patients compared with the general population. Thus, the risk of MS in family members of affected individuals is estimated at 4% for siblings, 2% for parents, and 2% for children. Moreover, it is now established that about 20% of MS patients have a family member affected by the disease [22]. Altogether, these results provide strong support for a significant but complex genetic etiology in MS [23].

In 2019, the International Multiple Sclerosis Genetics Consortium published a meta-analysis of 15 genome-wide association studies. This led to the identification of 233 statistically independent associations with MS susceptibility, which are genome-wide significant: 32 risk associations are on the major histocompatibility complex (MHC) locus, one is on chromosome X and 200 risk associations are in the autosomal non-MHC genome [24]. These results confirmed the involvement of many components of both adaptive and innate immunity [25]. In the polymorphic MHC region, the HLA gene cluster on chromosome 6p21 has been identified as the strongest genetic locus for MS [26]. The evidence accumulated from familial studies suggested that MS had one locus with moderate effect (HLA-DRB1*15:01) and many loci with small effects [22]. Furthermore, carriers of the HLA DRB1*15:01 allele are about three times more likely to develop MS than non-carriers [3]. While non-MHC MS risk alleles outnumber MHC MS risk alleles, some studies have shown that their impact on the disease is generally modest with an odd ratio of less than 1.2. In contrast, the 32 MHC effects contribute to approximately 4% of the total heritability in MS [25]. However, functional interpretation and translation to an understanding of the pathophysiology remain a challenge, since the exact mechanism by which HLA-coded products contribute to MS susceptibility is still unknown [25]. Recently, HLA-DRB1:15 has been associated with phenotypic markers of disease progression but progress still has to be made on this topic [23]. In 2023, the same consortium published a genome-wide association study that identified a significant association with rs10191329 in the DYSF-ZNF638 locus, where the risk allele is linked to a reduction in the median time to requiring a walking aid by approximative 3.7 years in homozygous carriers. Additionally, a suggestive association was found between rs149097173 in the DNM3-PIGC locus with a shortening in the median time to requiring a walking aid of a median of 3.3 years [27].

Finally, environmental factors can also interact with genetic risk loci through epigenetic factors such as DNA methylation, histone modification, and non-coding RNA alterations [28]. Thus, a

link between smoking and DNA methylation involved in MS pathogenesis has been established, as explained in the previous section [19].

## III. PATHOPHYSIOLOGY OF MULTIPLE SCLEROSIS

It is now well-established that the adaptative immune cells play a key role in the clinically defined pathology of the disease. However, the lingering question is whether these immune cells initiate the primary pathology (outside-in model) or represent a normal immune response targeted against a preceding oligodendrocytosis (inside-out hypothesis) (Figure 2) [29]. In the outside-in model, a dysregulation of the peripheral immune system leads to the infiltration of T- and B-cells into the central nervous system through a disrupted blood-brain barrier (BBB) [30]. These immune cells attack the myelin, leading to the degeneration of oligodendrocytes and the loss of myelin. This results in the release of myelin debris, which are then engulfed by the innate immune cells such as microglia. These cells act as antigen-presenting cells to T-cells, which will lead to an exacerbation of demyelination and oligodendrocytosis [29]. This model highlights the crucial role of immune cells in promoting inflammation and demyelination in multiple sclerosis [30]. However, it has been shown that the presence of inflammatory cells alone cannot induce demyelination as they can be found in both demyelinated and non-demyelinated sites. Moreover, it has been observed that peripheral inflammatory cells were absent in one-third of the CNS brain lesions [29]. The second hypothesis that may explain the etiopathogenesis of multiple sclerosis is the inside-out hypothesis. In this model, the oligodendrocyte degeneration is governed by an internal metabolic dysfunction of microglia and astrocytes. This leads to demyelination, gliosis, and release of cytokines and chemokines, which compromise the integrity of the BBB [29, 31]. Additionally, the gradual degradation of oligodendrocytes results in the release of antigenic myelin proteins into the circulatory and lymphatic systems. Altogether, this allows the entry of peripherally circulating T- and B-cells within the CNS [30]. When T-cells interact with antigen-presenting cells through major histocompatibility complex, they become activated. This activation causes the release of inflammatory mediators such as cytokines, nitric oxide, and glutamate, which exacerbate the degenerative process. This hypothesis is consistent with the observation of a low presence of parenchymal T- and B-cells in active demyelinating sites during the early stages of the disease. However, the degeneration of oligodendrocytes in MS seems to be more heterogeneous than currently understood [29]. Furthermore, if this hypothesis is correct, it is essential to elucidate the underlying mechanisms of primary oligodendrocyte death. It is presumed that these two hypotheses operate simultaneously in MS patients to contribute to the formation of inflammatory demyelinating lesions.

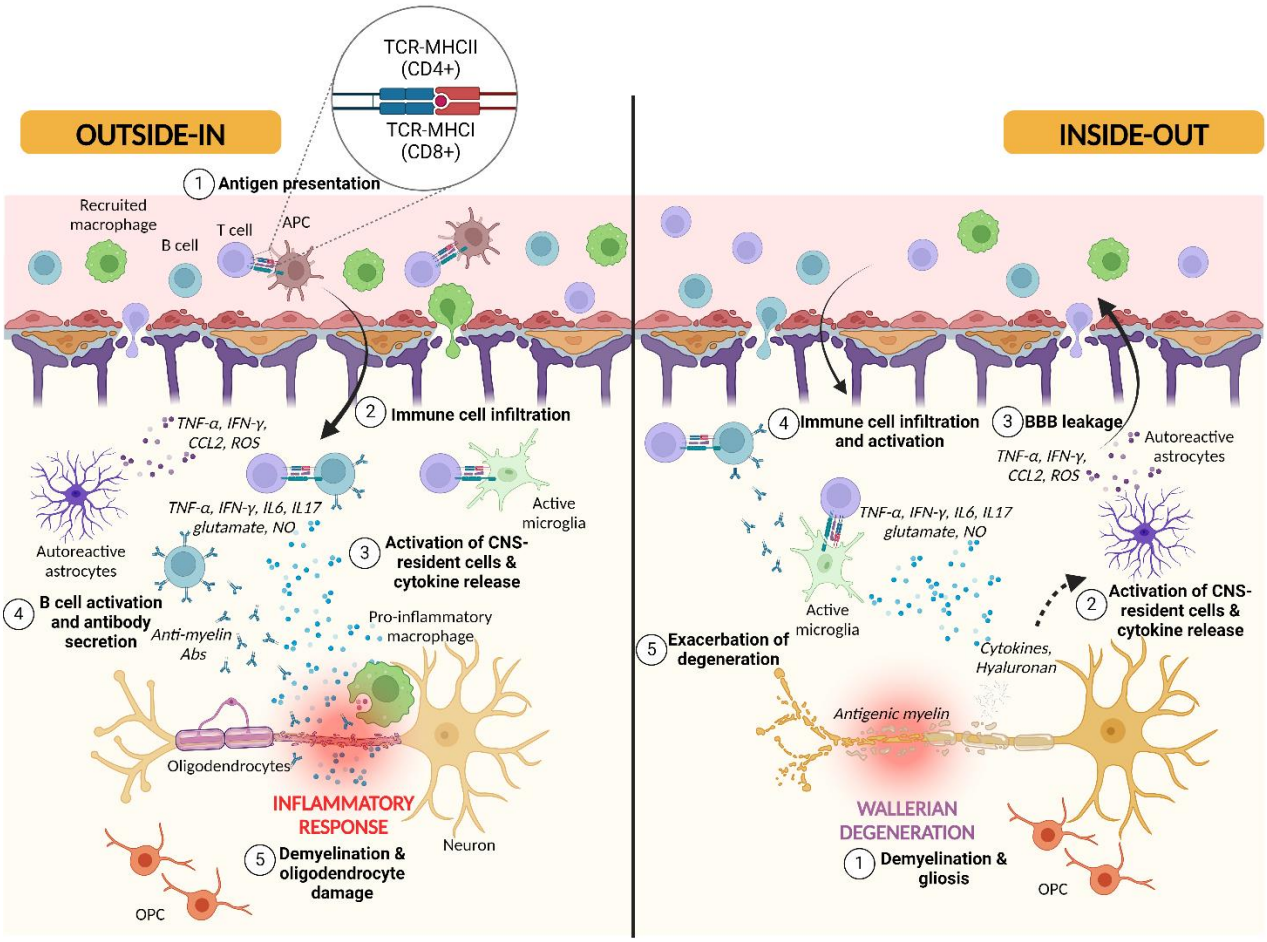



**Figure 2. Outside-in and inside-out mechanisms during the pathogenesis of MS, adapted from [30].** The outside-in mechanism illustrates the infiltration of reactive CD4+ and CD8+ T-cells, along with B-cells, from the periphery across a leaking blood-brain barrier into the central nervous system. Once inside the CNS, resident cells and infiltrating B-cells become activated, releasing pro-inflammatory cytokines and anti-myelin antibodies, respectively. This immune response leads to demyelination, oligodendrocyte damage, and neurodegeneration. Conversely, the inside-out mechanism is initiated by axonal injury resulting in Wallerian degeneration. This process activates microglia and induces oligodendrocyte apoptosis, followed by demyelination. The activation of CNS-resident cells triggers the release of cytokines, causing the BBB to become leaky. Immune cells from the periphery can then infiltrate the CNS, exacerbating the degeneration process. APC: antigen presenting cells; BBB: blood-brain barrier; ROS: reactive oxygen species; NO: nitric oxide**.** Created with Biorender.

While disruption of the BBB is the main mechanism by which immune cells infiltrate the brain, recently identified osseous channels connecting skull or vertebral bone marrow to the brain provide an alternative route. These channels enable the migration of myeloid cells including neutrophils and B-cells directly from the bone marrow into the CNS, though the exact mechanism remains unclear. Additionally, it has also been demonstrated that autoreactive T-cells can migrate to the bone marrow and amplify myelopoiesis, thereby enhancing CNS inflammation [32, 33].

In any case, the pathophysiology of MS involves numerous cells including adaptive immune cells (T- and B-cells), as well as glial cells (microglia, astrocytes, and oligodendrocytes).

## III.1. CELLS INVOLVED IN MS

### III.1.1. T-cells

T-cells, also known as T lymphocytes, play a pivotal role in the adaptive immune response by producing cytokines that mediate inflammation and regulate immune cells [34]. T-cells express the T-cell receptor (TCR) on their surface, which recognizes antigen fragments presented on MHC molecules. This recognition involves CD4 or CD8, depending on the T-cell subtype. For decades, CD4+ T-cells were considered the main adaptive immune effectors in MS, as experimental autoimmune encephalomyelitis, the most widely used model of MS, is driven by Th1 CD4+ cells [35]. Both Th1 and Th17 CD4+ cells are found deep within MS lesions, with a higher prevalence of Th1 cells. Th1 cells produce cytokines such as IFN-γ and TNFα, with IFN-γ levels increasing prior to disease exacerbation. This cytokine enhances the antigen-presenting activity by increasing MHC molecule expression and activates microglia, leading to phagocytosis and direct oligodendrocyte killing. Th17 CD4+ cells, on the other hand, secrete IL-17, IL-21 and IL-22. While IL-17 is essential for normal defense mechanisms, its role in MS may be linked to glutamate excitotoxicity. IL-21 may affect lymphocyte infiltration in lesions while IL-22 promotes BBB disruption [36]. Recent evidence has highlighted the secretion of IL-17 by CD8+ T-cells in active MS lesions, in equivalent amounts to CD4+ T-cells [37]. Furthermore, a phase II study has shown that the therapeutic depletion of CD4+ T-cells did not yield any therapeutic benefits in MS patients, while depletion of all T-cells significantly reduced MS relapses and new lesion development [38]. Additionally, analysis of infiltrating T-cells in human brain lesions revealed the presence of CD8+ T-cells in a greater number than CD4+ T-cells, regardless of disease stage [39]. This suggests a potentially significant role of CD8+ T-cells in MS disease. These cells recognize antigens presented by MHC class I molecules and induce their target cell death by introducing granzymes and perforin into their cytosol [36]. In MS, the expression of the MHC-I is gradually upregulated in astrocytes, oligodendrocytes, neurons, and axons, depending on the lesion activity [40]. The pathogenicity of CD8+ T-cells has been supported by various studies, which have demonstrated their increased numbers in acute and chronic lesions, up-regulation of cytotoxic mediator granzyme B, correlation with axonal injury severity, and higher prevalence in MS patients compared to healthy individuals [39].

While MS was initially viewed as being driven by T-cells, recent advancements in B-cell therapies have demonstrated significant efficacy in treating all forms of MS, highlighting the substantial contribution of B-cells in the CNS of MS patients [41, 42].

### III.1.2. B-cells

B-cells, also known as B lymphocytes, are the main effector of humoral immunity. Their precise role in MS is unclear but their crosstalk with T-cells is considered a central aspect of the disease pathogenesis [41-43]. Indeed, T-cells support B-cell proliferation and differentiation, and B-cell depletion significantly diminishes the pro-inflammatory responses of CD4+ and CD8+ T-cells [42, 44].

Studies have revealed abnormalities in B-cells in MS, including their propensity to produce excessive pro-inflammatory cytokines and chemokines (such as TNFα, IL-6, GM-CSF, lymphotoxin-α), which contribute to inflammation propagation [45, 46]. Additionally, B-cells exhibit defective regulatory functions with a low number of transitional B regulatory cells, produce soluble toxic factors that harm oligodendrocytes and neurons, present antigens to T-cells driving brain-homing T-cell proliferation, and contribute to the formation of tertiary lymphoid-like structures in the CNS, which seem to act as local sites of antigen presentation and lymphocyte activation [42, 43]. In early and active lesions, CD20+ B-cells predominate, while effector B-cells are present at later stages. Although these cells may have potential anti-inflammatory functions, studies have shown that B-cells in MS patients exhibit deficient production of IL-10 compared to healthy controls, which may explain why B-cells in patients are less capable of downregulating immune responses [42].

While B-cells are involved in MS, the role of autoantibodies and their link to pathogenesis remain uncertain. Studies suggest the formation of autoantibodies against various CNS components like neurons, glia, and immune cells [44, 47]. Currently, no autoantibody has been identified for diagnostic use [48]. The causal antigen also remains unknown, though some studies hypothesized the involvement of the Epstein-Barr virus in B-cell overactivation [43].

In addition to the adaptive immune system, the innate immune system also contributes to multiple sclerosis through the microglia cells, which are the most predominant immune cells within lesions [49].

### III.1.3. Microglia cells

Microglia cells are the resident macrophages of the CNS. They represent 10 – 20% of the glial cells and are thus the most abundant immune cells in the CNS [50]. Under normal conditions, these cells maintain CNS homeostasis by continuously surveying the environment through their motile protrusions [51]. They stand prepared to respond to insults such as viral and bacterial infections, toxins, and local tissue injury [52].

In MS patients, activated microglia are found in the CNS lesions (Figure 3). They are the major source of reactive oxygen species and produce nitric oxide radicals. Oligodendrocyte progenitor cells are particularly vulnerable to oxidative stress due to their lower expression levels of antioxidant enzymes. Microglia cells also contribute to excitotoxicity by producing glutamate [50]. Moreover, they act as antigen-presenting cells through MHC class I and II molecules, which are expressed at low levels in physiological conditions [51]. Microglia cells can also form small clusters of at least 4 up to 50 cells that are in contact with each other, named microglia nodules. These nodules are considered to precede lesion formation and are associated with axons undergoing Wallerian degeneration [53]. Microglia nodule as putative precursors of lesion formation in MS is consistent with the inside-out hypothesis.

Early after demyelination, microglia cells secrete many pro-inflammatory cytokines in active lesions, including IL-6, IL-1β, IL-12, IL-18, IL-23, and TNFα but also chemokines such as CCL2, CCL3, CCL4, CCL5, CCL7, CCL12, and CCL22. They can cause damage to the myelin sheath and oligodendrocytes, thereby contributing to CNS inflammation in MS patients [52]. Pro-inflammatory microglia persist within the lesion until resolution or inactivity occurs. Subsequently, a transition to an immunoregulatory phenotype is observed, promoting remyelination through the expression of anti-inflammatory markers such as CD206, arginase-1, IL-4, and IL-10. TNFα and IL-1β, also produced by these cells, stimulate OPC survival and proliferation during this phase [51, 54]. Furthermore, microglia cells are involved in the phagocytosis and clearance of myelin debris through the receptor TREM2. This process is critical in remyelination. Indeed, reduced myelin debris clearance has been associated with impaired remyelination [52]. Microglia also produce IGF1 and FGF2, which promote oligodendrocyte proliferation and support neuronal repair [50]. Recently, single-cell RNA sequencing has expanded our understanding on microglia in MS. Thus, it led to the identification of two functional subtypes of "microglia inflamed in MS" (MIMS) at the chronic active lesion edge. The first subtype, MIMS-foamy, is involved in myelin phagocytosis and clearance, while the second subtype, MIMS-iron, may contribute to the breakdown of immune tolerance in the presence of immunoglobulin immune complexes in MS [55, 56].

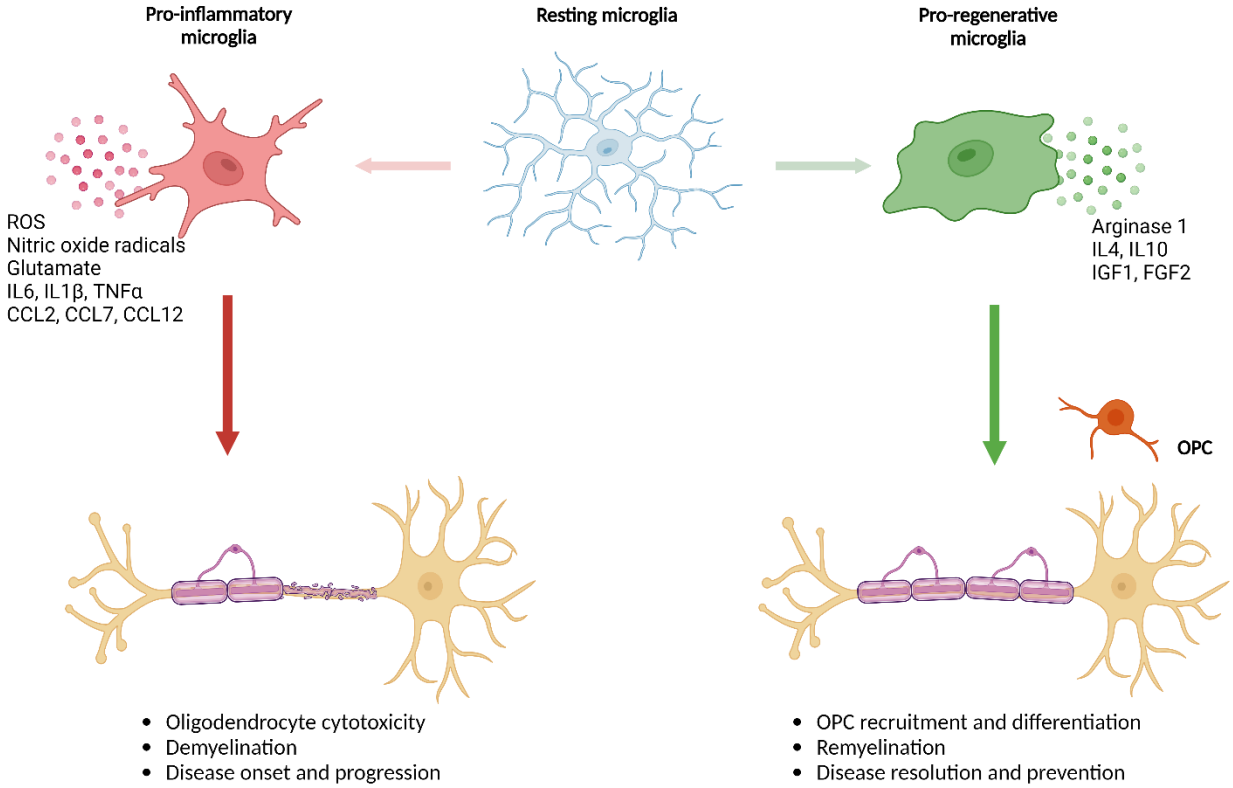



**Figure 3. Effects of microglia-derived factors on oligodendrocyte lineage depending on microglial subsets, adapted from [57].** Pro-inflammatory microglia secrete cytokines and reactive oxygen species that can directly damage oligodendrocytes, resulting in demyelination and disease progression. At the opposite, immunoregulatory microglia secrete cytokines and growth factors which promote OPC migration and differentiation, resulting in remyelination and disease prevention. ROS: reactive oxygen species; OPC: oligodendrocyte progenitor cells. Created with Biorender.

Thus, microglia cells have a complex role in balancing the risk of potential harm to CNS tissue and supporting tissue repair.

### III.1.4. Astrocytes

Astrocytes are the most abundant cell type in the CNS, as they represent approximately 30% of the glial cells [58, 59]. They support the neurons and the oligodendrocytes by providing energy substrates and trophic factors but also maintaining extracellular medium composition, pH, and electrolyte balance [59]. Additionally, astrocytes contribute to maintaining BBB integrity and are a major component of the glia limitans, regulating molecule entry into the brain parenchyma [58].

In MS, astrocytes exhibit a hypertrophic morphology in the active margins of demyelinating lesions, suggesting their early and active involvement in lesion development [60]. Initially, astrocytes

promote an inflammatory environment in MS lesions by releasing inflammatory mediators such as CCL2, IL-1β, TNFα, glutamate, and nitric oxide, leading to endothelial cell apoptosis and downregulation of tight junction protein expression, thereby increasing BBB permeability [61-64]. Astrocytes also actively recruit T-cells into the CNS parenchyma by producing chemoattractant molecules such as CCL20, CXCL10, IL-6, and tissue plasminogen activators which increase T-cell binding to endothelial cells [65, 66]. Activation of NF-κB pathway by IL-1β and TNFα is pivotal for establishing neuroinflammation [29]. Furthermore, the reactive astrocytes contribute to excitotoxic injury as the expression of glutamate-degrading enzymes is reduced, leading to excessive extracellular glutamate [59]. In patients, astrocytes also form glial scars, which act as a physical barrier that blocks OPC migration into demyelinated areas. Thus, OPC are almost sequestered at the lesion edges, leading to a failure of remyelination [67, 68]. Moreover, secretion of substances that interfere with remyelination (e.g. hyaluronan, FGF2, TNFα, IL-6) is also upregulated in the glia scar [69]. However, this scar may support demyelinating axons by confining inflamed areas and preventing the spread of immune cells and toxic levels of extracellular ions, metabolites, or DAMPs into the areas of repair [31, 58].

Over time, astrocytes transition to a more protective phenotype, secreting retinoic acid and anti-inflammatory cytokines (IL-4, IL-10, and IL-27), which limit BBB damage and inflammation [59]. Similar to microglia cells, astrocytes possess detoxification activities and express BDNF in lesions, promoting neuronal survival and regulating oligodendrocyte generation and remyelination [31].

### III.1.5. Oligodendroglial cells

Oligodendroglial cells include oligodendrocytes and oligodendrocyte progenitor cells (OPC). Oligodendrocytes are the CNS resident cell types responsible for myelin production. Myelin, consisting of oligodendroglial plasma loops tightly wound concentrically around the axon, facilitates saltatory conduction of neuronal action potential between nodes of Ranvier, enhancing nerve conduction speed and energy efficiency. Myelin also provides trophic support to neurons [70, 71]. Oligodendrocytes derive from OPCs. These stem cells, which represent 5 to 8% of the glial population, possess self-renewal potential and rapidly proliferate in response to injury [71, 72]. They are distributed throughout the CNS in both white and grey matter and require various molecules such as Olig1, Olig2, Nkx2.2, Nkx2.6, MyT1, and Sox-10 for differentiation [73-75].

In MS, pro-inflammatory and oxidative injuries lead to demyelination and oligodendrocyte loss, associated with progressive axonal degeneration and neurological decline. However, recovery from relapses in acute inflammatory lesions involves several mechanisms including remyelination.

Remyelination is a spontaneous regenerative process wherein demyelinated axons are ensheathed with new myelin sheaths, leading to functional recovery and clinical remittance [70, 71]. Prior to oligodendrocyte formation, the lesions are repopulated by OPCs. This migration is strongly governed by chemotactic cues such as PDGF, CXCL12, and semaphorin 3F which are chemo-attractants, and netrin-1, semaphorin 3A, ephrins, CXCL1, and tenascin C which are chemo-repellents [70, 71, 76, 77]. Activated by the pro-inflammatory environment and cytokines (e.g. TNFα, IL-1β, IL-11, and CXCL12), OPCs mature into pre-oligodendrocytes expressing oligodendrocyte marker O4 and transition into non-motile and proliferative cells with a complex morphology. Subsequently, pre-oligodendrocytes differentiate into immature oligodendrocytes [78]. These cells have undergone cell cycle arrest while oligodendrocyte marker O4 but not OPC markers such as A2B5 and PDGFRα. Immature oligodendrocytes elaborate arborizations and acquire maturity-associated markers (e.g. GalC) without forming myelin. The final step of remyelination involves the differentiation of immature oligodendrocytes into mature cells, synthesizing myelin components, and compacting myelin membranes around axons [70] (Figure 4). In MS, completely remyelinated lesions, termed shadow plaques, exhibit a relative decrease in myelin thickness and internodal length compared to the original parameters [70, 71]. Despite this, the myelin sheaths provide full functional recovery for axons and act as protective physical barriers against inflammatory molecules. However, remyelination becomes insufficient as MS progresses in most patients due to various factors [70, 79] :

- Interruption of remyelination due to inflammation.
- Secretion of CXCL1 and ephrins by glia scars, impeding OPC migration into lesions.
- Exhaustion of OPC sources following repeated demyelination episodes. However, contrary evidence suggests a failure in differentiation rather than recruitment.
- Reactivation of Wnt signaling pathways and developmental myelination regulators (e.g. LINGO-1, PSA-NCAM), leading to impaired OPC differentiation.
- Physiological aging and epigenetic age acceleration of glial cells. The exact mechanism underlying the accelerated aging remains to be clarified but could be linked to the exhaustion of glial cells [80, 81].

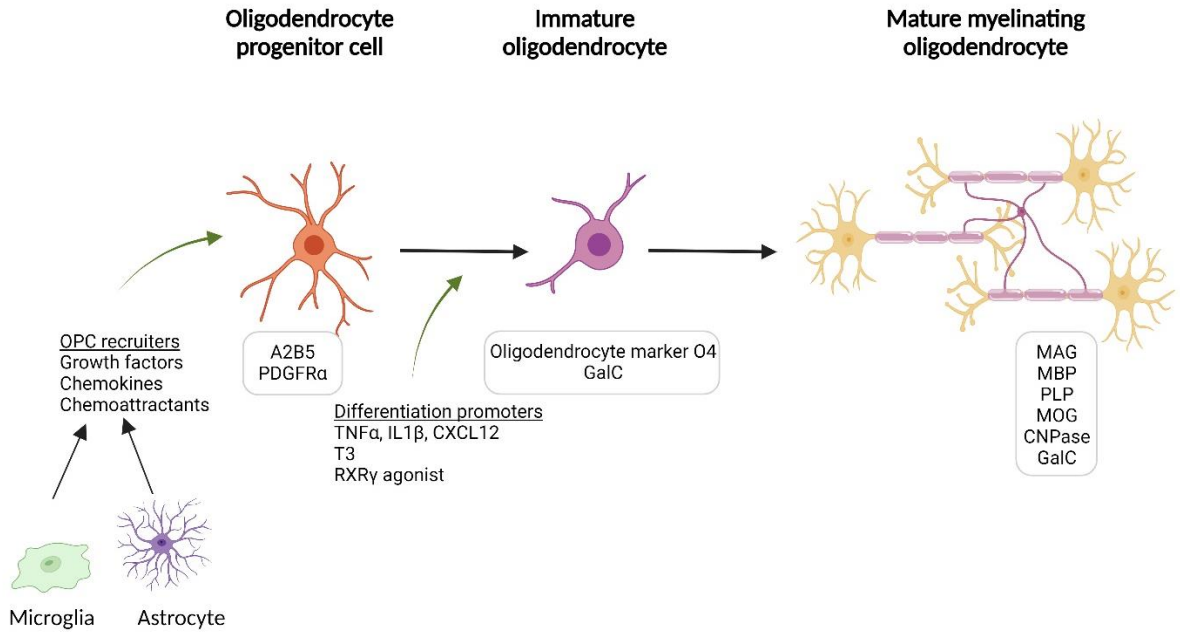



**Figure 4. OPC differentiation, adapted from [82].** In MS lesions, microglia, and astrocytes express factors that recruit OPCs to the lesion site. These OPCs, identified by markers such as A2B5 and PDGFRα, are attracted to the area of damage. Once at the lesion, OPCs differentiate into immature oligodendrocytes in response to differentiation-promoting signals. These immature cells further mature into myelinating oligodendrocytes, which produce and restore myelin. Created with Biorender

## III.2. LESIONS IN MS

MS is characterized by focal demyelinated areas in the white and grey matter of the CNS called lesions or plaques [83]. They indicate a loss of myelin sheath [84]. Lesions in MS patients are associated with axonal and neuronal loss that correlates with patient disability, astrocytic gliosis, and BBB breakdown [3, 83, 85].

In white matter, focal lesions include demyelinating lesions, inactive lesions, chronic active lesions, and slowly expanding lesions [86]. Active demyelinating lesions characterize the early disease stages [85, 87]. These lesions present an active lymphocyte infiltration (mainly CD8+ T-cells) associated with microglial activation mostly at the edge and in the periplaque [85]. Macrophages are principally in the centre and contain remnants of the destroyed myelin sheaths [87]. More precisely, minor myelin proteins such as MOG and MAG are rapidly degraded within macrophages while MBP and PLP persist in macrophages for 6 to 10 days [49]. The presence of large reactive astrocytes is also observed as well as a perivascular inflammation. Axons are relatively preserved but loss or

fragmentation occurs where damage is the most severe [49]. Inactive lesions mainly characterize later disease stages [85]. These lesions are sharply circumscribed and hypocellular [49, 85]. They present well-defined demyelination with dense fibrillary scars between the demyelinated axons [87]. A reactive astrocyte gliosis is observed as well as a decrease in mature oligodendrocyte number [49]. Finally, the number of microglia is reduced in the centre of the demyelinated plaque while activated microglia are in the periplaque [85, 87]. The density of lymphocytes is lower than in active lesions. In patients with a longer disease duration, chronic active lesions are observed. Macrophages are recruited at the edges. Fewer macrophages are present at the centre [85].

Slowly expanding lesions are also lesions, that affect patients in later disease stages [87]. These lesions consist of an inactive demyelinated centre surrounded by a rim of activated microglia. Few macrophages containing myelin debris are present at the edge, as well as an active axonal injury, leading to transected axons. Altogether, this suggests a very slow rate of ongoing demyelination and axonal damage [85].

Besides the focal lesions described above, macroscopically normal white matter with signs of diffuse inflammation and neuro-axonal damage can also be observed. They are called normal-appearing white matter (NAWM) and are pronounced in patients with progressive MS while they are spared in early MS stages [87]. Contrary to what researchers think, NAWM occurs independently from focal lesions. They are characterized by axonal degeneration and demyelination, diffuse T-cell infiltration (mostly CD8+), widespread microglia activation, diffuse axonal injury, and astrocytic gliosis.

Extensive cortical demyelination can also be observed in grey matter, more specifically in the forebrain and the cerebellum [85]. Grey matter lesions may appear as subcortical white matter lesions or may occur independently from white matter damages [49]. These lesions present some differences compared to white matter lesions: the degree of inflammation, the microglia activation, and the macrophage recruitment are much less compared with what happens in white matter [85, 87]. In consequence, there is less global tissue injury. However, cortical demyelination may have an impact on neuronal, dendritic, and axonal functions, viability, and survival [49].

# IV. CURRENT TREATMENTS FOR MS

## IV.1. SUBTYPES OF MS

The International Advisory Committee on Clinical Trials in MS defined four clinical courses of MS (Figure 5):

- Relapsing-remitting MS (RRMS): this form is characterized by recurrent relapses, which are the clinical expression of acute focal or multifocal inflammatory demyelination in the CNS [88]. It affects around 85% of the patients. Between relapses, a complete or incomplete neurological recovery is observed [85].
- Clinically isolated syndrome (CIS): this is the first clinical presentation of a disease that shows characteristics of inflammatory demyelination that could be MS but has yet to fulfill the criteria of dissemination in time [89]. In 2/3 of cases, CIS evolves in RRMS while in one-third of cases, the patients remain with monophasic illness, at least clinically [90].
- Primary progressive MS (PPMS): this form is characterized by a progressive accumulation of disability from the onset, leading to progressive and permanent neurological deficits for more than one year without relapses [85, 89]. PPMS affects around 15% of the MS population and is most commonly observed in males and older patients [88].
- Secondary progressive MS (SPMS): this form is characterized by a progressive accumulation of disability after an initial relapsing course [89]. In this case, a progressive and irreversible disability occurs independently of the presence of relapses [85]. SPMS commonly follows a period of well-defined RRMS (three or more years) with 90% of untreated RRMS patients becoming SPMS after 25 years [88]. With a more complete diagnosis of benign MS, the use of disease-modifying therapies, and lifestyle modifications, the disease course has changed, resulting in a lower risk of conversion to SPMS and a delay in the age at which patients reached disease milestones [91-93]. However, there are no clear clinical, imaging, immunologic, or pathologic criteria to determine the transition point when RRMS converts to SPMS.

CIS and RRMS can be active or not active, with activity determined by clinical relapses and or MRI activity. Progressive disease (PPMS and SPMS) can be active and with progression, active but

without progression, not active but with progression, or not active and without progression (stable disease). The progression is measured by clinical evaluation assessed at least annually [89].

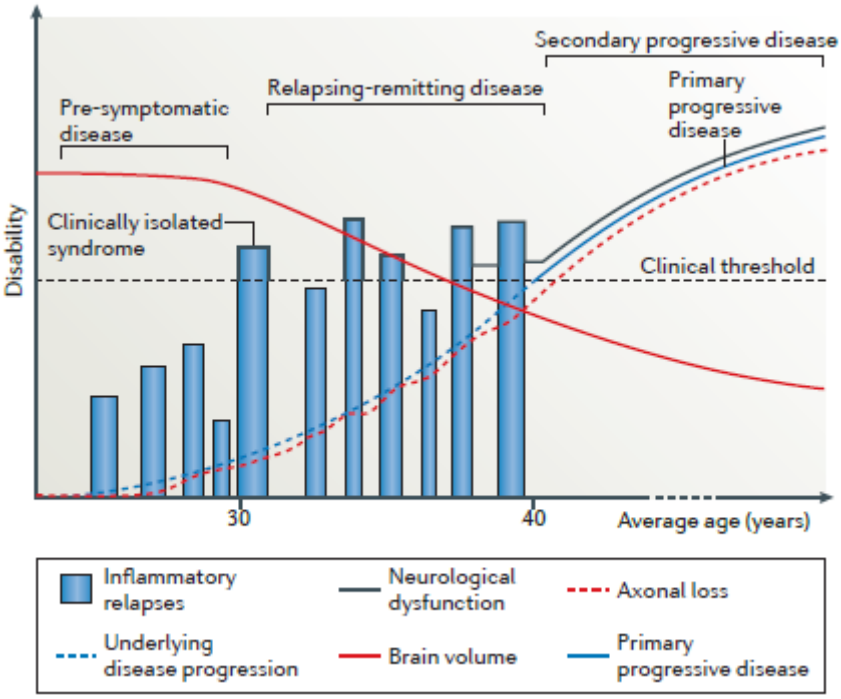



**Figure 5. MS clinical course** [83]. Four clinical courses have been described for MS: Relapsing-remitting MS in which relapses are separated by periods characterized by a lack of disease progression; Clinically isolated syndrome, which is the first clinical presentation of the disease showing characteristics of inflammatory demyelination but without dissemination in time; Primary progressive MS in which the disease progresses from the onset; Secondary progressive MS which characterizes a progressive accumulation of disability after an initial relapsing course.

## IV.2. APPROVED THERAPIES FOR MS

The current therapeutic approach for MS includes relapse treatment to accelerate clinical recovery and disease-modifying therapies (DMTs) to reduce MS relapses, delay the progression of disability, and limit new inflammation in the CNS [94].

### IV.2.1. Acute relapses

Relapses are defined as clinical episodes manifested as discrete episodes of neurological dysfunction lasting at least 24h, occurring in the absence of fever, infection, or acute concurrent medical illness. Symptoms usually peak within 1 to 2 weeks and then gradually ameliorate over the following 2 to 4 weeks without intervention [95, 96]. Not all relapses require treatment. However, when treatment is needed, most neurologists recommend high doses of oral or intravenous corticosteroids, such as

methylprednisolone, administered for a short duration to reduce inflammation and end the relapse faster [94]. In cases where corticosteroid therapy proves ineffective, plasmapheresis, which leads to a positive response in 72% of patients, should be performed [3, 95, 96].

### IV.2.2. Disease-modifying therapies

Advances in understanding the pathogenesis and progression of MS have led to major progress in its treatment. Current management strategies focus on reducing disease activity and slowing disease progression using DMTs [96]. DMTs alter the course of relapsing MS by exerting immunomodulatory or immunosuppressive effects. They reduce the rate of relapses, decrease the accumulation of MRI lesions, and stabilize disability. However, they do not confer benefits in the later secondary progressive phase (SPMS), during which neurodegenerative mechanisms become clinically predominant [96, 97]. Only Ocrelizumab has demonstrated efficacy in slowing progression in patients with PPMS [98].

The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have approved 17 DMTs for relapsing MS (Table 1).

Two different therapeutic approaches are used in the treatment of MS: escalation therapy and induction therapy [99]. The escalation approach involves the early initiation of first-line DMTs such as IFN-β-1a and 1b, glatiramer acetate, dimethyl fumarate, and teriflunomide. If the treatment is ineffective or partially effective, the therapy is switched to second-line drugs, including mitoxantrone, natalizumab, and fingolimod. In contrast, the induction approach starts with the early use of immunosuppressant drugs typically recommended as a second-line or third-line treatments in patients with very aggressive or active MS disease. This initial step is followed by long-term maintenance treatment, usually with immunomodulatory agents. A cohort study with prospective data showed that the escalation approach was associated with a lower risk of conversion to SPMS compared to induction therapy [91].

**Table 1**. **Overview of DMTs approved by EMA and FDA for multiple sclerosis**. Nrf2: nuclear factor erythroid 2–related factor 2; NF-ϰB: nuclear factor-kappa B; S1P: Sphingosine-1-phosphate; APC: antigen-presenting cell; MHC: major histocompatibility complex; PML: progressive multifocal leukoencephalopathy; ITP: immune thrombocytopenia; CNS: central nervous system; BBB: blood-brain barrier

| Drug | Year of licensing | Mechanisms of action | Indication | Frequency of administration | Common side effects | Ref. |
|---|---|---|---|---|---|---|
| **Oral administration** | | | | | | |
| Fingolimod (Gilenya®) | 2011 | S1P receptor modulator, inhibition of lymphocyte egression from lymphatic tissues | RRMS | 0.5 mg / day | Bradyarrhythmia, heart, block, increased risk of, infections, lymphopenia, liver dysfunction | [100] |
| Teriflunomid (Aubagio®) | 2013 | Pyrimidine synthesis inhibitor (dihydroorotate dehydrogenase inhibitor), inhibition of autoreactive B- and T-cell proliferation | RRMS | 7 or 14 mg / day | Nausea, diarrhea, alopecia, skin rash, hepatotoxicity | [3] |
| Dimethyl fumarate (Tecfidera®) | 2014 | Possible Nrf2 pathway activator and NF-ϰB inhibitor, reduction of inflammatory cytokine release | RRMS | 2 x 240 mg / day | Flushing, gastrointestinal symptoms, lymphopenia | [3, 96] |
| Cladribine (Mavenclad®) | 2017 | Synthetic deoxyadenosine analog, B- and T- cell depletion | RRMS | 3.5 mg/kg, cumulative dose over 2 years | Lymphopenia, increased risk of infection, headache | [96] |
| Siponimod (Mayzent®) | 2019 | S1P receptor modulator, inhibition of lymphocyte egression from lymphatic tissues | RRMS | 0.25 – 2 mg / day | Headache, nasopharyngitis, urinary tract infection, falls | [101] |
| Ozanimod (Zeposia®) | 2020 | S1P receptor modulator, inhibition of lymphocyte egression from lymphatic tissues | RRMS | 0.92 mg / day | Upper respiratory tract infection, elevated liver enzymes, orthostatic hypotension | [102] |

| | | | | | | |
|---|---|---|---|---|---|---|
| Ponesimod (Ponvory®) | 2021 | S1P receptor modulator, inhibition of lymphocyte egression from lymphatic tissues | RRMS | 20 mg / day | Upper respiratory tract infection, elevated liver enzymes, hypertension | [103] |
| **Subcutaneous administration** | | | | | | |
| IFN β-1b (Betaferon®, Extavia®) | 1995 | Downregulates expression of MHC molecules on APC, alters cytokine expression, inhibits T-cell proliferation, and blocks trafficking of inflammatory cells to CNS | RRMS | 250 μg / 2 days | Influenza-like symptoms, injection site reactions, lymphopenia | [3] |
| IFN β-1a (Rebif®) | 1998 | Downregulates expression of MHC molecules on APC, alters cytokine expression, inhibits T-cell proliferation, and blocks trafficking of inflammatory cells to CNS | RRMS | 3 x 44 μg / week | Influenza-like symptoms, injection site reactions, rhinitis, headache | [3] |
| Glatiramer acetate (Copaxone®) | 2003 - 2015 | Amino acid copolymer, alters T-cell differentiation inducing proliferation of anti-inflammatory lymphocytes | RRMS | 20 – 40 mg / day | Injection-site reactions, lipoatrophy, post-injection general reaction | [3, 104] |
| PegIFN β-1a (Plegridy®) | 2014 | Downregulates expression of MHC molecules on APC, alters cytokine expression, inhibits T-cell proliferation, and blocks trafficking of inflammatory cells to CNS | RRMS | 125 μg / 2 weeks | Injection-site reactions, influenza-like symptoms | [96] |
| Ofatumumab (Kesimpta®) | 2021 | Human anti-CD20 monoclonal antibody, depletes B-cells | RRMS | 20 mg / 4 weeks | Injection-site reactions, nasopharyngitis, headache, upper respiratory tract and urinary tract infections | [105] |
| **Intramuscular administration** | | | | | | |
| IFN β-1a (Avonex®) | 1997 | Downregulates expression of MHC molecules on APC, alters cytokine expression, inhibits T-cell proliferation, and blocks trafficking of inflammatory cells to CNS | RRMS | 30 μg / week | Influenza-like symptoms, muscle aches, asthenia, chills, fever | [3] |

| Intravenous administration | | | | | | |
|---|---|---|---|---|---|---|
| Natalizumab (Tysabri®) | 2006 | Humanized monoclonal antibody, α4β1 integrin inhibitor, prevents lymphocytes from entering the CNS across the BBB | RRMS | 300 mg / 4 weeks | PML, upper respiratory tract and urinary tract infections, infusion reactions | [96] |
| Alemtuzumab (Lemtrada®) | 2013 | Humanized anti-CD52 monoclonal antibody, depletes B- and T-cells (CD4+ and NK cells) | RRMS | 12 mg/ $m^2$ / year (2 courses in total) | Autoimmune thyroid disease, ITP, Goodpasture's syndrome, infusion reactions | [96] |
| Ocrelizumab (Ocrevus®) | 2017 | Humanized anti-CD20 monoclonal antibody, depletes B-cells | RRMS/PPMS | 600 mg / 6 months (4 courses in total) | Infusion reactions, chest infection, herpes infection | [106] |
| Mitoxantrone (Noventrone®) | 2000 | Synthetic anthracenedione derivative, neoplastic and immunomodulatory agent, suppresses macrophage, T- and B- cell proliferation, decreases proinflammatory cytokine secretion | RRMS | 12 mg/ $m^2$ / 3 months | Cumulative dose-dependent cardiomyopathy, acute leukemia | [107, 108] |

# V. THE FUTURE OF MS TREATMENT

MS is a complex disease with no definitive cause and effective cure. Therefore, animal models are indispensable for unraveling the underlying mechanisms of MS and evaluating new treatments.

## V.1. ANIMAL MODELS OF MS

Various animal models are commonly used in MS research. For a long time, these models were categorized into two groups: disease models and mechanism models. Disease models attempt to replicate the disease as accurately as possible. This category regroups the inflammatory models such as experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus (TMEV). In contrast, mechanism models provide a more reductionist approach and are used to study a specific aspect of the pathology like demyelination and remyelination process. This category includes toxin-induced models such as focal injection of toxins (lysolecithin or ethidium bromide) and oral administration of cuprizone.

### V.1.1. Experimental autoimmune encephalomyelitis

EAE is an autoimmune model characterized by inflammation, myelin damage, and neurodegeneration following immunization with CNS antigen (frequently MOG) emulsified in an adjuvant to elicit T-cell responses and thus increase immune response [109, 110]. It particularly models the inflammatory aspects of MS [70, 109]. However, the remyelination is not extensive, which may be due to the dense infiltration of macrophages and microglia in the lesion over a prolonged time, and to the extensive axonal lost which occurs in this model [111] [70].

Although this model has significantly contributed to our understanding of MS mechanisms and led to the development of several therapeutics for early MS (IFN-β-1α, IFN-β-1β, glatiramer acetate, natalizumab, and mitoxantrone) [29, 83, 109, 110], it was not able to predict significant side effects of the immunotherapeutic approaches [29]. Furthermore, it is important to use caution when interpreting the data obtained from this model, as it has a few drawbacks. Firstly, while EAE animals have a localized inflammation in the spinal cord, inflammation in MS is usually spread within the brain. Secondly, EAE is a monophasic disease, while MS has a more relapsing-remitting disease course. Thirdly, there are differences in the immune system between species. Lastly, the T-cell response in EAE is heavily biased towards CD4+ T-cells, whereas CD8+ T-cells dominate in MS [83, 109].

### V.1.2. Theiler's murine encephalomyelitis virus

TMEV is a single-stranded RNA picovirus able to induce immune-mediated death of oligodendroglial cells, axonal damage, and demyelination in both white and grey matter of the CNS [70]. This natural pathogen of mice results in a neurological disease that has a biphasic course [110]. The first phase, known as the acute phase, occurs one week after the injection and is characterized by the infection and the apoptosis of neurons in the grey matter of the brain. The second phase, known as the chronic phase, occurs one month after injection and is characterized by the infection of glial cells and macrophages, inflammatory demyelination, apoptosis of oligodendrocytes, and axonal degeneration in the white matter of the spinal cord [112, 113]. Although the CD4+ and CD8+ T-cell response is important in the neuropathogenesis of this disease, it is not sufficient to induce demyelination, as the persistence of infectious virus is needed for that to happen [110].

### V.1.3. Lysolecithin-induced focal lesion

Lysolecithin, also known as lysophosphatidylcholine (LPC), is a phospholipid commonly used to study remyelination in white matter tracts [114]. It induces demyelination in specific areas of the CNS, which can be observed within a few days. Spontaneous remyelination is typically observed by 14 days and becomes extensive by 6 weeks [70, 110]. The exact mechanism of action is not yet fully understood but it is now assumed that LPC disrupts membranes, including myelin, by inserting into lipid bilayers to form micelles [114, 115]. This insertion can lead to cell membrane permeability and necrotic cell death even below the critical micellar concentration. Recently, alternative mechanisms have been described including those involving receptor-mediated responses. Although no receptor is known to directly bind LPC, it has been shown that LPC could act through G-protein coupled receptors [114]. Additionally, LPC may act via lysophosphatidic acid (LPA). Indeed, *in vivo*, LPC is hydrolyzed into LPA by autotaxin, and it has been demonstrated that injection of LPA into spinal cord white matter was sufficient to induce demyelination [70, 114]. However, these two mechanisms are believed to be secondary to membrane destabilization and are unlikely to drive primary demyelination following LPC injection [114]. Furthermore, LPC may trigger an inflammatory response by acting as a chemo-attractant for monocytes [110]. However, there are confounding issues of toxin-induced axonal damage, BBB breakdown, and moderate traumatic injury with consequent immune cell infiltration at the injection site [70].

### V.1.4. Ethidium bromide injection

Ethidium bromide is a DNA intercalating agent that induces demyelination in predefined areas of the CNS by inducing oligodendrocyte death at the site of injection [70]. The remyelination is very slow, with very little remyelination at 14 days, largely incomplete at 6 weeks, and extensive remyelination around 3.5 months. Due to its DNA intercalating properties, the effects observed with ethidium bromide are not specific to myelinating cells, and depletion of astrocytes is observed [110].

### V.1.5. Cuprizone

Bis-cyclohexanone oxaldihydrazone, also known as cuprizone, is a copper chelator, frequently used as a model for toxic demyelination [116]. In this model, young mice are fed with cuprizone, which leads to the death of oligodendrocytes and subsequent reversible demyelination in specific white matter tracts of the brain [30]. Acute demyelination typically manifests after 5 – 6 weeks of treatment, characterized by nearly complete demyelination of corpus callosum and significant oligodendrocytosis. Chronic demyelination is observed after 12 – 13 weeks of treatment, characterized by progressive ablation of oligodendrocytes, massive demyelination, and axonal injury [110]. Following cessation of cuprizone treatment, robust spontaneous remyelination of demyelinated lesions is observed [116]. However, the mechanisms involved in cuprizone's action are not yet fully elucidated. Copper is an essential component of the mitochondrial enzyme cytochrome oxidase. Cuprizone treatment induces a decrease in the production of energy in oligodendrocytes and an increase in oxidant production, allowing the recruitment of microglia cells. These cells secrete high levels of inflammatory cytokines and may induce oligodendrocyte death. Cuprizone does not cross the epithelial barrier, suggesting its effect may be indirect by interfering with intestinal cooper absorption and reducing copper bioavailability [110, 116]. Additionally, cuprizone induces atrophy of peripheral immune organs such as the spleen and thymus, resulting in a diminished adaptive immune response [29]. The BBB remains intact in the cuprizone model, further limiting the capability of T-cells to infiltrate the CNS [70]. Several therapeutics are now being tested in the cuprizone model to develop drugs that promote new myelin formation and minimize progressive demyelination [29].

## V.2. STEM CELL THERAPIES

Despite the increasing number of therapeutics approved for MS, clear unmet needs exist for effective DMTs in some sub-groups of MS such as aggressive MS, treatment-refractory MS, and progressive MS. In this context, cell-based therapies including autologous hematopoietic stem cell transplantation (AHSCT) and mesenchymal stem cell (MSC) therapies have been explored for several years [117, 118].

### V.2.1. Autologous hematopoietic stem cell transplantation

AHSCT is a well-established multistep procedure designed to replace a patient's immune system with a new one derived from hematopoietic stem cells (HSC). The procedure involves four main steps. Firstly, HSC mobilization is initiated using granulocyte-colony stimulating factors alone or in combination with cyclophosphamide. This process aims to stimulate the release of precursor cells into the bloodstream. Following mobilization, the next step involves the harvesting of HSCs. Then, the immune cells, including those thought to be autoreactive in MS, are depleted through myeloablative or lymphoablative treatment. Finally, the immune system is rebuilt through the transplantation of HSCs, which will develop into a new immune system. While growing evidence supports the efficacy and safety of AHSCT, with two-thirds of treated patients maintaining no evidence of disease activity up to 4-5 years post-treatment [117], AHSCT has not been yet integrated into most national clinical guidelines [119], probably due to the limited number of randomized clinical trials. The results of the only randomized clinical trial conducted thus far, comparing AHSCT with standard DMT in RRMS patients, demonstrated a significant advantage in terms of time to disease progression and quality of life impairments [120]. Ongoing randomized clinical trials, such as RAM-MS and BEAT-MS are performed [121]. In Sweden, a retrospective observational study revealed promising outcomes, with AHSCT associated with a maintenance of "no evidence of disease activity" over 5 years in 73% of RRMS patients without compromising safety [119]. While no malignancies were reported, thyroid disorders were described. Similar findings have been reported in studies conducted in Norway and Denmark [122, 123]. Currently, AHSCT is recommended for young individuals (under 45 years old) with active RRMS or progressive MS at high risk for future disability. Eligibility criteria typically include recent clinical inflammatory activity, short disease duration (no longer than 10 years), and failure of approved high-efficiency DMTs [117, 118].

### V.2.2. Mesenchymal stem cell therapy

Mesenchymal stem cells (MSC) have emerged as a promising therapeutic option for individuals with MS due to their immunomodulatory and neuroprotective properties.

MSCs are multipotent adult stem cells capable of self-renewal and differentiation into various cell lineages, including mesordermal, ectodermal, and endodermal cells. They can be isolated from multiple sources such as bone marrow, adipose tissue, dental tissues, umbilical cord, and placenta. One of the key therapeutic mechanisms of MSC is their immunomodulatory properties, associated with their differentiation capacity and their paracrine effects through growth factor and cytokine secretion and their extracellular vesicles. *In vitro*, MSC can promote regulatory T-cell differentiation, inhibit the differentiation of Th1 and Th17 cells, and induce an anti-inflammatory phenotype in macrophages by secreting anti-inflammatory factors. Additionally, MSC can differentiate towards oligodendrocyte expressing OPC markers such as A2B5 and Olig2. Finally, MSC possess the capacity to move forwards the damage area following chemical gradients, making them attractive candidates for MS treatment.

Experimental models of MS, such as EAE and cuprizone-induced demyelination, have demonstrated the beneficial effects of MSC in reducing inflammatory infiltration, and demyelination and promoting OPC differentiation and remyelination. These preclinical studies have shown improvements in clinical scores and motor functions [124, 125].

Up to date, 29 clinical trials are registered on Clinicaltrials.gov, investigating the safety and the feasibility of MSC transplantation for MS, primarily focusing on SPMS. These trials are mainly phase 1 or 2 studies. Overall, MSC transplantation was feasible and has been well-tolerated, with no serious adverse events reported. Common adverse effects include fever and headache [124].

However, the efficacy of MSC therapy in MS remains uncertain, and conflicting results have been reported. While a double-blind randomized phase I/IIa study reported no significant differences in the rate of relapses, clinical score, and cognitive conditions, and a double-blind randomized phase I/II study highlighted the absence of treatment's effect on the number of gadolinium-enhancing lesions with the active form of MS [126, 127], a few phase I/IIa studies reported short-term beneficial effect (50% of patients had no evidence of disease activity in one trial), improvements in the clinical score and muscle strength [128-131]. Intrathecal administration has been suggested to be more efficacious than intravenous infusion [128]. However, studies including larger cohorts are needed to really understand the therapeutic potential of MSC therapy.

### V.3. REMYELINATION THERAPY

While the currently approved therapies for MS are predominantly immunomodulating, no therapeutics directly repair damage and restore functions. This last decade, research efforts, aimed at developing therapies for MS, have increasingly focused on remyelination of existing lesions, which could potentially restore function and prevent axonal degeneration. Several candidates have been validated in animal models such as EAE and toxin-induced demyelination, leading to several clinical trials. Here are some of the notable candidates:

- Monoclonal antibodies
    - **Opicinumab (anti-LINGO antibody)**: inhibits LINGO-1 (leucine rich repeat and Ig domain containing 1), a protein able to inhibit differentiation and axonal regeneration. Despite interesting results in preclinical models and phase II trial on optic neuritis, the SYNERGY trial included RRMS, and active SPMS participants did not show improvement in disability compared with placebo [132].
    - **Temelimab** (formerly known as GNbAC1, **anti-HERV-W-Env antibody**): neutralizes the envelope protein of human endogenous retrovirus W (HERV-W-Env) which inhibits immune cell activation and OPC maturation [133]. However, a randomized double-blind phase II trial did not meet its primary endpoint [134].
- **Biotin (MD1003):** a cofactor for carboxylases expressed on oligodendrocytes. In a definitive phase III trial, high-dose biotin supplementation did not show significant improvement in disability outcomes compared with placebo [132].
- **GSK239512**: antagonist of the histamine H3 receptor, able to stimulate OPC differentiation in vitro [132]. A randomized double-blind phase II study reported a small but significant effect on magnetization transfer ratio. However, this positive effect did not translate into clinical benefits [135].
- **Bexarotene**: agonist of the retinoid X receptor γ, promotes OPC differentiation. While its remyelinating benefits have been demonstrated in animal models, bexarotene was not well-tolerated and failed to meet the primary endpoint of a randomized double-blind phase II trial in RRMS [136]. However, converging neurophysiological and MRI evidence of efficacy were observed [137].
- **Clemastine fumarate**: antagonist of the muscarinic M1 receptor, belongs to the first generation of antihistamines and able to stimulate OPC differentiation [138]. The phase II ReBUILD trial reported a significant visual evoked potential latency (neurophysiological marker allowing the evaluation of remyelination) suggesting the possible achievement of

myelin repair [139]. The translation of this observation in clinical improvement is yet to be established [140].

- **Domperidone**: inhibitor of dopamine receptor, increases serum prolactin levels, which promotes remyelination [141]. A randomized open-label phase II trial demonstrated reasonably good tolerance and could not reject futility in reducing disability progression in SPMS [142].
- **Bazedoxifene**: selective modulator of estrogen receptor, able to enhance OPC differentiation and remyelination in focal demyelination models of MS [143]. A randomized double-blind phase II clinical trial is ongoing to assess its safety and efficacy in women with RRMS [144].
- **Metformin**: already approved for type II diabetes, shown to increase myelin levels and OPC differentiation in animal models. As metformin has multiple molecular actions, its mechanism of action in remyelination involves various molecular pathways such as AMPK-aPKC-CBP pathway or NMDA receptor [145-147]. A randomized double-blind phase IIa clinical trial assessing the effect of metformin and clemastine therapy on remyelination promotion in RRMS patients already on DMTs is ongoing [148]. A randomized triple-blind clinical trial is also ongoing to evaluate whether metformin, as an add-on treatment, is superior to placebo in delaying disease progression in patients with non-active PMS [149].

Other compounds have also shown promising results *in vitro* and *in vivo* such as antifungal drugs (miconazole [150]), hormone signaling modulators (liothyronine [151], tamoxifen [152]), steroids (clobetasol [153]), or already approved sphingosine-1-phosphate receptor modulators (fingolimod [154] and siponimod [155]). Their efficacity in patients still must be determined.

Despite promising results in preclinical studies, remyelination trials were widely unsuccessful in clinical settings [156]. The search for effective pro-remyelinating drugs remains thus a major unmet medical need and further research is needed to identify novel targets and develop strategies that can promote successful remyelination.

# CHAPTER II: SIPONIMOD AND SPHINGOSINE-1-PHOSPHATE MODULATORS

Since mid-1990s, sphingosine-1-phosphate receptor modulators have raised a lot of interest for MS treatment due to their immunomodulatory effects. This led to the commercialization of fingolimod in 2011, followed by siponimod and ozanimod in 2020, and ponesimod in 2021. Research on these compounds has also indicated a direct effect on the CNS, suggesting a possible involvement in remyelination.

## I. SPHINGOSINE-1 PHOSPHATE AND SPHINGOSINE-1 PHOSPHATE RECEPTOR EXPRESSION

Sphingosine-1 phosphate (S1P) is a bioactive lipid regulating various cellular processes including immune response, angiogenesis, heart rate, vascular tone, endothelium integrity, and cell migration [157, 158]. Its effects are mediated by the G-protein coupled receptor subtypes $S1P_{1-5}$ [159]. Although these receptors are broadly expressed, their expression patterns and roles vary across tissues (Figure 6). $S1P_1$, $S1P_2$, and $S1P_3$ are ubiquitously expressed while $S1P_4$ is predominantly found in lymphoid tissues and lungs, and $S1P_5$ in the spleen. S1P receptors are also widely expressed within the CNS, exhibiting cell-specific distribution patterns. Endothelial cells of the BBB express $S1P_1$, $S1P_3$, and $S1P_5$, which contribute to maintaining BBB integrity and preventing vascular permeability. Astrocytes and microglia express $S1P_1$ and $S1P_3$, with upregulated expression during neuroinflammation. Neurons express $S1P_1$ and $S1P_3$, while their precursor cells only express $S1P_1$, involved in neuronal development and neurite outgrowth. Oligodendroglial cells express four S1P receptor subtypes: $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$, with varying expression patterns during maturation stages. OPCs preferentially express $S1P_1$, which decreases during differentiation, while $S1P_5$ becomes the dominant subtype in mature oligodendrocytes [160-162].

The production of S1P can be initiated by external or internal signals, leading to the metabolization of membrane sphingomyelin to ceramide by sphingomyelinases. Ceramide is then metabolized to sphingosine (Sph) by ceramidases. Finally, Sph is phosphorylated by one of two Sph kinases (SphK1 and SphK2) to generate S1P. S1P is often transported out of cells where it can act in an autocrine or paracrine manner on S1P receptors. The concentration of S1P is relatively high in blood and lymph, but low in lymph nodes. This concentration gradient plays an important role in lymphocyte trafficking [163].

Given S1P's role in regulating lymphocyte trafficking and the presence of the S1P/S1PR complex in the CNS, the effects of S1P receptor modulators can theoretically be categorized into 2 groups: peripheral effect and central effect.

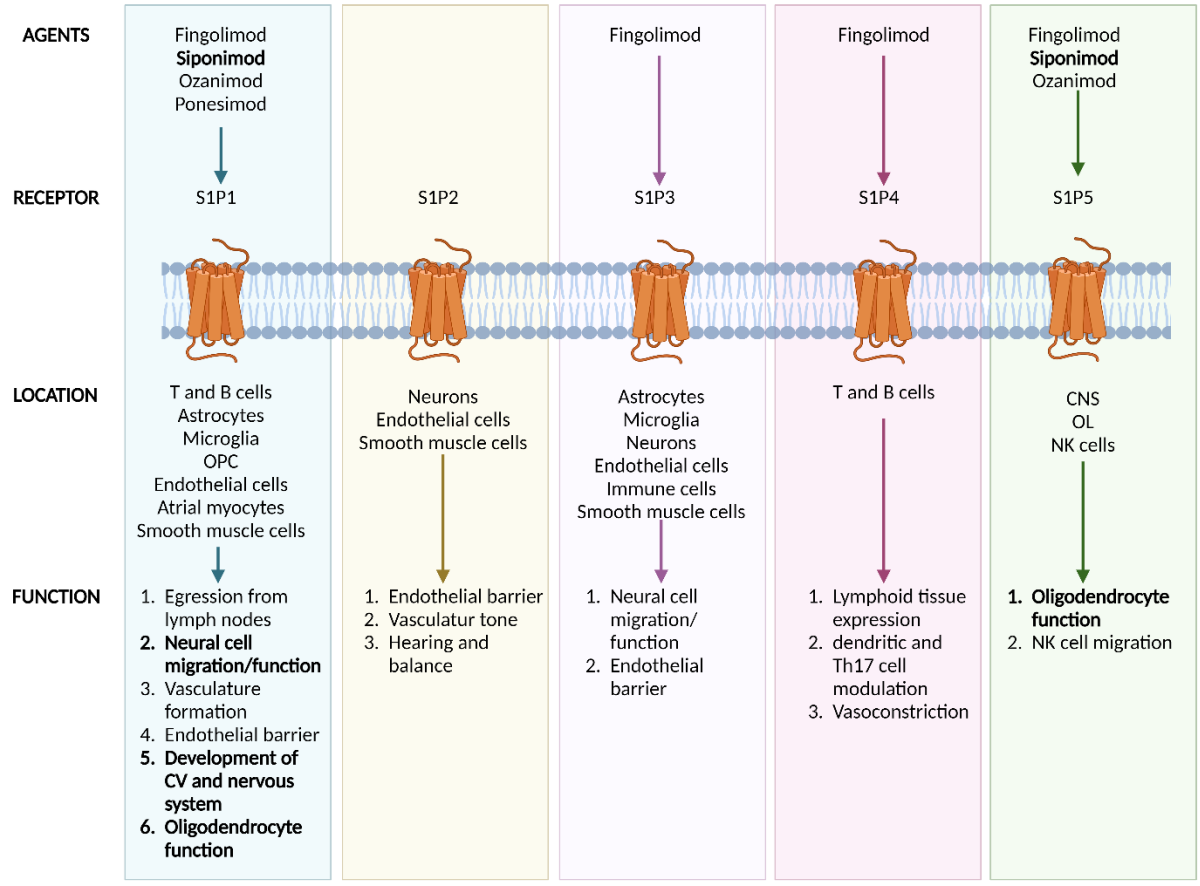



**Figure 6. Interaction of sphingosine-1-phosphate (S1P) receptor modulators with S1P receptor subtypes, adapted from [164].** OPC: oligodendrocyte progenitor cells; CNS: central nervous system; OL: oligodendrocytes; NK cells: natural killer cells; CV: cardiovascular. Created with Biorender.

## II. EFFECTS OF SPHINGOSINE-1-PHOSPHATE RECEPTOR MODULATORS ON THE IMMUNE SYSTEM

In the last decade, the FDA and the EMA have approved four DMTs targeting S1P receptors for the treatment of MS: fingolimod, siponimod, ozanimod, and ponesimod [160]. These modulators act as S1P receptor agonists, bearing structural resemblance to the endogenous S1P. However, they also behave as "functional antagonists" as their binding to the receptors induces receptor desensitization, internalization, and subsequent degradation [165]. This mechanism has been recognized as the primary driver of the immunomodulatory effects of these compounds, supported by accumulating evidence demonstrating the essential role of $S1P_1$ in lymphocyte recirculation [161].

## II.1. EFFECTS OF S1P ON LYMPHOCYTE RECIRCULATION

Naïve T-cells and central memory T-cells ($T_{CM}$) are initially activated and reactivated in the secondary lymphoid organs (SLOs) upon encountering an antigen presented by an antigen-presenting cell. Following activation, T-cells return to the blood circulation and reach the sites of inflammation [166]. This egress of lymphocytes from SLOs into the systemic circulation is driven by the interaction between S1P and $S1P_1$, along with a concentration gradient of S1P between lymphoid tissues and blood, as S1P binding induces receptor internalization (Figure 7) [162]. While S1P is highly expressed in the blood and afferent lymph, promoting $S1P_1$ internalization, its expression is low in SLOs, resulting in the re-expression of $S1P_1$ following clonal expansion of T-cells [159]. This upregulation enables the cells to exit the SLO and reach the efferent lymph, followed by the bloodstream. Several studies using knock-out mice lacking $S1P_1$ have demonstrated that mature T- and B-cells were unable to exit SLOs, highlighting the importance of $S1P/S1P_1$ complex in this process [167-169].

## II.2. EFFECTS OF SPHINGOSINE-1-PHOSPHATE RECEPTOR MODULATORS ON LYMPHOCYTE RECIRCULATION

### II.2.1. Fingolimod (FTY720)

Fingolimod is the first FDA-approved oral therapy for MS. It is a structural analog of natural sphingosine that needs to be phosphorylated *in vivo* by sphingosine kinase 2 to produce fingolimod-phosphate, able to bind $S1P_{1,3-5}$ with high affinity (0.3 – 3.1 nM) [170]. While the binding of fingolimod to $S1P_1$ causes aberrant phosphorylation of the receptor leading to prolonged internalization, ubiquitination, and subsequent degradation, S1P only induces internalization of $S1P_1$ without triggering its degradation [163, 171]. As lymphocytes do not express $S1P_1$ in patients treated with fingolimod, they become unresponsive to normal S1P gradient, causing their functional sequestration, and subsequently reducing their infiltration into CNS [172].

Fingolimod affects both T- and B-cells, with T-cells experiencing a greater impact compared to B cells [173]. Fingolimod treatment leads to a reduction in both CD4+ and CD8+ T-cell numbers, with a more pronounced effect observed on the CD4+ T-cell subset [174]. Moreover, the selectivity of fingolimod towards T-cells expressing the lymph-node homing CC chemokine receptor CCR7 has been demonstrated in a study involving patients with RRMS. Fingolimod prevents the egress of CCR7-positive naïve T-cells and $T_{CM}$, including Th17 cells, from the SLOs, while the levels of CCR7-negative effector memory T-cells ($T_{EM}$) remain largely unaffected by the

treatment [175] (Figure 7). Given that $T_{EM}$ may play a crucial role in memory immune responses in peripheral tissues, their preservation during fingolimod exposure maintains immunosurveillance and the ability to respond to invading pathogens, thereby helping to retain desirable immunological functions [159, 170]. The effect of fingolimod is reversible as cell count typically returns to normal values within 4 – 8 weeks after stopping treatment [176].

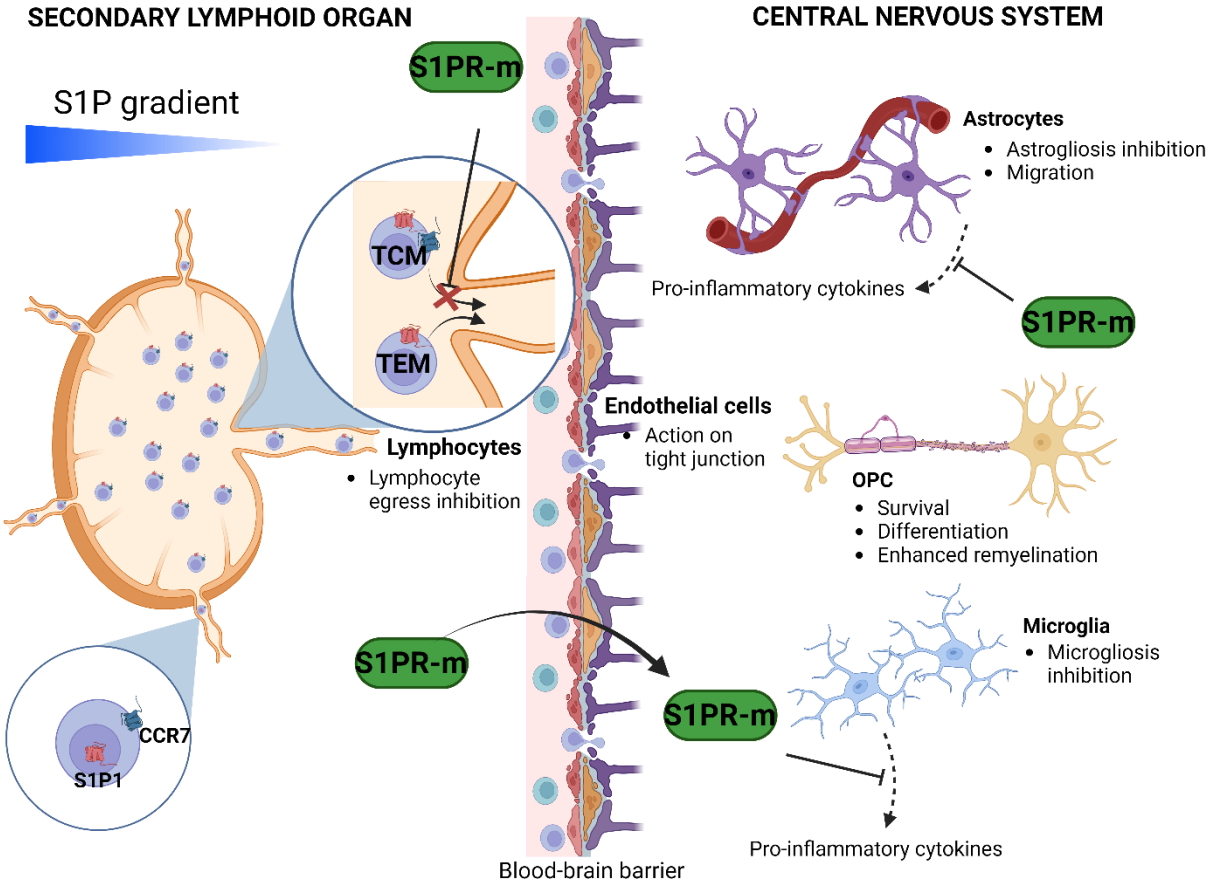



**Figure 7. Peripheral and CNS mechanism of action of S1P receptor modulators (S1PR-m).** In SLOs, S1PR-m bind $S1P_1$ on $T_{CM}$, which causes the engulfment of $S1P_1$. Any new S1P receptors being produced inside the cell remain in a state of arrest until S1P receptor modulation is removed. Therefore, $T_{CM}$ do not leave the lymph node in response to S1P signals, and, by inhibiting the movement of $T_{CM}$ into the circulation, S1PR-m prevents their migration into the CNS. $T_{EM}$, which do not express CCR7 are largely unaffected by S1PR-m, thus preserving immunosurveillance and the capacity to respond to and contain locally invading pathogens. S1PR-m can cross BBB and have direct effects on S1P receptors expressed throughout the CNS which may explain their potential efficacity in remyelination. SLO: secondary lymphoid organ; $T_{CM}$: central memory T-cell; S1P: sphingosine-1-phosphate; $T_{EM}$: peripheral effector memory T-cell; CCR7: CC chemokine receptor 7; CNS: central nervous system; OPC: oligodendrocyte progenitor cell. Created with Biorender.

### II.2.2. From fingolimod to siponimod

In 2020, the EMA approved a new sphingosine-1-phosphate receptor modulator, siponimod (BAF312, Mayzent®) for the treatment of adult patients with SPMS with active disease evidenced by relapses or imaging features of inflammatory activity. Similar to fingolimod and other S1P receptor modulators, siponimod's therapeutic effects are primarily mediated by rapid internalization, degradation, and functional antagonism of $S1P_1$, leading to lymphocyte sequestration in the lymph nodes [177]. However, there are several distinctions between the two compounds, including their chemical structures. Contrary to fingolimod, siponimod does not require phosphorylation and exhibits selective binding to $S1P_1$ and $S1P_5$ with nanomolar affinity (EC50 of 0.39 nM and 0.98 nM, for $S1P_1$ and $S1P_5$, respectively), potentially alleviating some side effects [177]. However, acute bradycardia has been observed with siponimod, whereas this adverse effect was initially attributed to $S1P_3$ based on findings in rodents [161]. In humans, activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels mediated by siponimod in atrial myocytes can fully explain the bradycardia [177]. This could be attenuated through dose titration [178]. Siponimod has also been engineered to possess a relatively short elimination half-life, enabling the reversion of its effects within 1 week following the end of the treatment while still allowing once-daily oral dosing [177].

As previously mentioned, S1P receptors are expressed by various types of brain cells. In addition, S1P receptor modulators can cross BBB following oral administration. Therefore, by modulating the S1P receptors expressed on CNS cells, S1P receptor modulators may exert a direct impact on neuropathological processes such as neurodegeneration, gliosis, and remyelination [170].

## III. EFFECTS OF SPHINGOSINE-1-PHOSPHATE RECEPTOR MODULATORS ON THE CENTRAL NERVOUS SYSTEM

### III.1. OLIGODENDROCYTE PROGENITOR CELLS AND OLIGODENDROCYTES

S1P receptors have been associated with protection against demyelination and the promotion of remyelination in both *in vitro* and *in vivo* studies using S1P, fingolimod, or siponimod.

Both OPCs and oligodendrocytes express $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$. OPCs predominantly express $S1P_1$, while $S1P_5$ is abundantly expressed on oligodendrocytes. Fingolimod and siponimod, acting as "functional antagonists" upon binding to $S1P_1$, act as agonist on $S1P_5$ on both OPCs and oligodendrocytes [179, 180].

In OPC culture, fingolimod has been shown to enhance oligodendrocyte survival, inhibiting apoptosis induced by serum and glucose deprivation [181]. S1P receptors have been associated with cytoprotective activity through the activation and phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase B (Akt). However, this activity has been observed in oligodendrocytes but not in OPCs, suggesting the involvement of S1P receptor subtype $S1P_5$ [182]. This has been confirmed by studies on animals lacking $S1P_1$ and $S1P_5$, which have shown that $S1P_1$ plays a pivotal role in early differentiation and OPC differentiation [183, 184], while $S1P_5$ was found to be more involved in regulating OPC migration and oligodendroglial survival [185, 186].

Additionally, studies have highlighted the regenerative and promyelinating effects of fingolimod, which follow the dynamics of a bell-shaped curve with high concentrations being less efficient than low concentrations [160, 187]. Thus, *in vitro* studies on oligodendroglial lineage cells have shown that OPC differentiation into pre-oligodendrocytes and mature oligodendrocytes, and MBP protein levels were increased with nanomolar concentrations of fingolimod while OPC differentiation was inhibited with micromolar concentrations [154, 188, 189]. In line with these results, *ex vivo* studies on LPC-induced demyelinated cerebellar brain slices have shown the enhanced remyelination capacity of fingolimod at low concentrations (0.1 nM) but not at high concentrations (1 μM) [190]. The mechanism behind this effect remains unclear. However, a time-dependent regulation of S1P receptor expression has been observed following prolonged treatment with high fingolimod concentrations, suggesting a change of $S1P_1$ and $S1P_5$ expression levels in mature oligodendrocytes and thus a different functional response to fingolimod [181].

Following the interesting results obtained *in vitro* and *ex vivo*, *in vivo* studies using EAE and cuprizone models have yielded contradictory results. While some studies have observed a potential effect of oral fingolimod (1mg/kg) on OPC differentiation or migration without apparent effects on remyelination [191], others have reported accelerated remyelination following oral fingolimod administration (0.3 mg/kg) [192]. Notably, early intervention with fingolimod (1 mg/kg) was found to prevent mature oligodendrocyte death in corpus callosum, whereas these effects were not recapitulated with a later treatment, suggesting the importance of time-dependent intervention to prevent demyelination [193]. However, in EAE model, no effect of oral fingolimod administration (0.3 mg/kg) on MBP level was observed at day 7 post-onset, while the treatment significantly increased MBP at 30 post-onset [194]. Furthermore, the efficacy of fingolimod on remyelination has also been observed in LPC-induced demyelination rat model, in which oral low doses of

fingolimod (0.3 mg/kg) but not high doses (1 mg/kg) increased the number of OPCs 6 days after LPC administration [195].

The contradicting results observed *in vivo* could be explained, at least partially, by different models, dosage, timing of treatment, and analysis of the results used in experimental procedures [160].

While research on remyelination using S1P receptor modulators has primarily focused on fingolimod, limited evidence suggests a potential direct involvement of siponimod in reducing demyelination and promoting oligodendrocyte survival. Siponimod (3 or 10 μM) has been shown to increase MBP levels and attenuate LPC-induced demyelination in spheroid cell cultures and organotypic slice cultures, respectively [155, 196]. Moreover, a small but significant reduction of IL-6 levels was also observed [155]. The capacity of siponimod to penetrate and distribute inside the brain has been observed in rats and mice [197]. Additionally, siponimod demonstrated pro-remyelinating effects in xenopus tadpole and cuprizone-induced demyelination, as well as a beneficial effect of prophylactic treatment on demyelination prevention in EAE-associated neuritis [187, 198].

However, further investigation is warranted to elucidate the direct impact of siponimod on OPC/oligodendrocyte proliferation, differentiation, migration, and survival [160].

S1P receptors are also present in other CNS cell types such as astrocytes and microglia, suggesting a potential effect of S1P receptor modulators on these cells.

### III.2. ASTROCYTES AND MICROGLIA

Astrocytes mainly express $S1P_1$ and $S1P_3$, alongside other subtypes at lower levels [163]. Analysis of post-mortem brain tissue from MS patients has revealed a significant increase in $S1P_1$ and $S1P_3$ expression on reactive astrocytes in both active and chronic inactive MS lesions [199]. Furthermore, the direct involvement of $S1P_1$ in astrocyte function has been demonstrated in animal model lacking $S1P_1$ [200].

*In vitro* studies on human astrocytes or EAE mice have shown that fingolimod can stimulate the production of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF), and heparin-binding EGF-like growth factor (HBEGF), all of which are involved in OPC and oligodendrocyte differentiation and survival [201-203]. Additionally, fingolimod has been found to limit secretion of proinflammatory cytokines and chemokines by primary culture of human astrocytes [199].

In EAE model, fingolimod treatment led to a significant suppression of NF-ϰB p65 translocation in activated astrocytes, accompanied by decreased production of Il-6, TNF-α, GM-CSF, CCL2 and nitric oxide [204]. However, *in vitro* studies using fingolimod on astrocytes have shown conflicted results, as fingolimod induced the release of GM-CSF, which reduces the effect of cytokines on endothelium [205].

Furthermore, proinflammatory cytokine expression was also reduced in microglia cells treated with fingolimod. While *in vitro* experiments suggested that fingolimod acted as a functional agonist of $S1P_1$ on astrocytes [206], *in vivo* experiments conducted on EAE mice supported functional antagonism of astrocytic $S1P_1$ [200].

Studies have also highlighted the beneficial effects of siponimod on astrocytes and microglia, independently of peripheral effects. *In vitro* functional assays using astrocytes generated from human fibroblasts have demonstrated that siponimod inhibits NF-ϰB translocation, induces rapid nuclear translocation of Nrf2, an antioxidant pathway that may confer neuroprotection to astrocytes during neuroinflammation [207], and maintains high levels of glutamate transporters such as GLAST and GLT1 [208]. These effects were also observed with fingolimod, underscoring the involvement of $S1P_1$. Continuous intracerebroventricular infusion of siponimod significantly decreased GFAP and Iba-1 levels, markers of astrocytes and microglia cells, respectively, thereby attenuating astrogliosis and microgliosis in mice EAE model [209]. In addition, partial restoration of the cortical neuronal circuit has been observed after direct administration of siponimod into the CNS [210]. In a chronic EAE model, macrophage infiltration and microglia activation were decreased by orally administered siponimod [211].

These findings collectively suggest that siponimod exerts beneficial effects on astrocytes and microglia, contributing to its potential neuroprotective properties.

### III.3. ENDOTHELIAL CELLS OF THE BLOOD-BRAIN BARRIER

No patient data regarding the effect of S1P receptor modulators on BBB integrity in MS have been reported. However, endothelial cells express at least 3 subtypes of S1P receptors ($S1P_{1-3}$), which are required to develop and maintain a functional vascular system, thereby playing a role in BBB integrity. Although a proper equilibrium in the signaling between $S1P_1$ and $S1P_2$ is essential for the maintenance of a competent endothelial barrier at the BBB, *in vitro* studies using endothelial cell culture have shown that S1P can reduce cell death after cytokine exposition, with $S1P_1$ being the predominant receptor involved in this survival [205].

Recently, preservation of occludin and zonula occludens-1 expression following traumatic brain injury was observed in mice treated with intraperitoneal siponimod (1 mg/kg), suggesting positive effects on tight junction and BBB integrity [211]. Zonula occludens-1 expression in endothelial cells was also recovered 24h after siponimod treatment using an in vitro BBB model [212]. As siponimod does not act on $S1P_3$, this effect appears to be mediated by $S1P_1$. In this study, fingolimod treatment was not able to recapitulate these effects. Similarly, in another study, $S1P_1$ inhibition increased occluding and zonula occludens-1 levels [213].

## CHAPTER III: EXTRACELLULAR VESICLES

(Chapter adapted from "Extracellular vesicles for the treatment of central nervous system diseases" published in *Advanced Drug Delivery Review*) [214].

A wide array of nanocarriers has been extensively investigated for drug delivery to the CNS over the past few years. These nanoparticles can be used as vehicles to deliver drugs to the CNS following both invasive [215-218] and non-invasive [219-221] administration routes.

Nanoscale systems used for therapy and theragnostic applications, also known as nanomedicines, can be classified according to the nature of the nanomaterial from which they are made of. Some of the most common nanocarriers, as well as their advantages and disadvantages as drug delivery systems, are summarized in Table 2. Depending on the material selected to form the nanomedicines, it can allow passive crossing of the BBB, but also enables surface functionalization to further enhance transport across epithelial barriers and/or to target specific cell types and/or brain regions. Recent evidence support the use of extracellular vesicles (EVs) as nanomedicines. EVs are small vesicles (nanometers scale) consisting of an aqueous core surrounded by a lipid bilayer. They contain genetic materials (mostly miRNA and mRNA), proteins, small molecules and lipids, the nature of which depend on the parental cells. Compared to polymeric and lipid-based nanoparticles, EVs offer many additional advantages as drug vehicles, as they are less toxic and immunogenic and can cross biological barriers, including the BBB [222]. Osorio et al. showed that despite having lower encapsulation efficiency of miR-219a-5p (miRNA) compared to poly (lactic-co-glycolic acid) (PLGA) nanoparticles and liposomes, EVs were more effective crossing a BBB *in vitro* model [223]. Moreover, following intranasal administration they successfully decreased disease burden in experimental autoimmune encephalomyelitis mice [223]. In addition, EVs show great potential as drug delivery carriers but also as a therapeutic tool for CNS diseases due to their intrinsic physiological properties.

**Table 2. Different nanocarriers used as drug delivery vehicles for the CNS.**

| Type of nanocarrier | | Production method | Composition | Type of cargo | Therapeutic effects of the carrier | Advantages as drug carrier | Disadvantages | References |
|---|---|---|---|---|---|---|---|---|
| Polymeric | PLGA | Single or double emulsification-solvent evaporation technique, nanoprecipitation or spray drying | Composed of polymerized glycolic acid monomers and lactic acid monomers | Small hydrophobic drugs, proteins and nucleic acids | None reported | Biocompatible, biodegradable, suitable for lyophilisation, ease of preparation, controlled pharmacokinetic and surface functionalization | Use of organic solvents during formulation, poor drug loading, aggregation, cannot cross the BBB | [224, 225] |
| Lipid-based | Nanoemulsions | High pressure homogenization, phase inversion emulsification method and self-nanoemuslification method | Composed of oil, water and an emulsifier (most commonly a surfactant but also proteins or lipids) | Hydrophobic drugs | None reported | Ease of formulation, small size and no use of organic solvent | Destabilization by Ostwal ripening and risks of erythrocyte lysis | [226, 227] |
| | Lipid micelles | Self-assembly when concentration of surfactant is above the critical micelle concentration | Composed of oil (phospholipids or cholesterol), water and surfactant | Lipophilic drugs (classic micelles) and hydrophilic drugs (reverse micelles) | None reported | Spontaneous formation, solvent-free formulation, small size, sustained and controlled release and no neurotoxic effects reported, surface modification for targeting | Low stability and low drug-loading | [228] |
| | Liposomes | Thin film hydration with aqueous media or organic solvent | Mainly composed of phospholipids and an aqueous medium | Hydrophilic and hydrophobic compounds | Shown to exert intrinsic anti-proliferative activity on U-937 histiocytic cells | Highly biomimetic and semi-spontaneous, passively cross the BBB, possible surface functionalization | Use of organic solvents, low stability, big size, short half-life, rapid clearance, sterilization difficulties, neurotoxicity | [229-231] |
| | Solid lipid nanoparticles | High shear homogenization, double emulsification or microemlsion based method | Formed of solid-state lipids at room and body temperature, surfactant and aqueous medium | Lipophilic drug | None reported | Highly stable, solvent-free formulation, ease to scale-up, controlled release, suitable for lyophilisation, passively cross the BBB, possible surface modification | Not for fragile drugs, multi-step formulation, reduced hydrophilic drug entrapment efficiency, reported to induce inflammation | [232, 233] |
| | Nano-lipid carrier | High-pressure homogenization, solvent emulsification-evaporation methods or melt dispersion | Composed of a mix of solid and liquid lipids and surfactant. | Lipophilic and hydrophilic drugs by conjugation of drug functional groups to the lipid matrix | Shown to possess endothelial-protective effects in a murine acute lung injury model | High stability, high lipophilic drug loading, long-term storage stability, passively cross the BBB, possible surface modification | Low proteins and genes loading, lack of scale up methods, cytotoxicity due to surfactant amount | [234, 235] |
| | Lipid nanocapsules | Phase inversion temperature | Composed of a mixture of triglycerides, nonionic surfactants and saline | Lipophilic and amphiphilic drugs | Shown to trigger GLP-1 secretion in both human and murine cells as well as *in vivo* in mice | Solvent-free formulation, highly stable, ease to scale up, surface modification for targeting and PgP efflux pump inhibition | Multistep and use of high temperatures | [236, 237] |
| Biological | Extracellular vesicles | Isolation from most cell types of the body or body fluids by differential ultracentrifugation, density gradient centrifugation, size exclusion chromatography, immunoaffinity capture or PEG-mediated precipitation | Composed of particular lipids, proteins and nucleic acids specific to the parent cell | Small lipophilic and hydrophilic molecules, proteins and nucleic acids | Exert several therapeutic effects depending on the physiological characteristics of the parent cells | Small size, low immunogenicity, long half-life in the circulation, can cross the BBB, possible surface modification for high targeting efficiency | Low loading efficiency and need of high reproducible productivity and purification method to scale up | [222] |

# I. INTRODUCTION TO EXTRACELLULAR VESICLES

Research on EVs began in the mid-20th century, marked by their initial isolation [238]. Initially regarded as cellular waste products, the perception of EVs significantly changed in 1996 when their biological functions were first demonstrated. Recognizing the need for standardized guidelines in the burgeoning field of EV research, the International Society for Extracellular Vesicles (ISEV) was founded in 2011. ISEV published the first set of guidelines for EV-related studies in 2014 which were updated in 2018 and 2023 [239]. Figure 8 summarizes the important milestones in the field of EVs.

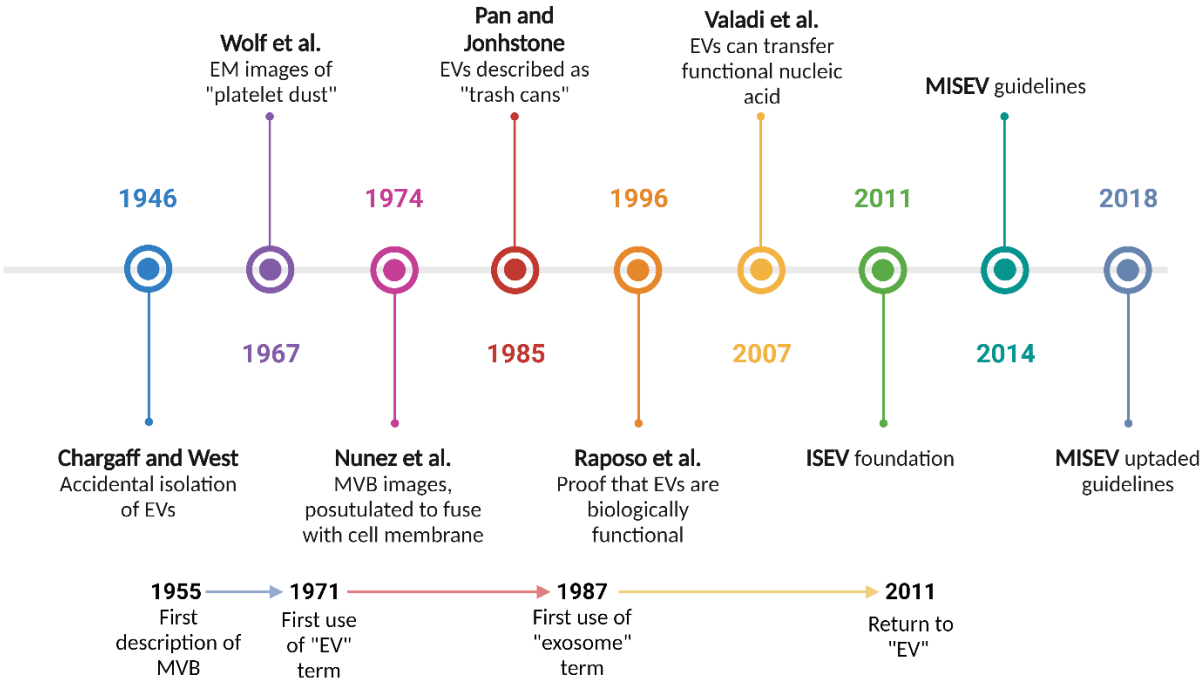



**Figure 8. Timeline of selected milestones in the EV field** [238, 240, 241]**.** Created with Biorender.

## I.1. NOMENCLATURE, BIOGENESIS, AND COMPOSITION

### I.1.1. Nomenclature

A variety of terms has been used in literature to describe secreted membrane-enclosed vesicles observed, from "pequenas particulas" (small particles) to extracellular microvesicles, microparticles and virus-like particles [241]. However, research progressed necessitating a distinct and precise nomenclature based on the biogenesis pathway rather than size [242].

In 2011, Gyorgy et al. suggested the term "extracellular vesicle" as a generic term for particles released from the cells that are delimited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus [243]. This term was later formalized in the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines in 2018 [244].

The term "exosome" refers to vesicles released following the fusion of multivesicular bodies with the plasma membrane [244, 245], while "microvesicle" describes vesicles that form by direct budding of the plasma membrane [240], and "apoptotic body" refers to membrane-bound vesicles derived from disassembly of apoptotic cells [246] (Table 3). However, the overlapping range of size, similar morphology, variable composition, and the absence of consensus on specific markers of EV subtypes make it extremely difficult to assign an EV to a particular biogenesis pathway [247]. To address this issue, MISEV recommends using operational terms that describe EVs based on their physical characteristics (e.g. small EVs vs medium/large EVs), biochemical composition (e.g. CD63+ EVs, CD81+ EVs, …), or the conditions under which they are produced (e.g. hypoxic EVs, IFNγ-stimulated EVs) [239, 244]. Despite these recommendations, the term "exosome" is still frequently overused, while most, if not all, EV samples contain heterogenous mixture of vesicle types [248, 249]. Furthermore, not all researchers agree with the current recommendations [241].

There is growing evidence of a wide diversity of non-vesicular extracellular particles (NVEPs) that are frequently co-isolated with EVs, due to their overlapping physicochemical properties. NVEPs are defined as all non-EV particles made from cell-derived components of one or more molecular classes such as proteins and nucleic acids; lipids, if present, do not form a delimiting bilayer membrane. The term "extracellular particles" thus serves as an umbrella term for all particles outside the cell, including both EVs and non-vesicular entities [239]. The recent ISEV guidelines, published in 2023, suggest using the term "EPs" or "EV-preparation" or "EV-containing preparation" when EVs and NVEPs cannot be fully distinguished.

**Table 3. Different subtypes of EVs and their main characteristics.**

| Vesicle type | Characteristics | | |
|---|---|---|---|
| | **Size (nm)** | **Biogenesis** | **Main markers** |
| **Exosome** | 40 – 120 | MVB fusion with plasma membrane | Tetraspanins (CD63, CD9, CD81), TSG101, ALIX |
| **Microvesicle** | 50 – 1000 | Direct budding of plasma membrane | Integrins, selectins, CD40 ligand |
| **Apoptotic body** | 500 – 2000 | Blebbing of apoptotic cell membrane | Histones |

### I.1.2. Exosome and microvesicle biogenesis

Exosomes are small vesicles formed within the endosomal system as intraluminal vesicles (ILVs), which are created by the inward budding of endosomal membrane (Figure 9). These vesicles are secreted when multivesicular endosome/body (MVEs or MVBs) fuse with the cell surface. MVBs may also fuse with lysosomes, leading to the degradation of their content. The factors determining the fate of exosomes are not fully understood, though cholesterol levels within MVBs appear influential. Specifically, MVBs enriched with cholesterol are typically directed towards exosome secretion whereas the cholesterol-poor MVB population is targeted for lysosomal degradation [250]. Exosome biogenesis is dependent on the cargo and cell type and can be influenced by various signals or pathological stimuli. The main mechanism for exosome formation involves the endosomal sorting complex responsible for transport (ESCRT), which includes four multiprotein complexes (ESCRT-0, -I, -II and -III) and accessory proteins such as Alix and VSP4. ESCRT-0, -I, and -II recognize and sequester ubiquitinated membrane proteins at the endosomal limiting membrane, while ESCRT-III facilitates membrane budding and ILV fission [247, 250]. Interestingly, even in the absence of ESCRT components, MVBs containing CD63+ ILVs can still form, indicating the presence of ESCRT-independent pathways for exosome generation [251]. One of these pathways involves ceramide production by neutral type II sphyngomyelinase, which creates membrane subdomains inducing spontaneous negative curvature and potentially influencing cargo sorting via S1P receptor activation [252]. Another ESCRT-independent

mechanism involves tetraspanin proteins (e.g. CD63, CD81, CD82, and CD9) which form cluster and dynamic membrane platforms, promoting inward budding. Additionally, MHC class II proteins can drive MVB formation, as these proteins are recruited to exosomes independently from their ubiquitination [253].

The cargo within exosomes varies based on cell type and the physiological and pathological state of the donor cell. While proteins sorting into exosomes involves syntenin, the small GTPase ADP-ribosylation factor 6 (ARF6), chaperones such as HSC70 and HSP70, and GPI-anchored proteins, mechanisms remain unclear. miRNA within exosomes often have specific sequence motifs acting as cis-acting elements for exosome targeting, with ESCRT-II potentially serving as RNA-binding complex sequestered by tetraspanin-enriched microdomains. The miRNA-induced silencing complex (RISC) and AGO2 are also implicated in this process [247, 250].

Following their formation, MVBs are transported to and fuse with the plasma membrane to release ILVs as exosomes into the extracellular space (Figure 9). This transport relies on the cytoskeleton (actin and microtubules), associated molecular motors (dynein, kinesins and myosins), and small GTPases. Docking and fusion with the plasma membrane involve various RAS-related proteins (Rab, such as Rab7, Rab27a, and Rab27b), actin, and SNARE proteins such as VAMP7. Intracellular $Ca^{2+}$ levels also regulate exosome secretion, possibly through the activation of SNARE complexes, although this mechanism is not yet fully understood [247, 250].

The formation of microvesicles from the plasma membrane involves several molecular rearrangements, including changes in lipid components (e.g. translocation of phosphatidylserine from the inner leaflet to the cell surface) and changes in protein composition and $Ca^{2+}$ levels via scramblase and calpain activation. Cytoskeletal elements and their regulators, such as actin, myosin, and small GTPases are also involved, along with cholesterol, which is abundant in microvesicles. For microvesicle cargoes, cytosolic components bind to the inner leaflet of the plasma membrane, while nucleic acid sorting may involve conserved zipcode RNA sequence motifs. The fission of microvesicles from the plasma membrane depends on actin and myosin interaction with subsequent ATP-dependent contraction [247, 250]. TSG101 and VSP4, which are part of ESCRT machinery, also participate in this process [254].

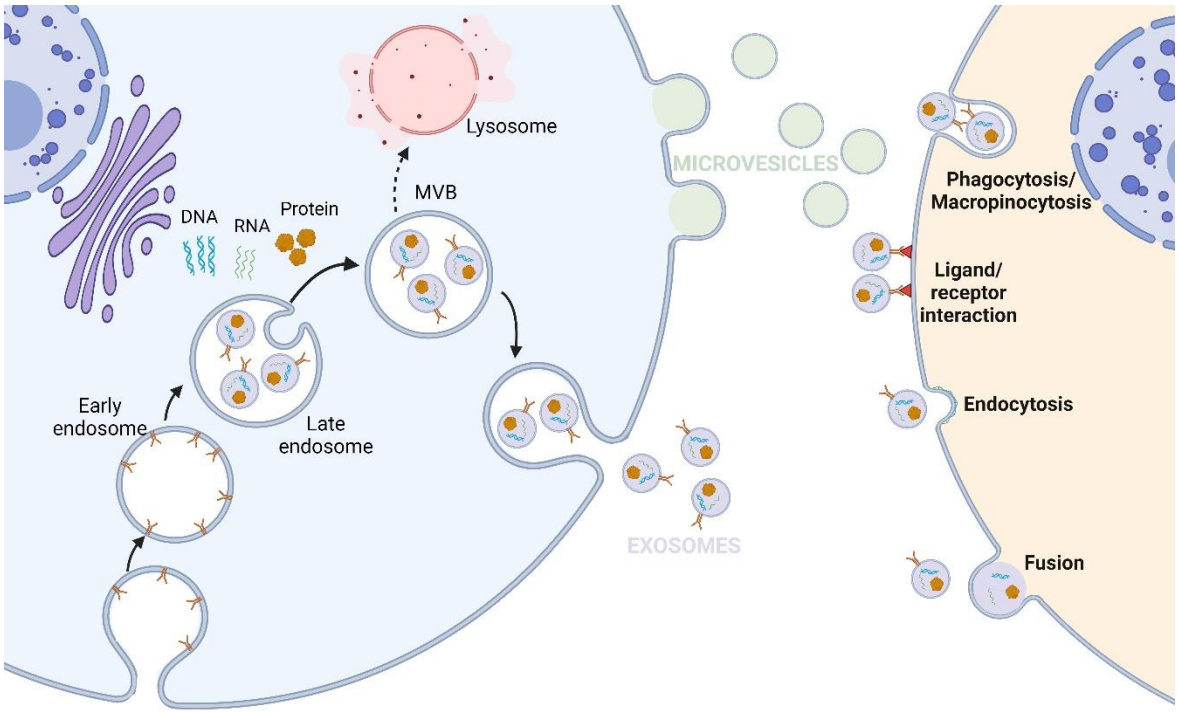



**Figure 9. Biogenesis of extracellular vesicle and their interaction with recipient cells**. Biogenesis of exosomes is initiated upon the formation of early endosomes as a result of membrane inward invagination. Subsequently, the endosome membrane invaginates inward to form nanoscale intraluminal vesicles (ILVs), leading to the development of late endosomes which become multivesicular bodies (MVBs) containing ILVs. MVBs then mature and fuse either with lysosomal membranes for degradation or with the cellular membrane to release exosomes into the extracellular space. In contrast, microvesicles originate from the outward budding of the plasma membrane. After their release, EVs interact with recipient cells through various mechanisms, including membrane fusion, ligand/receptor interactions, and endocytosis (clathrin or caveolin-dependent, phagocytosis or macropinocytosis). Created with Biorender.

### I.1.3. EV composition

EVs contain proteins, lipids, and nucleic acids which could explain their biological properties.

The protein composition of EVs is largely determined by biogenesis, leading to an enrichment of proteins such as Alix, TSG101, chaperones, and tetraspanins. Additionally, EVs often contain glycoproteins such as major histocompatibility complex class I and II molecules [255].

Compared to the plasma membrane, EVs from various cell types are highly enriched in cholesterol, ceramides, sphingomyelin, and disaturated lipids, suggesting that the EV lipid bilayer is more rigid than the plasma membrane. This may enhance their resistance to degradation and stability as carriers of biomolecules. Moreover, EVs contain enzymes involved in lipid metabolism, such as phospholipases D and A2 [256].

EVs are also enriched in small RNAs, predominantly miRNAs. Numerous studies have demonstrated that EV-associated miRNA can repress the expression of target genes in recipient cells and that EV-mRNA can be translated into proteins [226]. The amount of RNA in EVs varies depending on the cell type of origin. The mechanisms underlying RNA selection for incorporation into EVs are not yet fully understood. However, some studies suggest that EV-associated miRNAs undergo post-transcriptional modifications in their 3' untranslated regions, which may facilitate their selective packaging into EVs [257]. While there is evidence that EVs also contain DNA, several studies indicate that EVs do not protect DNA from DNAse degradation, implying that the DNA is likely located on the vesicle surface rather than inside the vesicles [256].

## I.2. EV INTERACTION WITH CELLS

EVs can interact with target cells through ligand-receptor mechanisms, leading to biological effects (Figure 9). In such cases, EVs act at the cell surface, without delivering their content, triggering intracellular signalling cascades [256, 258]. Besides this direct interaction, the biological effects of EVs depend on their ability to deliver their intraluminal cargo into target cells.

Direct evidence for the fusion of EVs with target cell membrane has been demonstrated in a few studies [250]. This process involves several protein families, such as SNAREs and Rab-proteins. Although this process would be the most efficient method of cargo transfer, EVs are typically internalized via endocytosis, an energy-dependent active process [259]. It is extremely rapid, with EVs entering cells within 15 minutes following initial introduction [260]. The main endocytosis pathway is clathrin-mediated endocytosis, where clathrin-coated vesicles deform the membrane into a vesicular bud, which matures and pinches off. The resulting intracellular vesicle undergoes clathrin uncoating and either fuses with the endosomal membrane or is targeted to lysosomes for degradation [260]. However, evidence suggests that clathrin-independent pathways also exist in cells, including caveolin-dependent endocytosis. Caveolae are subdomains of glycolipid rafts rich in cholesterol, sphingolipids, and caveolins, which form small cave-like invaginations in the plasma membrane that internalize and fuse with endosomes or lysosomes [258]. Another endocytic pathway, macropinocytosis, allows cells to internalize large quantities of EVs. This process involves membrane ruffling, where extensions of the plasma membrane envelop an area of extracellular fluid and internalize it as a result of the fusion of the membrane protrusions with themselves or back with the plasma membrane. This mechanism does not require direct contact with the internalized material [261, 262]. While some studies indicate that macropinocytosis plays a role in EV uptake, others using inhibitors suggest it is a minor pathway or specific to certain cell types

[260]. Phagocytosis is another pathway reported for EV uptake by specialized cells such as macrophages and dendritic cells. However, it remains unclear whether phagocytosis allows EV internalization for cell communication or if this process is used for EV degradation [250].

The primary route by which EVs deliver their contents into the cytosol of recipient cells is still debated. EV recognition and capture likely depend on their size and surface components [258].

Current knowledge of the fate of EVs post-endocytosis remains limited. After endocytosis, internalized EVs are directed to early endosomes, also known as sorting endosomes. From there, cargo may either be recycled back to the plasma membrane for excretion, either directly or via recycling endosomes, or be transported towards lysosomes through late endosomes for degradation [263]. This endo-lysosomal degradative pathway responsible for EV clearance requires further investigation to elucidate its role in eliciting cellular responses [263]. The fusion of endocytosed EVs with late endosomes, which leads to the release of cargo into the cytosol, has also been suggested and might be favored by the acidic pH of the late endosome environment [264, 265]. Moreover, a recent study has identified a new intracellular trafficking route for endocytosed EVs, which involves the transport from late endosomes to nucleoplasmic reticulum. This pathway enables EVs to deliver their cargo into the nucleoplasm through the entry of Rab7+ late endosomes containing endocytosed EVs into nuclear envelope invaginations, thus creating a subnuclear compartment [266].

## II. EXTRACELLULAR VESICLE ISOLATION

Prior to their use, EVs must be isolated using one or more separation techniques. These procedures exploit the biophysical characteristics of EVs such as size, density, charge, and surface composition [244]. Table 4 presents the most common EV isolation techniques described in the literature. Each isolation method has its advantages and disadvantages, which can affect downstream process. However, an ideal method has not yet been established within the EV research community [267]. The choice of separation method should be based on the known properties of specific EV source and the desired EV yield and purity (Figure 10) [239]. Nevertheless, a worldwide survey from 2016 found that ultracentrifugation, either differential or density gradient, was the most commonly used technique [268]. No similar survey has been conducted since then.

**Table 4. Common EV isolation techniques reported in the literature**

| Method | Details | Time | Advantages | Disadvantages | Ref. |
|---|---|---|---|---|---|
| **Based on ultracentrifugation** | | | | | |
| Differential ultracentrifugation | EV isolation from conditioned media by differential centrifugations to remove cells and cell debris, followed by ultracentrifugation at 100.000g to isolate EVs | 140 – 600 min | Simple, cost efficient, large volumes, no additional chemicals, large amounts of EV | Not for viscous samples, EV aggregation, lipoprotein contamination, low purity, low reproducibility | [268-270] |
| Density gradient ultracentrifugation *Sucrose or iodoxinol density gradient* | EV isolation based on buoyant density using a discontinuous gradient of a sucrose solution or iodoxinol | 250 min – 2 days | No additional chemicals | Time-consuming, low purity, complexity, sample loss during fractionation, co-purification of viral particles. | [269, 271] |
| **Based on size** | | | | | |
| Ultrafiltration | Passage of the medium through a filter membrane via a centrifuge force. Components with size lower than pore size are discarded while components (EVs) with a higher size are collected | 130 min | Simple, fast, no special equipment required, large volumes, no additional chemicals | Filter plugging, loss of sample, contamination with proteins, vesicle deformation | [271, 272] |
| Size exclusion chromatography | Separation of EVs into several fractions when passing through a gel column containing beads with known pore sizes | 0.5ml/min + column washing | Cost efficient, high purity, reproducibility, vesicle integrity preserved, prevents EV aggregation, no additional chemicals, scalable | Not for large volumes (require in that case additional concentration steps), long run time, specialized equipment | [268] |
| Tangentiel flow filtration | Crossflow filtration where the mainstream flows parallel to the membrane face allowing a continuous cycle with applied pressure | 120 min | Scalable, batch-to-batch consistency | Membrane fouling, reduced purity with high protein samples, specialized equipment | [273, 274] |
| **Based on precipitation** | | | | | |
| Precipitation with polymers (*PEG*) | EV precipitation caused by polyethylene glycol followed by conventional centrifugation to pellet EVs | 65 min | Cost efficient, simple, EV integrity preserved, pH close to physiological range, scalable | Contamination and retention of the polymer, co-precipitation of proteins | [275] |
| Commercial kits for polymer precipitations *ExoQuick and TEI* | EV precipitation caused by commercial reagent followed by conventional centrifugation to pellet EVs | 45 – 65 min (sometimes overnight) | Simple, EV integrity preserved, pH close to the physiological range | Cost, poor reproducibility, polymer retention, co-precipitation of proteins | [275] |
| **Based on immunoaffinity** | | | | | |
| Immunoaffinity techniques *ExoTEST™, ExoCap™* | EV capture based on specific interaction between EV surface markers (CD9, CD63, CD81, EpCAM) and immobilized antibodies | About 240 min | Selectivity, high purity, possibility of subtyping | Not for large volumes (require in that case additional concentration steps), cost, require knowledge of EV markers, limited scalability, difficulty with EV detachment | [276] |
| **Microfluidics** | | | | | |
| Microfluidics-based techniques | Microscale isolation based on a variety of EV properties like immunoaffinity, size, and density | 1-14 µl/min | Rapidness, purity, efficiency | Cost, complexity of devices, specialized equipment, low sample capacity | [277] |

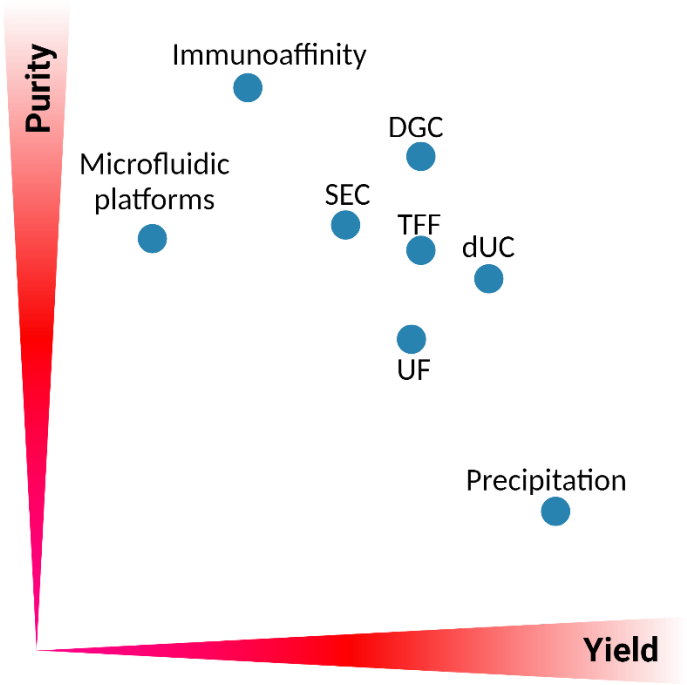



**Figure 10. Schematic illustration of commonly used EV isolation methods adapted from [278]**. DGC: density gradient centrifugation; SEC: size exclusion chromatography; TFF: tangential flow filtration; dUC: differential ultracentrifugation; UF: ultrafiltration. Adapted from Fricke et al., 2019. Created with Biorender.

## III. EXTRACELLULAR VESICLES AS THERAPEUTICS FOR CENTRAL NERVOUS SYSTEM DISEASES

Cell therapy is one of the avenues explored to find a cure for neurological diseases. As explained in the first chapter, MSC are promising therapies for MS and other CNS diseases [279]. More than 100 clinical trials with MSC are ongoing or completed for these diseases (ClinicalTrials.gov). However, mostly due to massive entrapment into the lungs after intravenous administration, cell therapy has shown limited efficacy during clinical trials [280]. Moreover, although mostly deemed safe, injecting exogenous cells could present a risk of malignant transformation [281].

It is accepted that the MSC therapeutic effect is mediated mostly by soluble factors, among which EVs [282]. EVs derived from MSC seem to recapitulate the therapeutic effects observed when using the cells and are considered safer than their mother cells [283]. As such, more and more studies report the therapeutic effects (mostly immunomodulatory properties) and use of MSC-derived EVs, the field of neuropathological diseases included (Table 5).

Chronic inflammation is an important part of MS pathophysiology, which explains the study of MSC-EVs as a potential MS treatment. Laso-Garcia et al. have shown that an intravenous administration of MSC-EVs in the TMEV model improved motor deficits and reduced brain atrophy [284]. Moreover, EVs were able to modulate the activation state of microglia and to decrease inflammatory infiltrates with a drastic reduction of plasma pro-inflammatory cytokine levels. Pusic et al. assessed the efficacy of EVs isolated from IFNγ-stimulated dendritic cells on *in vivo* and *ex vivo* models of remyelination [285]. An increase in myelin levels and a decrease in oxidative stress, respectively, were observed. The results were larger with EVs derived from IFNγ-stimulated dendritic cells than with EVs produced by unstimulated cells. An analysis of EV content showed the presence of miRNAs involved in oligodendrocytes differentiation and myelin production (e.g. miR-219). The presence of anti-inflammatory miRNAs (e.g. miR-181a, miR-451, miR-532-5p and miR-665) has also been reported. Similarly, Giunti et al. reported that intravenous administration of EVs isolated from IFNγ-stimulated MSC reduced the expression of neuroinflammation markers, possibly through miR-467f and miR-466q acting on microglia [286]. Riazifar et al. confirmed the presence of mRNAs encoding for anti-inflammatory molecules (e.g. IDO and CD74) and neuroprotective and anti-inflammatory proteins (e.g. MIC-1, Gal-1, and HSP70) in EVs derived from IFNγ-stimulated MSC [287]. When these EVs were administered to EAE mice, they were able to reduce the disease evolution and the spinal cord demyelination. They also decreased neuroinflammation and increased the number of Treg cells which are important cells that regulate disease progression. In line with these results, Fathollahi et al. observed an

increased Treg cell number associated with reduced clinical scores, decreased demyelinated lesions, and increased TGF-β levels in EAE mice treated with adipose-derived MSC-EVs [288]. Immunomodulatory effects of EVs were also reported by Jafarinia et al. and Koohsari et al., in EAE, treated with intravenous administration of EV from human adipose-derived MSC or human umbilical cord-derived MSC, respectively. However, in these two studies, no significant expression of MBP, Olig2, and GFAP was observed [289, 290]. In another study conducted by Zhang et al., EVs isolated from bone marrow MSC significantly increased the number of newly generated oligodendrocytes and mature oligodendrocytes, along with MBP levels, while decreasing neuroinflammation [291].

**Table 5. Use of EVs for CNS disorders treatment**

| Pathology | EVs source | Administration | Total dose | Treatment plan | Therapeutic effect | Ref. |
|---|---|---|---|---|---|---|
| Brain injury | Human bone marrow-derived MSC | Intravenous | EVs produced by $6*10^6$ MSC / mouse | 1 dose on day 1, 3 and 5 after ischemia | Angiogenesis, improvement of neurological impairment, long-term neuroprotection | [292] |
| | Murine bone marrow-derived MSC | Intravenous | EVs produced by $1*10^8$ MSC / mouse | 1 dose on day 1 after traumatic brain injury | Improvement of spatial learning, reduction of neurological deficits, angiogenesis, neurogenesis, reduction of inflammation | [293] |
| | Human bone marrow-derived MSC | Intravenous | $1,5*10^{10}$ EVs / mouse | 1 dose at 1 hour after traumatic brain injury | Improvement of spatial learning and pattern separation ability, decrease of neuroinflammation | [294] |
| | Human bone marrow-derived MSC | Intraperitoneal | EVs produced by $1*10^8$ MSC / kg / mouse | 1 dose at 1 hour before traumatic brain injury or 1 day after | Improvement of long-lasting cognitive functions, amelioration of inflammation, restoration of short-term myelination deficits | [295] |
| | Human bone marrow-derived MSC | Intravenous | 100 µg EVs (based on proteins) / mouse | 1 dose at 1 hour after traumatic brain injury | Improvement of cognitive functions, minimization of cortical lesion volume, decrease of glutamate levels | [296] |
| | Human bone marrow-derived MSC | Intranasal | $25.6 \times 10^9$ EVs/mouse | 1 dose at 1.5 hours after traumatic brain injury | Prevention of cognitive and mood dysfunctions, decrease of neuroinflammation | [297] |
| Epilepsy | Human bone marrow-derived MSC | Intranasal | $1,5*10^{10}$ EVs/mouse | 75µl (200µg/ml) on the day of status epilepticus or at 18h after | Neuroprotection, anti-inflammatory properties | [298] |
| | Umbilical cord derived MSC | Intravenous | 50 µg MSC-EVs / mouse | 1 dose at 2h after SE | Restoration of seizure-induced neuronal morphology alterations, neuroprotection against oxidative stress-induced damages | [299] |
| Multiple sclerosis | MSC | Intravenous | 25 µg MSC-EVs / mouse | 1 dose on day 60 post infection | Improvement of motor deficits, reduction of brain atrophy, proliferation of subventricular cells | [284] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | IFNγ-stimulated dendritic cells | *Ex vivo* (mature hippocampal slice cultures) | 100 µg of EVs (based on proteins) | Incubation for 3 days before demyelination<br>Incubation after demyelination | Increase of myelin levels | [285] |
| | IFNγ-stimulated human bone marrow-derived MSC | Intravenous | 150 µg (based on proteins)/ mouse | 1 dose at the peak of the disease (ca. 15-20 day post infection) | Reduction of disease evolution, decrease of spinal cord demyelination | [287] |
| | IFNγ-stimulated human bone marrow-derived MSC | Intravenous or intraperitoneal | EVs produced by $1*10^6$ (iv) or $3*10^6$ (ip) MSC | 1 dose / day for 6 or 8 days | Reduction of neuroinflammation marker expression | [286] |
| | Human adipose-derived MSC | Intranasal | 10 µg of EVs (based on proteins) | 1 dose / day for 12 days (15 days post infection) | Reduction of disease evolution and lesion volume, increase of immunomodulatory response | [288] |
| | Human adipose-derived MSC | Intravenous | 50 µg of EVs / mouse | 1 dose at day 10 post infection | Reduction of disease evolution, leukocyte infiltration and demyelination | [289] |
| | Human umbilical cord derived MSC | Intravenous | 50 µg of EVs / mouse | 1 dose at day 9 post infection | Amelioration of neuroinflammation | [290] |
| | Bone marrow derived MSC | Intravenous | $5*10^{10}$ particles | 1 dose twice a week for 4 weeks (for EAE model)<br>1 dose once a week for 2 weeks (for cuprizone model) | Improvement of neurological outcome, improvement of cognitive functions, decrease of neuroinflammation, and increase of remyelination | [291] |
| Alzheimer's disease | Human adipose tissue-derived MSC | *In vitro* | Not communicated | Incubation EV + cells for 24h | Decrease of intracellular β-amyloid peptide levels | [300] |
| | Neuroblastoma cells | Intracerebroventricular infusion | 4 µg of EVs (based on proteins) | Not communicated | Sequestration of β-amyloid peptide levels | [301] |
| | Murine neuroblastoma cells | Stereotaxic injection into the right hippocampus | 10 µg of EVs (based on proteins) / mouse | 2 mg of EVs (based on protein) /ml at 0.25µl/h for 14 days | Sequestration of β-amyloid peptide levels | [302] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Bone marrow derived MSC | Intracerebroventricular | 30 µg of EVs (based on proteins) | 1 dose / month for 2 months | Decrease of soluble of β-amyloid peptide levels, reduction of β-amyloid peptide deposition area, amelioration of inflammation | [303] |
| | Bone marrow derived MSC | Intravenous or intracerebroventricular | 0.5 or 25 µg of EVs / mouse | 1 dose / day for 5 days | Improvement of AD-like behaviors (only with intracerebroventricular injection), decrease of β-amyloid peptide levels | [304] |
| Parkinson's disease | Stem cells from human exfoliated deciduous teeth | *In vitro* | Not communicated | Not communicated | Suppression of 6-OHDA-induced apoptosis in dopaminergic neurons | [305] |
| | Stem cells from human exfoliated deciduous teeth | Intranasal | $43*10^8$ EVs / mouse | 1 dose ($2.85*10^8$ EVs) at day 8 to 22 after the lesion | Improvement of the rat gait parameters<br>Increase of Tyr hydrolase expression | [306] |
| | Human umbilical cord derived MSC | Intravenous | 200 µg of EVs (based on proteins) / rat | 1 dose every 3 days for 8 weeks | Reduction of dopaminergic neuron loss, improvement of behavioral deficits | [307] |

## IV. EXTRACELLULAR VESICLES AS NANOMEDICINES FOR CENTRAL NERVOUS SYSTEM DISEASES

In addition to their therapeutic effects, EVs have also been used as drug delivery system to administer RNA, small molecules, or proteins to the CNS. This can be explained by their many advantages, as summarized in Table 1. In order to load a therapeutic molecule in EVs, different methods have been explored. These can be mainly divided into 2 categories: modification of the parent cells before isolation (transfection or incubation with the compound of interest) or modification of EV post-isolation (sonication, extrusion, freeze-thaw cycles, electroporation, membrane permeation with saponin) [308] (Figure 11), each presenting their advantages and limitations (Table 6).

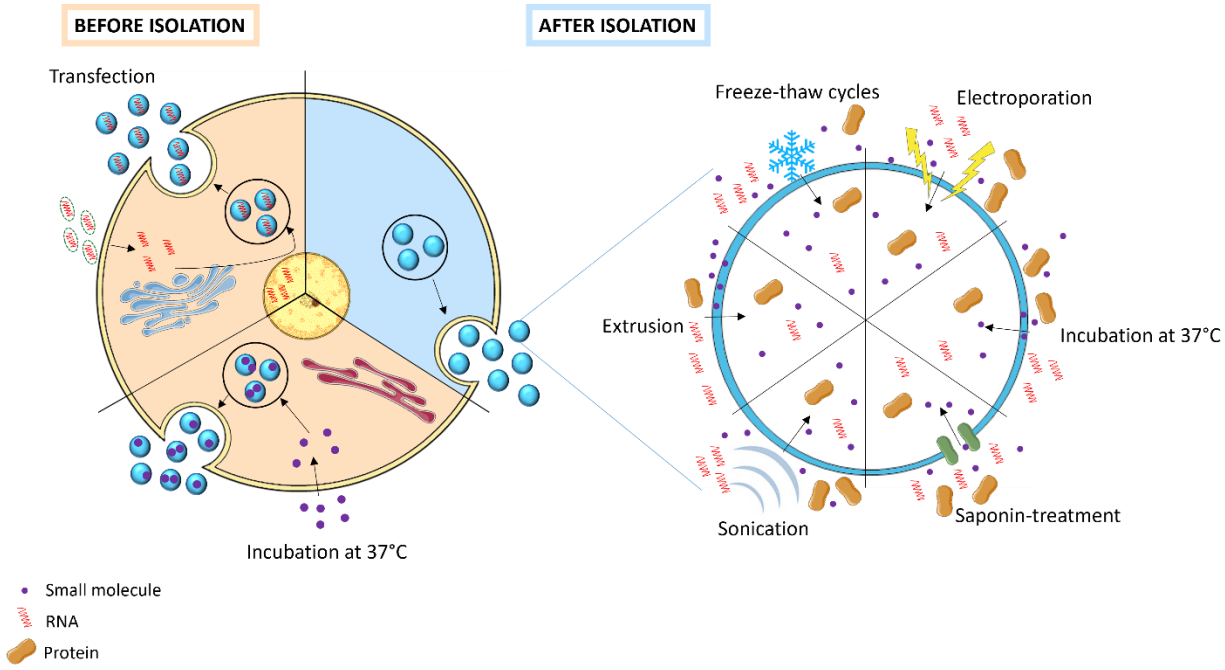



**Figure 11. Overview of extracellular vesicles loading strategy.** Further details are given in the main text.

**Table 6. Commonly used encapsulation methods after EV isolation**

| Methods | Principle | Cargo | Drug-loading rate | Advantages | Limitations | References |
|---|---|---|---|---|---|---|
| Sonication | Several cycles of sonication with a cooling phase between each cycle | Catalase | 26.1 ± 1.2% | Higher loading rate with hydrophilic drugs<br>No cytotoxicity of empty sonicated EVs | Disruption of EV integrity<br>Potential loss of intrinsic activity of the EVs<br>EV size increasing | [309, 310] |
| | | TPP1 | 70 µg into $10^{11}$ EVs | | | |
| | | Paclitaxel | 28.29 ± 1.38% | | | |
| Electroporation | Electric impulse allowing permeabilization of the membrane | RNAs (miRNA and siRNA) | Not communicated | Well-described method commonly used for cell transfection<br>Adapted for nucleic acid encapsulation | Disruption of EV integrity<br>Potential loss of intrinsic activity of the EVs<br>Risk of RNA aggregate formation | [311-313] |
| | | Paclitaxel | 5.3 ± 0.48% | | | |
| Extrusion | The compound and EVs are extruded through 200 nm-pores diameter | Catalase | 22.2 ± 3.1% | Higher loading rate than passive incubation | Disruption of EV integrity<br>Potential loss of intrinsic activity of the EVs<br>EV size increasing | [309] |
| Saponin-treatment | The EVs and the proteins are incubated with 0.2% saponin | Catalase | 18.5 ± 1.3% | Higher loading rate than passive incubation | Disruption of EV integrity<br>Potential loss of intrinsic activity of the EVs | [309, 310] |
| | | TPP1 | 50 µg into $10^{11}$ EVs | | | |
| Incubation | The compound and EVs are mixed and incubated at 22°C or 37°C for various amount of time | Curcumin | 2.9 g into 1 g EVs | No disruption of EV integrity<br>Mostly for hydrophobic drugs<br>No interaction with functional or physical properties of EV | Lower loading rate with hydrophilic drugs<br>Risk of adsorption on EV membrane | [309, 313-316] |
| | | Placlitaxel | 1.44 ± 0.38% | | | |
| | | Doxorubicin | 132.2 ± 2.9 ng into 1 µg EVs | | | |
| | | Dopamin | 15.97 ± 0.22% | | | |
| | | siRNA | 1000 – 3000 molecules / EV | | | |
| | | Catalase | 4.9 ± 0.5% | | | |
| Freeze-thaw cycles | Incubation of EVs and the protein for 30 min, followed by freezing at -80°C and thawing at RT (3 cycles) | Catalase | 14.7 ± 1.1% | Higher loading rate than passive incubation | Disruption of EV integrity<br>Potential loss of intrinsic activity of the EVs<br>Not frequently used | [309] |

### IV.1. RNA

The idea of exogenous RNA encapsulation in EVs originates from the observation that EVs can transfer genetic material from one cell to another [317, 318]. Various forms of RNA - siRNA, miRNA and mRNA - have been successfully encapsulated into EVs. Three main encapsulation methods have been reported in literature: electroporation, co-incubation with EVs and parent cell transfection. Alvarez-Erviti et al. are the first ones who encapsulated siRNA into EVs to treat CNS disorders [319]. They encapsulated beta-site APP cleaving enzyme 1 (BACE1) siRNA by electroporation in EVs isolated from dendritic cells. BACE1 is a protease that plays an important role in the formation of β-amyloid peptides. Thus, knocking down the expression of this gene in neurons could be a therapeutic approach against Alzheimer's disease. Intravenous administration of EVs containing BACE1 siRNA in mice has led to a significant decrease in the total β-amyloid 1-42 levels. These results support the ability of EVs to deliver cargo to neurons, microglia, and oligodendrocytes and also to cross BBB. Moreover, no cell toxicity or T-cell proliferation have been observed, which show that EVs have been well-tolerated. Cooper et al. also used electroporation to encapsulate α-synuclein siRNA into EVs isolated from murine dendritic cells [320]. α-synuclein aggregates are found in Lewy bodies, which are a characteristic of Parkinson's disease. *In vitro*, α-synuclein siRNA-EVs were able to decrease α-synuclein mRNA expression and consequently its protein level. These results have been confirmed *in vivo* after intravenous administration in mice overexpressing α-synuclein. Although electroporation seems to be a promising technique to encapsulate RNA into EVs, it also led to the extension formation of insoluble RNA aggregates which may cause overestimation of the amount of RNA actually loaded into EVs [312].

Incubation of siRNA with EVs can also be used to encapsulate genetic materials into EVs. Didiot et al. modified siRNA targeting Huntingtin mRNA by adding a cholesterol moiety in 3', which increased its hydrophobicity in order to encapsulate this siRNA into glioblastoma-EVs [313]. This modification led to a promotion of siRNA cellular and EVs internalization. However, no information about the impact on siRNA loading has been provided. Huntington disease is caused by the expansion of a polyglutamine tract at the N-terminus of the huntingtin protein, thereby altering its function. Thus, targeting this protein could slow down the development of the disease. siRNA-EVs silencing of huntingtin mRNA expression led to a significant decrease of protein level *in vitro* as well as *in vivo* following a stereotactic injection in mouse striatum. Furthermore, siRNA-EVs did not induce activation of microglia, which suggests that siRNA-EVs were well-tolerated. However, clinical improvement was not assessed. Incubation is also the method used by Guo et al.

to encapsulate siRNA coding for the silencing of phosphatase and tensin homolog (PTEN) into MSC-EVs [321]. This protein is expressed in neurons and regenerative axons and limits the synthesis of proteins sustaining axonal growth. Thus, knocking down its expression appears to be an interesting therapeutic strategy to repair spinal cord injury. A significant decrease of PTEN gene expression after intranasal administration of PTEN siRNA-EVs in a spinal cord injury model has been observed. As a result, significant recovery, enhancement of axonal growth and neovascularization and reduction of astrogliosis and microgliosis have been observed. Moreover, PTEN expression in the liver was not modified, suggesting that there is no accumulation of PTEN-EVs in this organ.

Electroporation and incubation are both effective methods to encapsulate RNA into EVs but their efficiency decreases when the size of the RNA increases. As an alternative, Kojima et al. transfected HEK-293T cells with a modified plasmid coding for catalase [322]. The plasmid modification allows a boost of EVs production, improves the delivery to the CNS, and increases encapsulation of mRNA into EVs. Catalase protects neurons from oxidative stress and attenuates the cell death that occurs in Parkinson's disease for instance. EVs isolated from these transfected cells contained catalase mRNA. *In vitro*, mRNA-EVs were able to attenuate the 6-OHDA-induced neurotoxicity. Furthermore, attenuation of neuroinflammation was observed in a murine Parkinson model. Cell transduction or transfection could also be used for miRNA encapsulation. Osorio-querejeta et al. transduced HEK293T cells in order to produce EVs loaded with miR-219a-5p [223], a miRNA that induces OPC differentiation and remyelination. Intranasal administration of miR-219a-5p-EVs in EAE mice resulted in their clinical score improvement, while incubation with OPC induced their differentiation. However, no impact on inflammation in this model has been reported. Similarly, Jahangard et al. transfected rat bone marrow MSC with recombinant expression vectors carrying miR-29a or miR-29b, both of which target BACE1 [323]. The presence of these miRNA in the EVs was confirmed and their administration in an AD rat model led to downregulation of BACE1, prevention of spatial learning deficits, and partial recovery of cognitive function.

### IV.2. SMALL MOLECULES

Several small molecules have already been reported in the literature as potential new drugs to treat CNS disorders. For example, curcumin is known to have anti-inflammatory properties [324]. Zhuang et al. encapsulated this compound into EVs (Exo-Cur) and assessed their efficiency after intranasal administration in different brain inflammatory disease models [325]. Exo-Cur inhibited inflammation, induced IL-1$\beta^{+}$-microglia apoptosis, and improved mouse clinical score.

Administration of dopamine to treat Parkinson's disease has only peripheric effects as dopamine cannot cross the BBB when administered by intravenous injection [326]. However, when loaded in blood EVs (Exo-DA), it significantly improved functional recovery in a murine Parkinson's disease model [316]. No toxicity in hippocampus, liver, spleen, and lung has been reported.

EVs as vehicle for small molecules have also been assessed for brain cancer therapy. Indeed, Zhuang et al. encapsulated JSI124, a bioactive small compound able to inhibit signal transducer and activator of transcription 3 (Stat3) signaling pathway, into EVs (JSI124-EVs) [325]. In a brain tumor model, JSI124-EVs significantly prolonged the mouse survival and inhibited the secretion of inflammatory cytokines (e.g. IL-1β and IL-6). Moreover, the tumor was less invasive compared to native EVs or free-JSI124. Yang et al. also encapsulated cytotoxic drugs (paclitaxel and doxorubicin) into EVs to treat brain cancer [315]. An intravenous administration of these EVs in zebrafish brain cancer model led to a significant reduction of tumor area and VEGF mRNA expression.

Additionally, Qi et al. encapsulated quercetin in plasma EVs (Que-EV) to enhance its bioavailability and brain targeting, as quercetin has demonstrated properties such as tau pathology prevention, neuroprotection, and cognitive improvement. In a rat model of Alzheimer's disease, Que-EV significantly enhanced bioavailability of quercetin, rescued cognitive dysfunction, and reduced the formation of insoluble neurofibrillary tangles [327].

### IV.3. PROTEIN/PEPTIDE

Proteins and peptides also have a lot of potential for the treatment of CNS disorders but their therapeutic potential is often limited by their fast degradation and inability to cross the BBB. One strategy to improve their pharmacological properties is to encapsulate them in a nanocarrier like liposomes or polymeric nanoparticles but, while this would improve their stability, most of these carriers have difficulties crossing the BBB and reaching the CNS. As EVs can cross this barrier more easily (see next section), Haney et al. encapsulated catalase into EVs. *In vitro*, an accumulation of catalase-EVs into neurons has been observed, as well as a decrease of reactive oxygen species in microglia cells activated with lipopolysaccharides. *In vivo*, administration of catalase-loaded EVs via the nasal route in a mouse model of Parkinson's disease led to antiinflammatory and neuroprotective effects [309]. The same group encapsulated a soluble lysosomal enzyme, tripeptidyl-peptidase-1 (TPP1), into murine EVs to treat lysosomal storage disorder [310]. An intraperitoneal administration of TPP1-EVs led to a decrease in neuroinflammation and astrocytosis and is thus a promising strategy for the treatment of Batten disease. In both cases, EVs

accumulated in the brain and significantly increased the stability of the protein against protease degradation. In another study, Izadpanah et al. encapsulated neprilysin, an active enzyme involved in the clearance of abnormal aggregated β-amyloid sheets in the brain, into bone marrow MSC-EVs. Intranasal administration of these EVs in a rat model of Alzheimer's disease significantly reduced neuronal damage and Aβ plaques, leading to improvements in brain-related behavioral functions [328].

As discussed here, the use of EVs as drug vehicles for CNS disorders is very promising and has been tested in several pre-clinical models of neurological diseases. However, there is currently a lack of quantitative information on the amount of EVs that actually reaches the CNS after administration. It is not excluded either that observed improvements following EV administration result, at least partially, from a peripheric effect of EV. Moreover, encapsulation methods should be improved in order to increase encapsulation efficiency and loading.

## V. EV ACCUMULATION IN THE CENTRAL NERVOUS SYSTEM

Evidence suggests that EVs can cross the BBB. Indeed, Morad et al. assessed how EVs derived from breast cancer cells could cross the BBB by using an *in vitro* BBB static model [329]. In this case, EVs were able to cross the cell monolayer easily. To verify if the observation was still valid in a dynamic system, a microfluidics organ-on-a-chip model of the BBB was used [329]. In 2017, Yuan et al. showed that EVs derived from macrophages could cross the BBB in an inflammatory state via integrin lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) in human hCMEC/D3 cell model of the BBB [330]. When injected intravenously in healthy or inflamed (intracranial injection of 10 μg of lipopolysaccharides) mice, 5 times more EVs were found in the brain in the inflamed condition compared to control. These results were confirmed when more BDNF was found in the brain parenchyma of inflamed animals after BDNF-loaded EVs intravenous injection compared to free-BDNF [330].

Only a limited number of studies about the mechanism by which EVs can cross the BBB have been conducted. Thus, the knowledge on this topic is very limited. One hypothesis is that EVs use the same mechanism as viruses, namely the endocytosis process and more precisely transcytosis [331].

While EVs seem to cross the BBB on their own, efforts have been devoted to further increase their accumulation in the CNS, mostly by using the nose-to-brain pathway and/or by modifying their surface.

### V.1. IMPACT OF ADMINISTRATION ROUTE

Various administration routes have been assessed for the delivery of EVs in the CNS: intravenous (IV), intranasal (IN), intrathecal, and intraperitoneal (IP) routes. All of them allow EVs to reach the brain parenchyma but the amount of EVs fluctuates according to the route [325, 332, 333]. To compare biodistribution, Betzer et al. administrated glucose-coated gold nanoparticles encapsulated in EVs intravenously and intranasally to C57/BL6 mice [334]. At 1h after injection, the quantity of gold in the brain was significantly higher with IN administration hinting that this route of administration led to a larger brain accumulation of EVs. Moreover, EVs were still detectable 24h after IN administration, which was not the case following IV administration. The same observation was made by Perets et al. after IN and IV administration of MSC-derived EVs to mice [335]. Although researchers initially thought that EVs had a better circulation time than synthetic nanoparticles, recent results rather indicate a rapid clearance of EVs from circulation after IV administration [336]. This might explain why a very small amount of EVs is able to reach the CNS after IV administration and why IN administration might be more promising for CNS delivery.

In a recent study, Haney et al. evaluated the biodistribution of macrophage-derived EVs by using DiR-labeled EVs in CLN2 knock-out mice (a Batten disease mice model) [337]. Four different routes were assessed: IV, IN, IP, and intrathecal. At 20 days after injection, the highest DiR fluorescent signal in the brain was achieved after intrathecal administration. The authors hypothesized that the intrathecal route allowed to bypass EV entrapment in peripheral organs, mainly the liver and the kidney, and thus improved brain bioavailability. Additionally, DiD-labelled EVs were also administered in CLN2 knock-out mice and EV accumulation in the brain was assessed using confocal microscopy. Among the four routes of administration, the intrathecal injection allowed the best DiD-EVs accumulation as expected, followed by IN administration. Moreover, all routes of administration allowed decreased astrocytosis and increased neuronal survival, especially when EVs were administered intrathecally and intranasally [337].

In light of the above, the intrathecal route seems to be the most effective way to administrate EVs for therapeutic purposes. Nevertheless, a less invasive route such as IN administration is preferred to treat CNS disorders that require chronic dosing. Moreover, a preferential accumulation of MSC-EVs in the brain has been observed after IN administration of EVs. Indeed, Perets et al. showed different distribution patterns of MSC-derived EVs in the brain of mice according to the pathology: they accumulated mostly in the striatum of traumatic brain injury and Parkinson's disease models and in the striatum and the hippocampus in an Alzheimer's disease model [338]. Guo et al. administered intranasally MSC-EVs to rats with a spinal cord lesion [321]. They observed a homing

of the MSC-EVs to the lesion and a higher uptake by neurons compared to astrocytes and microglia. The specific mechanisms underlying this homing are not fully understood yet, but it appears that it could depend on inflammatory events that occur at the site of the lesion [321, 334].

Currently, the route followed by the EV after IN administration has not been studied.

### V.2. SURFACE MODIFICATION OF EV TO IMPROVE BIODISTRIBUTION

As discussed, EVs seem to be able to cross the BBB by themselves. However, some research groups modified their surface to further increase their accumulation in the CNS or to target specific cells. Two main strategies have been used to achieve this: parent cell genetic modification to produce modified EV pre-isolation or EV post-isolation surface modification (Table 7).

Parent cell genetic modification is the most popular technique to modify EV surface to enhance brain accumulation. Álvarez et al. delivered BACE1 siRNA to the CNS using EVs decorated with rabies viral glycoprotein (RVG) that targets nicotinic acetylcholine receptors largely distributed in the CNS neurons [319]. To do so, they transfected a plasmid coding for Lamp2B, an EV membrane protein, fused with the targeting RVG peptide, in primary dendritic cells derived from mice bone marrow. The presence of the fusion protein was assessed by western blot and qPCR. EVs-Lamp2B-RVG containing silencing BACE1 siRNA were injected intravenously into mice. Compared to the untreated mice and mice that received siRNA-RVG, siRNA-RVG-EV treated mice had a lower expression of BACE1 mRNA, suggesting that RVG can improve siRNA delivery to the CNS. However, the impact of siRNAs encapsulated in undecorated EVs on BACE1 mRNA expression has not been assessed [319].

Peptides displayed on EV surface using this method can be degraded during EV biogenesis in the parent cell, for example, by endosomal proteases. Thus, Hung et al. used an improved version of neurons-targeted EVs by adding a glycosylation motif (GNSTM) on their surface [339]. Lamp2B-GNSTM-RVG-EVs were also produced by using engineered cells. A higher expression of Lamp2B-GNSTM-RVG in cell lysate and EVs was observed. Therefore, it seems that the addition of the glycosylation motif enhanced the peptide stability by avoiding degradation by cell protease. Lamp2B is not the only EV surface protein used to modify EV surface via parent cell genetic modification. The C1C2 domain of lactadherin was also used to decorate EVs in the context of cancer therapy [340]. Based on the examples mentioned above, it seems that parent cell modification via plasmid transfection is an effective technique to display targeting peptides at the surface of EVs.

Concerning post-isolation techniques for EV surface modification in the context of CNS therapy, very few studies have been published. Ye et al. grafted a KLA-low-density lipoprotein (KLA-LDL) at EV surface, using a simple coincubation with the peptide of interest. LDL has been grafted in order to improve EV passage across the BBB (by binding to the LDL receptor) and KLA is a pro-apoptotic peptide that aims to enhance the therapeutical effect of this anti-glioma formulation [341]. The grafting efficiency of LDL and KLA-LDL was approximately 1.98 and 1.77%, respectively. *In vitro*, LDL-EVs were taken up to a greater extent by U87 cells than unmodified EVs. However, when KLA was added prior to LDL-EVs no difference in uptake was observed [341]. After intravenous administration of DiR-EVs and KLA-LDL-DiR-EVs, the highest fluorescent signal was achieved with the targeted EVs. Moreover, it has been shown that the encapsulation of methotrexate into KLA-LDL-EVs increased the survival rate of mice bearing a glioma. Another strategy to graft a peptide at the surface of EVs after EV isolation is to use bio-orthogonal copper-free azide alkyne cyclo-addition, which is a type of click chemistry [342]. Using this technique, Tian et al. grafted the cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide [c(RGDyK)] on the murine bone marrow MSC-EV surface. This peptide has an affinity for the αvβ3 integrin which is overexpressed in cerebral vascular endothelial cells after ischemia. They aimed to enhance curcumin-loaded EV delivery in the ischemic part of the brain. To achieve this functionalization, firstly dibenzocyclooctyne- sulfo-N-hydroxysuccinimidyl ester (DBCO-sulfo-NHS) was added to EVs as a linker. NHS creates a chemical bound with amines at the surface of the EVs and the alkyne in the DBCO can be linked to an azide moiety to give birth to a triazole linkage. For this, an azide moiety has been added to the [c(RGDyK)] lysine. To evaluate the grafting efficiency, a fluorescent dye has been conjugated to [c(RGDyK)] before grafting on EVs. The unbound fraction of the [c(RGDyK)] has been removed by ultracentrifugation. Using this technique, they calculated that an average of 263 peptides were attached at the surface of one EV [342].

It has also been reported that EVs have intrinsic interesting surface properties that allow them to cross the BBB. Indeed, Qu et al. showed that EVs produced by blood cells have transferrin receptor (TfR) on their surface. TfR is also expressed on the apical side of the endothelial cell of the BBB and is often targeted in studies that aim to improve CNS delivery with a different type of nanovectors. Dopamine-loaded blood EVs and free-dopamine were injected into mice intravenously. The biodistribution in the brain was compared 6 hours after injection, and the results showed that delivery with blood EVs allowed a 15-fold higher brain distribution of dopamine compared to free dopamine [316].

**Table 7. Representative examples of the main EVs surface functionalization approaches for CNS delivery**

| EVs origin | Surface modification | Targets | Grafting technique | Outcomes | Ref |
|---|---|---|---|---|---|
| Primary dendritic cells | RVG | Nicotinic acetylcholine receptors | Transfection of plasmid encoding for Lamp2B-RVG peptide | *In vivo*: IV injection of BACE1 siRNA in RVG-EVs lowered BACE1 mRNA expression in striatum compared to the control | [319] |
| Human embryonic kidneys (HEK293T) cells | GNSTM-RVG | Nicotinic acetylcholine receptors | Transfection of plasmid encoding for Lamp2B-GNSTM-RVG | *In vitro:* increased uptake of GNSTM-RVG-EVs compared to control on neuroblastoma cells | [339] |
| Human embryonic kidneys (HEK293T) cells | GNSTM-RVG | Nicotinic acetylcholine receptors | Transfection of plasmid encoding for Lamp2B-GNSTM-RVG | *In vitro*: enhanced passage of EVs-GNSTM-RVG through bEnd.3 cells monolayer as a BBB model.<br>*In vivo:* enhanced accumulation of GNSTM-RVG-EVs in brain tissue after IV injection compared to unmodified EVs | [343] |
| Murine fibroblast (L929) cells | KLA-LDL or LDL | LDL receptor at the BBB | Coincubation of peptide with EVs post isolation. | *In vitro*: LDL-EVs and KLA-LDL-EVs had a higher uptake than EVs on U87 cells.<br>*In vivo*: higher accumulation in the brain of KLA-LDL-EVs compared to EVs. | [341] |
| Bone marrow-derived MSCs | c(RGDyK) | $\alpha_v\beta_3$ integrin overexpressed in the endothelial cell in the ischemic brain lesion | Copper-free click chemistry | *In vitro:* Higher accumulation of EV-RGD in U87 cells expressing $\alpha_v\beta_3$ integrin compared to EVs<br>*In vivo:* higher accumulation of RGD-EVs in the brain and ischemic lesion after IV injection. | [342] |
| Mouse macrophages<br>Raw264.7 cells | RGE | Neuropilin-1 (NRP-1) overexpressed in glioma cells | Copper-free click chemistry | *In vivo:* curcumin-loaded RGE-EVs decreased glioma tumor volume to a higher extent compared to curcumin-loaded EVs | [344] |
| Blood cells | TfR-Transferrin | Transferrin receptor at the BBB | The TfR-Transferrin complex was already present at the surface EVs post-isolation | *In vitro:* higher accumulation of blood-EVs in bEnd.3 cells compared to HeLa-EVs<br>*In vivo:* dopamine-loaded blood EVs had a higher accumulation in brain tissue than free dopamine after IV administration. | [316] |

## VI. THE CHALLENGE OF EV-THERAPEUTIC TRANSLATION TO THE CLINIC

While EVs have raised a lot of enthusiasm as a drug delivery system, only 3 clinical trials with EVs in CNS were ongoing in 2020 (https://clinicaltrials.gov, Table 8). However, due to COVID-19, 2 of them have been suspended and thus, in 2024, only one trial is ongoing. This reflects the youthfulness of the field and the many challenges that still need to be addressed before fully exploiting the potential of EVs. The main current limitations are: scale-up of the production, production of highly purified EVs, standardization for isolation and characterization methods, low encapsulation efficiency, shelf stability, and poor understanding of the mechanisms of action [345, 346].

Scale-up of the production is currently hindered by the limitations related to cell expansion. Indeed, to produce a large amount of EVs required for clinical and industrial applications, the first solution is to grow a large amount of cells. To achieve this, flasks, multilayer culture flasks, or bioreactors (fixed-bed, stirred-tank with microcarriers or continuous perfusion hollow fiber reactors) can be used [347] but it requires space, manipulation of large quantities of liquids, sophisticated equipment, and extensive skills and optimization. An alternative is to stimulate cells to produce more EVs with external signals [348]. These signals can be biological (e.g. serum starvation [349], cytokines activation [350] or hypoxia [351]), chemical (e.g. ethanol or cytochalasin B [352]) or physical (e.g. extrusion, centrifugation, filtration or slicing). For instance, the start-up EVerZom has launched a platform in order to increase EV production yield by using turbulence to stimulate cells.

However, external signals may have an impact on cell properties and modify EV content. For example, cytochalasin B induces the production of membrane vesicles, that do not use the same mechanism of cargo sorting as EVs [352]. Cytokine activation often leads to the production of EVs with different properties than EVs produced under normal conditions. Thus, a full characterization of these EVs should be done.

Another approach would be to use the extensive knowledge and experience researchers have in the field of nanomedicines to produce EV-mimicking nanosystems, with the aim to combine, ideally, the advantages of both carriers. The objective would be to preserve EV's inherent ability to cross barriers and their biological activity with a more controlled, flexible, and reliable production process. To do so, researchers developed liposomes with lipid composition similar to EVs [353]. Liposomes can be functionalized with proteins of interest or genetic materials. However, this method requires a complete knowledge of EVs content and more precisely, what is responsible for their therapeutic effects. Fusion of EVs with liposomes has also been assessed in order to improve

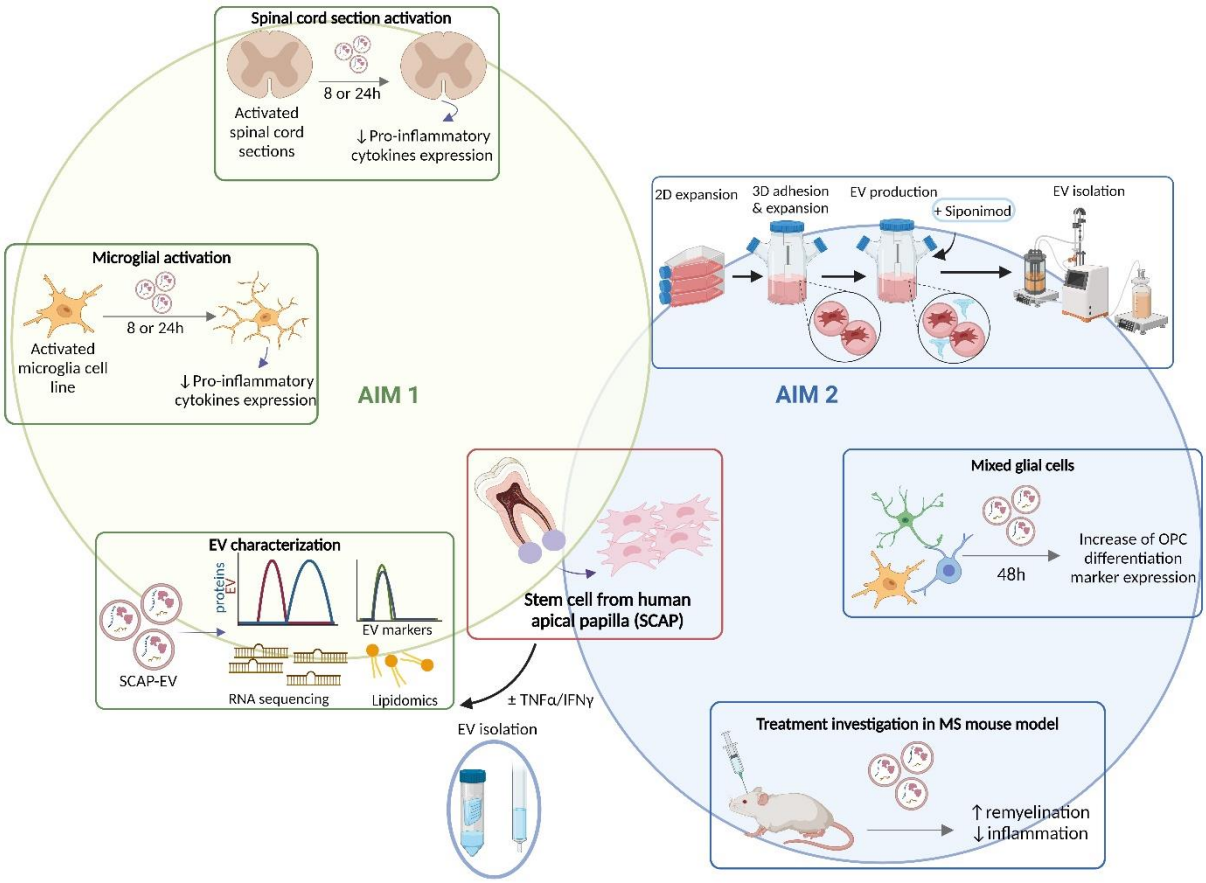
the encapsulation of a hydrophilic or a lipophilic compound into EVs or to decorate the surface of EVs [354]. With this technique, encapsulation of a lipophilic drug could be 30 times more important compared to incubation of the parental cells with the drug of interest.

**Table 8. Clinical studies using EVs for treatment of CNS disorders**. IV: intravenous, MSC: Mesenchymal stem cell

| Indication | Phase | Study size | EV source | Administration | EV manipulation | Results/status | NCT number | Ref. |
|---|---|---|---|---|---|---|---|---|
| Depression, anxiety and dementias | Not applicable | 300 | MSC | IV (in association with focused ultrasound) | None | Suspended (pending COVID-19 pandemic) | NCT04202770 | [305] |
| Craniofacial neuralgia | Not applicable | 100 | Unknown | IV (in association with focused ultrasound) | None | Suspended (pending COVID-19 pandemic) | NCT04202783 | [306] |
| Acute ischemic stroke | 1 – 2 | 5 | MSC | IV | Enrichment by miR-124 | Passed completion date | NCT03384433 | [355] |

# PART II
# AIM OF THE THESIS

Extensive research conducted during the last decade has highlighted the therapeutic potential of mesenchymal stem cells for various pathologies, including neurological disorders such as MS. This therapeutic effect is likely mediated by their soluble factors but also by their EVs. Our main objective is thus to develop nanomedicines that will contribute to resolve chronic inflammation and stimulate remyelination in the central nervous system. We aim at exploiting our know-how in the field of nanomedicines and stem cells to deliver siponimod, a compound that can promote the differentiation of oligodendrocyte progenitor cells, encapsulated within mesenchymal stem cell-derived EVs and deliver them to the CNS.




As this work was the first in our laboratory dedicated to EVs, the first objective of this project was to isolate and characterize EVs from human dental mesenchymal stem cells. Another objective was to use these EVs as a drug delivery system to encapsulate siponimod and evaluate its efficacy *in vitro* and *in vivo*.

Therefore, the result section is divided in two chapters:

**Chapter I: Influence of a pro-inflammatory stimulus on the miRNA and lipid content of human dental stem cell-derived extracellular vesicles and their impact on microglia activation**.

Previous experiments conducted in our laboratory have demonstrated that stem cells from human apical papilla (SCAP) exhibit anti-inflammatory and neuroprotective effects via their secretome, notably when subjected to a pro-inflammatory environment. SCAP are a promising source of MSCs compared to bone marrow-derived MSCs due to their accessibility, neural crest origin, higher proliferation rate, and greater expression of specific neural transcripts and proteins. The SCAP secretome includes both soluble factors and EVs. In this study, our objective was to determine whether SCAP-EVs were able to replicate the therapeutic properties of their parent cells. We thus isolated and characterized EVs from non-activated SCAP and from SCAP activated with TNFα and IFNγ. Additionally, we assessed the ability of these EVs to reduce pro-inflammatory cytokine expression in both *in vitro* and *ex vivo* models.

**Chapter II: Siponimod-loaded EVs increased oligodendrocyte progenitor cell differentiation**.

This chapter focused on the use of EVs as drug delivery system for siponimod. As demonstrated in the previous section, SCAP-EVs were able to slightly reduce neuroinflammation in both *in vitro* and *ex vivo* models. Our objective was to encapsulate siponimod in SCAP-EVs and evaluate the impact of this system on oligodendrocyte progenitor cell differentiation and inflammation resolution. To achieve this, we encapsulated siponimod into SCAP-EVs using an innovative technique called turbuloporation followed by isolation of siponimod-loaded EVs. We then assessed their efficacy *in vitro* by measuring their ability to reduce pro-inflammatory cytokine expression in microglia cells and to induce OPC differentiation. The therapeutic efficacy of siponimod-loaded EVs was subsequently tested in a demyelination model.

# PART III
# RESULTS

# CHAPTER I: INFLUENCE OF A PRO-INFLAMMATORY STIMULUS ON THE MIRNA AND LIPID CONTENT OF HUMAN DENTAL STEM CELL-DERIVED EXTRACELLULAR VESICLES AND THEIR IMPACT ON MICROGLIAL ACTIVATION

## Adapted from


Research article

Influence of a pro-inflammatory stimulus on the miRNA and lipid content of human dental stem cell-derived extracellular vesicles and their impact on microglial activation

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## ABSTRACT

Neuro-inflammation occurs in numerous disorders such as multiple sclerosis, Alzheimer's disease and Parkinson's disease. However, anti-inflammatory drugs for the central nervous system have failed to show significant improvement when compared to a placebo in clinical trials. Our previous work demonstrated that stem cells from the apical papilla (SCAP) can decrease neuro-inflammation and stimulate oligodendrocyte progenitor cell differentiation. One hypothesis is that the therapeutic effect of SCAP could be mediated by their secretome, including extracellular vesicles (EVs). Here, our objectives were to characterize SCAP-EVs and to study their effect on microglial cells. We isolated EVs from non-activated SCAP and from SCAP activated with TNFα and IFN-γ and characterized them according to their size, EV markers, miRNA, and lipid content. Their ability to decrease pro-inflammatory cytokine expression *in vitro* and *ex vivo* was also assessed. We showed that the miRNA content was impacted by a pro-inflammatory environment but not their lipid composition. SCAP-EVs reduced the expression of pro-inflammatory markers in LPS-activated microglial cells while their effect was limited on mouse spinal cord sections. In conclusion, we were able to isolate EVs from SCAP, to show that their miRNA content was impacted by a pro-inflammatory stimulus, and to describe that SCAP-EVs and not the protein fraction of conditioned medium could reduce pro-inflammatory marker expression in LPS-activated BV2 cells.

# I. INTRODUCTION

Once it became apparent that extracellular vesicles were involved in local and systemic cell communication [250, 356], extensive studies were conducted to elucidate their role in pathology development and regulation, making them potential biomarker of various diseases, mostly cancer [357-359]. Furthermore, EVs play a crucial role in physiological processes such as immune regulation, leading to the development of therapeutic approaches using these vesicles [360]. Recently, the use of EVs as a drug delivery vehicle has also raised a lot of interest. Indeed, EVs are nanoscale vesicles that can be loaded with a specific bioactive molecule (i.e. miRNA, lipid, small molecule [308]), can cross biological barriers, including the blood-brain barrier, are characterized by low immunogenicity [214] and have no toxicity [361], and are able to deliver their cargo into recipient cells [362]. EV can be isolated from diverse sources, such as eukaryotic cell and bacteria conditioned media, biological fluids, and plants. Eukaryotic cell-conditioned medium is the preferred source of EVs as nanomedicines, as it offers the possibility to scale-up EV production processes [363], to have a better reproducibility of EV isolation compared to other sources, to modify parent cells in order to load a bioactive molecule into EVs but also to modify EV composition by changing cell culture conditions [364].

As mesenchymal stem cell (MSC)-derived EVs can present similar therapeutic properties as their parent cells [365], they have become a source of choice to produce EVs for therapeutic uses. Indeed, recent studies have shown that, like MSC, EVs produced by MSC were able to decrease inflammation in mice [366] but also to reduce neurological impairment in neurodegenerative diseases [214]. Our previous work has shown that stem cells from the apical papilla (SCAP) can exert an anti-inflammatory and neuroprotective effect [367] via their secretome [368], notably when subjected to a pro-inflammatory environment. This could be mediated by soluble factors but also by their EVs [287].

To the best of our knowledge, no one ever isolated EVs from SCAP based on size differentiation, characterized them according to their miRNA and lipid content, and studied their impact on the secretion of inflammatory markers in activated glial cells. We thus optimized a protocol to obtain EVs from SCAP-conditioned medium and we characterized them according to MISEV2018 recommendations [244]. Then, as the secretion of immunomodulatory factors by SCAP increased upon exposure to a pro-inflammatory stimulus, we hypothesized that their EV content could be affected as well. Since EVs are known to be one of the major vehicles for miRNA trafficking and as lipids are major components of EV membrane, we focused on those two components. Finally, we evaluated whether SCAP-secreted EV (SCAP-EV), produced in a steady state or a pro-

inflammatory environment, would have an immunomodulatory action on glial cells. Thus, the aim of this study was to better understand the impact of a pro-inflammatory environment on EV composition and determine whether SCAP-EVs would recapitulate SCAP anti-inflammatory effect on glial cells.

## II. MATERIALS AND METHODS

All relevant data have been submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV220308).

### II.1. CELL CULTURE AND ISOLATION OF EVS

Previously characterized human SCAP from healthy tooth were used [369]. SCAP were cultured at 37°C and 5% $CO_2$ in minimum essential medium Eagle (MEM, Sigma-Aldrich, St Louis, USA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine 200 mM (Thermo Fisher Scientific, Waltham, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific). Non-activated and activated SCAP were cultured for 3 days in serum-free MEM and in serum-free MEM containing TNFα (20 ng/ml) and IFN-γ (20 ng/ml), respectively [368]. Conditioned media were then collected and EVs were isolated by centrifugation and ultrafiltration followed by size exclusion chromatography (SEC) (Figure 1). Briefly cells, dead cells and cell debris were removed by successive centrifugations (300 g for 10 min, 1,000 g for 20 min and 10,000 g for 30 min, respectively). The supernatant was then filtrated on 0.22 µm filter and concentrated 200 – 400 times using an ultrafiltration device with a 30 kDa or a 100 kDa cut-off (Centricon® Plus-70, Merck Millipore, Burlington, USA) until a volume lower than 500 µl was obtained. Finally, small EVs (exosomes and small microvesicles) were separated from contaminating proteins using a SEC column (qEV Original® 35 nm or 70 nm, Izon Science, Lyon, France). Fractions from 1 to 30 (500 µl each) were collected. Endotoxin detection and quantification were performed with ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, USA), according to the manufacturer's instructions.

### II.2. EV CHARACTERIZATION

#### II.2.1. Protein quantification

Proteins in fractions 1 to 30 were quantified using a Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific).

### II.2.2. Nanoparticle tracking analysis (NTA)

Particle concentration was quantified using a ZetaView in all fractions (1-30) (Particle Metrix, Inning am Ammersee, Germany) with a recording video frame set at 60 s. EVs were diluted (1:1000 – 1:2000) in ultrapure water to a concentration ranging between $10^7$ – $10^8$ particles/mL. Sensitivity was set to 79 and camera shutter to 100. Measurements were averaged from particles counted in 11 different positions for 2 repeated cycles with camera at medium resolution modes. Size distribution and zeta potential were also measured by NTA.

### II.2.3. Dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA®)

Fifty µL of each SEC fraction (1-30) were bound to protein-binding ELISA plate (ELISA Strip Plate, 771261; Greiner Bio-One, Frickenhausen, Germany). After overnight incubation at 4°C, the plate was shaken on a tilting shaker at 30 rpm at 4°C. Then the plate was washed 3 times with Delfia buffer (#1244-111; PerkinElmer, Wellesley, USA) diluted to 1x in PBS and blocked for 90 min with 1% BSA in PBS. Primary antibodies against CD9 (MAB1880, R&D Systems, Minneapolis, USA), CD81 (#349502; Biolegend, San Diego, USA), CD63 (MCA2142; Serotec Bio-Rad), Flotillin-1 (#610821; BD Biosciences, San Jose, USA) and ApoB (sc-13538; Santa Cruz) (1 µg/mL in 1% BSA) were then incubated for 90 min. After 3 washes in the Delfia buffer, goat anti-mouse biotinylated antibody (NEF8232001EA; PerkinElmer) diluted at 0.2 µg/ml in 0.1% BSA was added for 60 min. After 3 washes, Europium-conjugated Streptavidin (#1244-360; PerkinElmer) diluted at 1:1000 was added for 45 min. After 6 washes, the Delfia enhancement solution (#1244-105; PerkinElmer) was incubated for 15 min. Quantification of the signal was performed using time-resolved fluorometry with excitation/emission 340/615 nm, flash energy/light exposure high/medium, and integration lag/counting time 400/400µs (Victor X4 multilabel plate reader; PerkinElmer).

### II.2.4. Western Blot

Proteins from $10^9$ EVs (fractions 7 to 11) and SCAP were extracted using a RIPA buffer containing EDTA and protease inhibitors. Protein concentration was measured with a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were then denatured with Laemmli sample buffer 6x (375 mM Tris-HCl, pH 6.8; 30% glycerol; 9% β-mercaptoethanol; 9% SDS; 0.03% bromophenol blue). Positive (CD81) and negative (Calnexin) markers were analyzed by western blot after running 12.5 µg of proteins/sample on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 4–20 % gradient gel (Bio-Rad). Separated proteins were then transferred with a semi-

dry method to nitrocellulose membranes (Thermo Fisher Scientific) that were blocked with 5 % dry milk dissolved in Tris-buffered saline. Membranes were then incubated with an anti-calnexin antibody (mAb2679, Cell Signaling Technology, Danvers, USA) and an anti-CD81 antibody (sc-166028, Santa Cruz), diluted at 1:1000. Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase and diluted at 1:10000 were used for the detection. Membranes were revealed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) and images were acquired using Fusion Solo S (Vilber Lourmat, Collégien, France).

### II.3. IMPACT OF A PRO-INFLAMMATORY STIMULUS ON SCAP-EV MIRNA CONTENT

Total RNA from 5 x $10^{10}$ non-activated SCAP-EVs and 5 x $10^{10}$ activated SCAP-EVs was extracted using miRNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA concentration was measured by Qubit RNA HS assay kit (Q10211, Thermo Fisher Scientific). Small RNA sequencing has been performed using 50 ng of RNA by Genewiz (Azenta Life Sciences, Leipzig, Germany).

Small RNA sequencing data were processed using mirdeep2 pipeline [370]. Differential expression analysis was done using DESeq2 v1.32.0 Bioconductor package [371]. miRNA with a p-adjusted value < 0.05 and showing an absolute log-foldchange > 1.5 between activated and non-activated samples were considered differentially expressed. multiMiR v1.14.0 Bioconductor package [372] was used to predict the miRNA target genes. miRNA-target pairs predicted by at least 3 of the 4 predictions databases interrogated (DIANA-microT, miRDB, PicTar, and TargetScan) were selected. Over-representation analysis were done using clusterProfiler 4.0.0 [373] on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Raw data can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE208577

### II.4. INFLUENCE OF SCAP ACTIVATION ON EV LIPID CONTENT

The lipid content of the different samples was analyzed by Liquid Chromatography coupled to Mass Spectrometry (LC-MS). Briefly, lipids from EV were analyzed after liquid/liquid extraction ($CH_2Cl_2$-MeOH-$H_2O$) in acidic condition in the presence of internal standards (17:0-LPC, 17:1-LPE, 17:1-LPG, 17:1 LPI, 17:0 Sulfatide, 17:0 sphingomyelin, 17:0 Ceramide). A Xevo-TQS (from Waters) coupled to an UPLC was used to analyze the samples with three different methods, according to lipid family:

- For the lysophospholipids, phospholipids, sulfatides, and sphingomyelin lipids, an HSS LC-18 column 100x2.1mm, 1.8µm (Waters) at a temperature of 40°C was used. The mobile phase consisted in a gradient between A: MeOH-ACN (9/1, v/v) 75% - $H_2O$ 25%; B: MeOH- ACN (9/1, v/v) and C: IpOH, all containing ammonium acetate (5mM). An ESI probe operated in negative mode was used for sample ionization.
- For the ceramide, we used a BEH LC-18 column 50x2.1, 1.7µm (Waters) at a temperature of 40°C. The mobile phase consisted in a gradient between A: $H_2O$ 25% - MeOH 75%; B: MeOH 100%, all containing acetic acid (0.1%). An ESI probe operated in negative mode was also used for sample ionization.
- For arachidonic acid derivatives and related compounds, an Acquity UPLC BEH C18 (150 x 2.1 mm; 1.7 µm) column was used. Mobile phases consisted in $H_2O$-ACN-acetic acid (94.9:5:0.1; v/v/v) and ACN-acetic acid (99.9:0.1; v/v). An ESI probe operated in negative mode was used for sample ionization.

For all the lipids, the relative quantification was based on the ratio between the area under the curve (AUC) of the lipid of interest and the AUC of the respective internal standard. The data were then normalized to the number of EVs in the samples.

### II.5. IMPACT OF SCAP-EVS ON BV2 CELL ACTIVATION

Murine microglial cells (BV2 cells) were cultured at 37°C and 5% $CO_2$ in high-glucose DMEM medium with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. BV2 cells were seeded overnight into 24-well plates ($2.5 \times 10^5$ cells per well) and incubated for 1h with fresh culture medium containing 100 ng/mL LPS and 1% EV-depleted FBS (FBS centrifuged for 1h at 100,000 g to remove EV). LPS concentration was chosen based on previous studies conducted in the lab [374-376]. Then, EVs isolated from non-activated or activated SCAP ($5 \times 10^9$ EV/well) were added to the cells and incubated for 8h or 24h before RNA extraction. In a subsequent experiment (same setting: BV2 cells activated with 100 ng/mL of LPS, EVs incubated for 8h before RNA extraction), EVs isolated from activated SCAP (Fractions 7-11) were compared to a pool of the protein fractions (Fractions 12-30) obtained from the same sample after SEC.

Total RNA from BV2 cells was extracted using TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcription was performed using the GoScript Transcription System (Promega, Madison, USA) from 1 µg of total RNA. qPCR was performed with qPCR Master Mix (Promega) and a STEPone PLUS instrument and software (Applied Biosystems, Foster City, USA) as [377]. Data were analyzed with the ΔΔCt method using

the 60S ribosomal protein L19 (RPL19) as a reference gene. Primer sequences are given in Table 9.

**Table 9**. List of primers sequences

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| Mouse RPL19 | TGACCTGGATGAGAAGGATGAG | CTGTGATACATATGGCGGTCAATC |
| Mouse IL-6 | ACAAGTCGGAGGCTTAATTACACAT | TTGCCATTGCACAACTCTTTTC |
| Mouse IL-1β | TCGCTCAGGGTCACAAGAAA | CATCAGAGGCAAGGAGGAAAAC |
| Mouse iNOS | AGGTACTCAGCGTGCTCCAC | GCACCGAAGATATCTTCATG |
| Mouse TNFα | AGCCCCCAGTCTGTATCCTT | GGTCACTGTCCCAGCATCTT |

### II.6. IMPACT OF SCAP-EVS ON MOUSE SPINAL CORD ACTIVATION

Animal experiments were declared to the ethical committee for animal cares (07-OTLADDB-2023-7). Spinal cord slices were obtained from female C57BL/6J mice. Briefly, the spinal cords were hydro extruded, cut into 400 µm slices in a tissue chopper. The slices (4 – 5 per well) were incubated in DMEM-F12 media (containing 1 % EV-depleted FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) overnight. They were then stimulated with LPS (100 ng/ml) for 1 hour prior to incubation with EV isolated from non-activated or activated SCAP ($5 \times 10^9$ EV/well) or the vehicle (PBS) for 8 or 24 hours. Slices were then recovered and stored at -80°C until processed for mRNA extraction. Gene expression was analyzed as described in 2.5. TNF-α and IL-6 levels were quantified in the culture medium using respectively Mouse TNF alpha uncoated ELISA kit and Mouse IL-6 uncoated ELISA kit (Invitrogen), according to the manufacturer's instructions.

### II.7. STATISTICAL ANALYSIS

Statistical analysis was performed using PRISM (GraphPad Software, CA, USA). Error bars represent the standard error of the mean (SEM) in all figures. One-way ANOVA followed by Tukey, or Kruskal-Wallis followed by Dunn's test were performed to compare different conditions ($p < 0.05$). The number of experiments and the number of replicates are indicated in the figure legends.

## III. RESULTS

### III.1. CHARACTERIZATION OF SCAP-EVS

To isolate the EVs secreted from SCAP, a combination of 2 methods based on the size of the vesicles was used: ultrafiltration and SEC (Figure 12A). Two ultrafiltration cut-offs were compared (30 kDa and 100 kDa) as well as two SEC columns (35 nm and 70 nm) to optimize the yield of EVs and the elimination of contaminating proteins.

SEC allowed to separate EVs from soluble proteins and to obtain EVs with a very low soluble protein contamination (Figure 12B). The pore size of the ultrafiltration units had an impact on the size (Figure 12C) and on the number of isolated EVs per cell (Figure 12D). Indeed, 2.5 times more vesicles were recovered with units presenting a cut-off of 30 kDa than with units with a cut-off of 100 kDa. No impact of the column type on particle number or size was observed (Figure 1C & 1D). For further EV isolations in this work, 30 kDa ultrafiltration units and 35 nm SEC columns have been used.

As the highest concentration of particles was observed in fractions 7 to 11 (Figure 12B), experiments were performed on a pool of these fractions. The mean size of EVs in the pool was 137.5 nm (SD = 49.7 nm).

In order to confirm that the particles in the pool were indeed EVs, samples were analyzed by DELFIA® and Western Blotting for positive and negative EV markers (as per MISEV2018 recommendations [244]). Isolated particles from factions 7 to 11 were positive for tetraspanins (CD63, CD81, and CD9) and flotillin (all positive markers of EVs) (Figure 12E & 12F) while they were negative for calnexin (Figure 12F) and ApoB (analyzed DELFIA®, no signal), 2 negative markers of EVs.

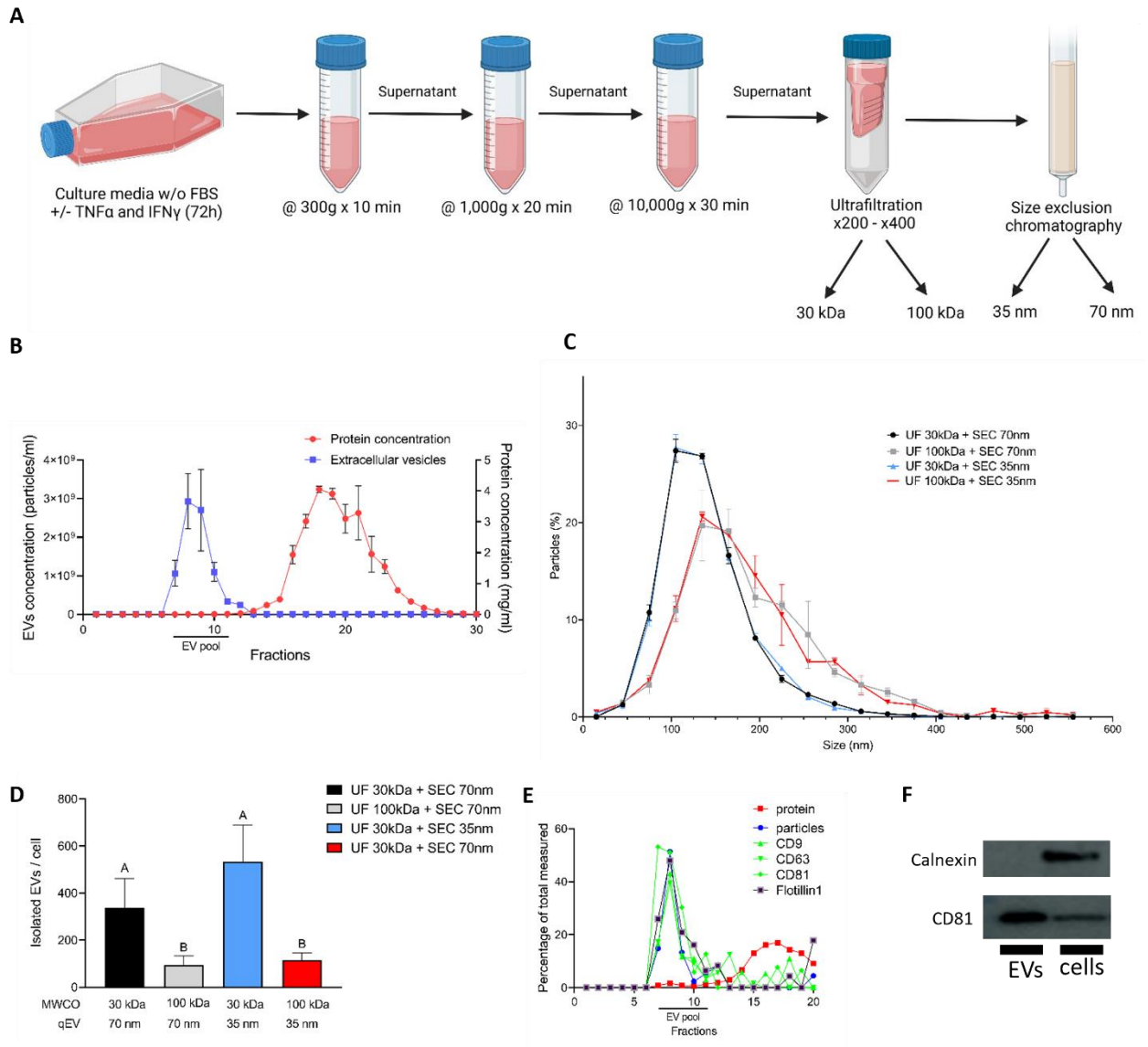



**Figure 12. Isolation and characterization of SCAP-EV**. A. EV isolation workflow B. Quantification of protein (micro BCA) and number of EVs (NTA) in each fraction after the SEC with a pore size of 35 nm (n = 3) C. Size distribution of isolated EV comparing 2 ultrafiltration cutoffs (30 and 100 kDa) and 2 size exclusion chromatography pore sizes (35 and 70 nm) (n = 3) D. Quantification of isolated EV per cell (n =3) E. Delfia Immunoassay on EV positive markers (CD9, CD63, CD81 and Flotillin 1) F. Western Blot on a negative marker of EVs (calnexin) and a positive marker of EVs (CD81) (full image in Supplementary data S1).

## III.2. IDENTIFICATION OF SCAP-EV MIRNA CONTENT

As EVs are thought to be one of the main miRNA transporters [378], we first analyzed the miRNA content of steady-state SCAP-EV.

Around 100,000 mapped reads were detected from non-activated SCAP-EVs, while 236 miRNA were identified (Supplementary data S2). The identified small RNAs were mainly miscellaneous small RNA, long non-coding RNA, and small nuclear RNA (Figure 13A). Among the miRNA

contained in EVs isolated from non-activated SCAP, 22 had a count higher than 1,000 (Supplementary data S3). A miRNA-target prediction analysis revealed that 1868 genes are potential targets of these miRNA (Supplementary data S4). When a KEGG enrichment analysis was performed on the targeted genes, 36 pathways that could be impacted by the 22 miRNA were identified (Figure 14). The 5 more affected pathways were MAPK signaling pathway, pathways in cancer, neurotrophin signaling pathway, regulation of actin cytoskeleton, and focal adhesion pathways.

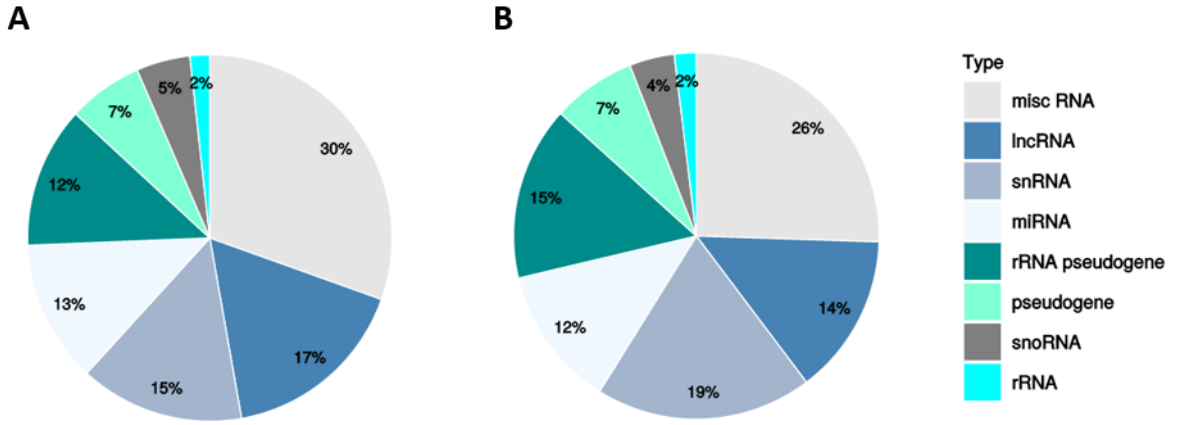



**Figure 13. Distribution of small RNAs identified in SCAP-EV**. Content of RNA in A. non-activated SCAP-EV and B. in activated SCAP-EV. miscRNA: miscellaneous small RNA; lncRNA: long non-coding RNA; snRNA: small nuclear RNA; miRNA: microRNA; rRNA: ribosomal RNA; snoRNA: small nucleolar RNA

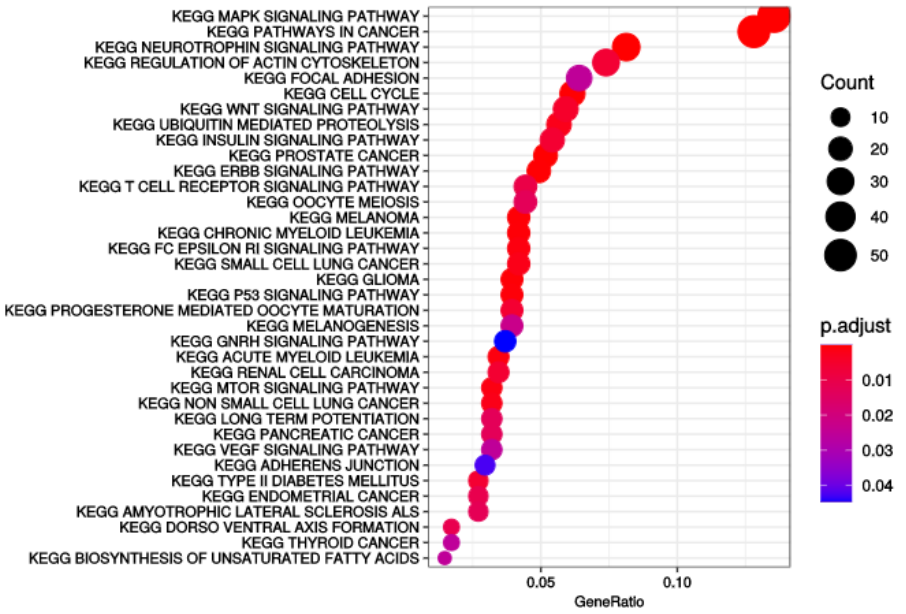



**Figure 14. KEEG pathway analysis of genes potentially targeted by the miRNA identified in EVs from non-activated SCAP**. KEGG analysis was performed on the 1868 potential targets of the 22 miRNA identified in EVs with a count higher than 1000.

### III.3. IMPACT OF SCAP ACTIVATION ON EV COMPOSITION

Then, to study how SCAP activation would impact their EV content, miRNA and lipid from EVs produced by SCAP subjected to a pro-inflammatory stimulus (namely TNFα and IFN-γ) were analyzed and compared to EVs produced from non-activated SCAP.

In terms of protein concentration, no differences were observed between non-activated SCAP-EVs and activated SCAP-EVs (2631 ± 125 μg/ml and 2483 ± 237 μg/ml for $10^9$ EV, respectively).

#### III.3.1. miRNA content

The proportion of the different small RNAs, including miRNA, was not impacted by the activation of SCAP (Figure 13B). More precisely, 120,000 mapped reads were detected from activated SCAP-EVs, while 248 miRNA were identified in activated SCAP-EVs (Supplementary data S5).

To evaluate the impact of pro-inflammatory cytokines on the miRNA content of SCAP-EVs, a differential expression analysis comparing non-activated and activated SCAP-EV miRNA was performed. Principal component analysis (PCA) showed a clear separation between non-activated and activated samples along PC1 (78% of variance), suggesting a significant effect of SCAP activation on the miRNA content of their EVs (Figure 15A). Among the differentially expressed miRNA, 25 and 15 were significantly up-regulated and down-regulated, respectively (Figure 15B). More precisely, miR-155-5p, miR-324-5p, miR-92b-3p, miR-221-3p, and miR-146a-5p were the most up-regulated in EVs of activated SCAP, while let-7a-5p, miR-150-5p, miR-204-5p, and miR-142-5p were the most down-regulated. A KEEG analysis was performed on the potential targets of these 40 miRNA (Supplementary data S6) that identified 33 pathways potentially affected by SCAP activation (Figure 15C).

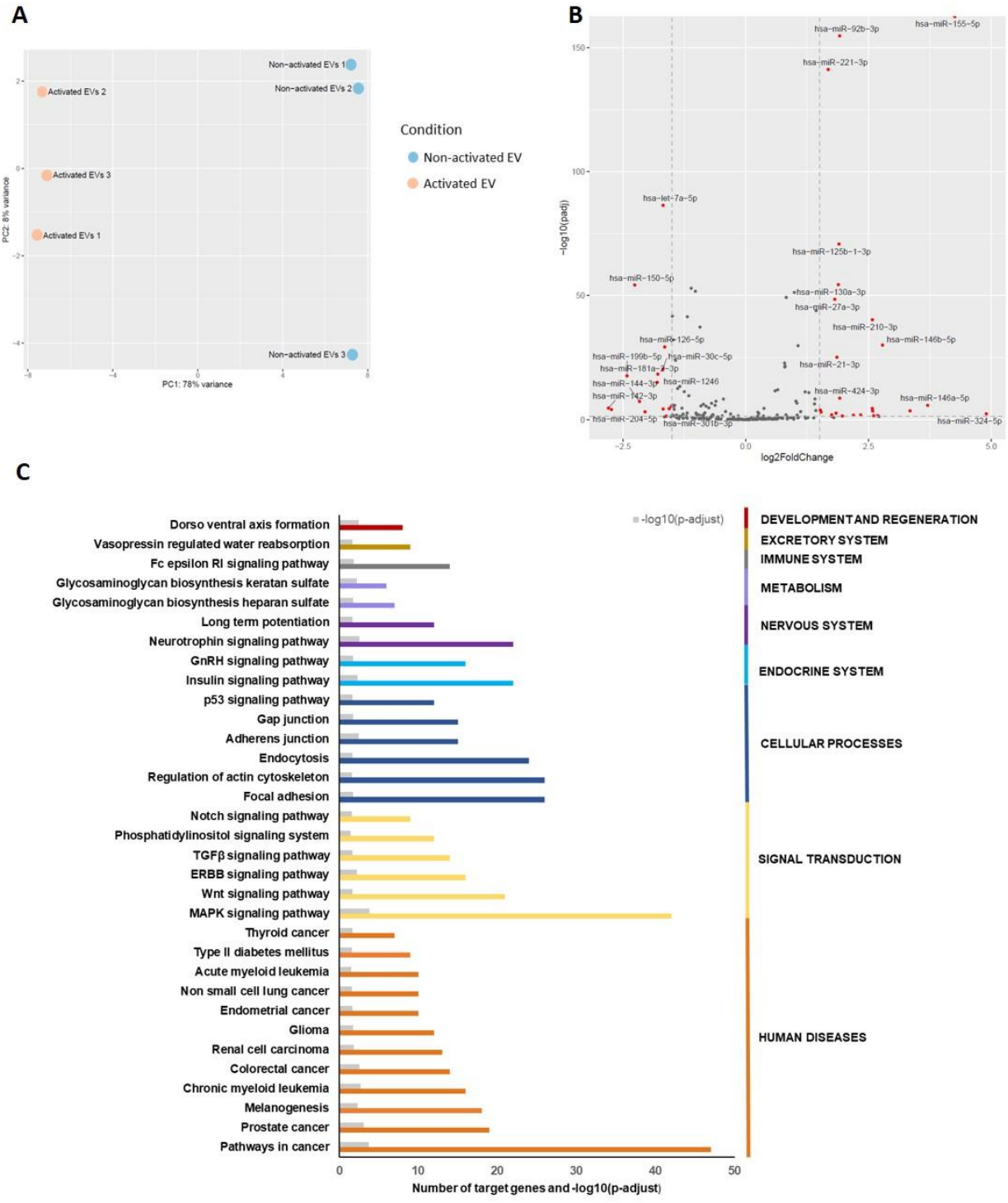



**Figure 15. Impact of SCAP activation on the miRNA content of EVs**. A. Principal component analysis. B. Volcano-plot of miRNA differentially expressed in EVs isolated from activated SCAP versus non-activated SCAP. C. KEGG enrichment analysis of targeted genes of differentially expressed miRNA and the BRITE hierarchy. The color bars show the number of target genes involved in the pathway while the grey bars represent the –log10(p-value).

### III.3.2. Lipid content

In order to study the impact of SCAP activation on their EV lipid content, the proportion of phospholipids, ceramides, sulfatides, and sphingomyelin was compared between EVs isolated from activated SCAP and non-activated SCAP. The lipidomic analysis allowed the identification of numerous lipids present in SCAP-EVs (Figure 16) but no significant difference in the lipid composition was observed between non-activated and activated SCAP-EVs (Figure 16A-E).

The impact of SCAP activation on the EV content of lipid mediators like eicosanoids or their precursors was also assessed. In our experimental setting, only leukotriene B4, 11-hydroxyeicosatetraenoic acid, arachidonic acid, and 2-arachidonoylglycerol (2-AG) were detected. While 2-AG tended to increase in activated SCAP-EVs (Figure 16F), while arachidonic acid (Figure 16G) and leukotriene $B_4$ (Figure 16H) tended to decrease, only 11-HETE was significantly decreased in activated SCAP-EVs (Figure 16I).

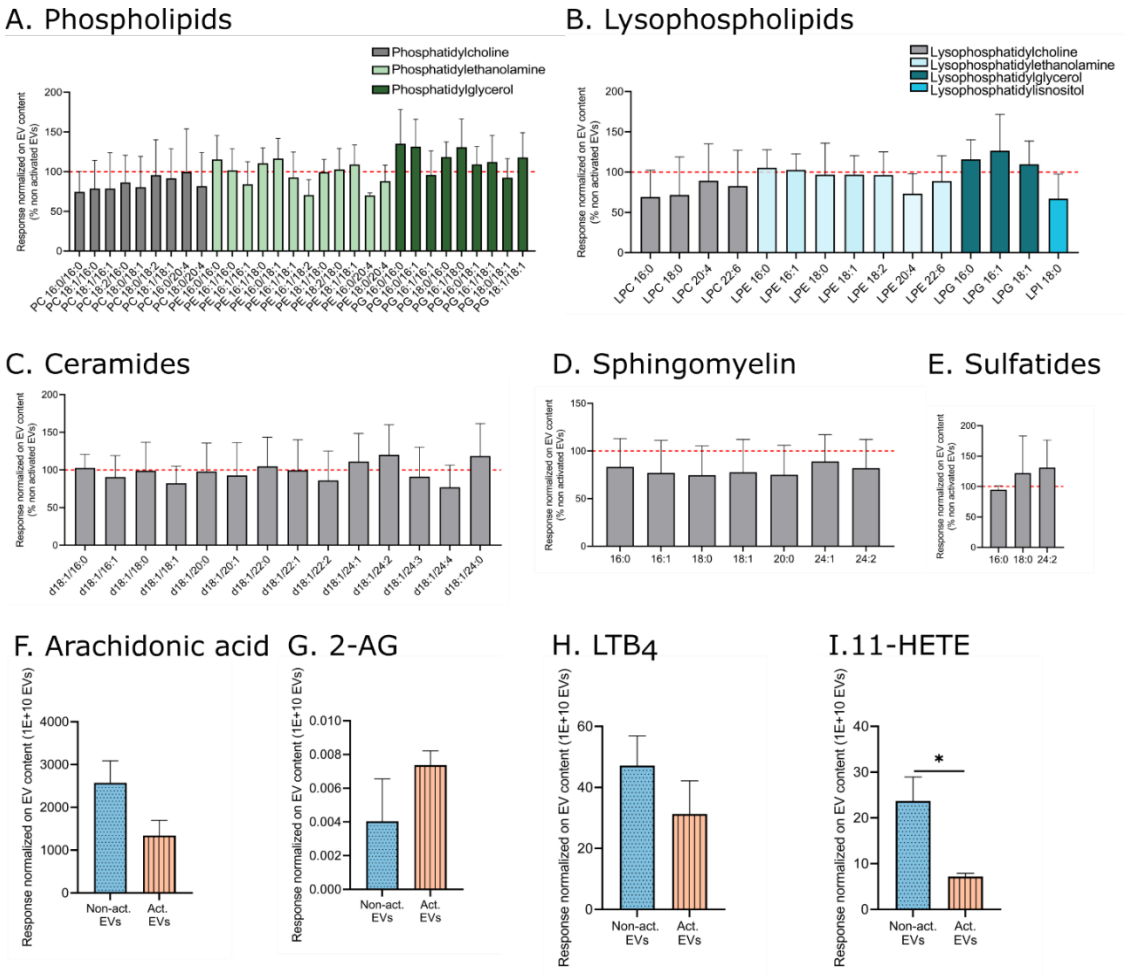



**Figure 16. Lipid content of non-activated SCAP-EVs and activated SCAP-EVs.** A. Phospholipids B. Lysophospholipids C. Ceramides D. d18:1 Sphingomyelins E. d18:1 Sulfatides F. Arachidonic acid G. 2-Arachidonoylglycerol (2-AG) H. Leukotriene $B_4$ I. 11-HETE. The dotted red line represents the detection in non-activated SCAP-EV.

### III.4. IMPACT OF SCAP-EVS ON MICROGLIAL PRO-INFLAMMATORY CYTOKINE EXPRESSION

To assess whether SCAP-EVs, non-activated and activated, would have an immunomodulatory effect, as observed for their parent cells [368], LPS-stimulated BV2 microglial cells were incubated with EVs to study their impact on pro-inflammatory cytokine gene expression was studied.

First, EVs were incubated with steady-state BV2 cells for 8h and 24h. Treating BV2 cells with SCAP-EVs, isolated either from activated or non-activated cells, significantly reduced interleukin (IL)-1β gene expression after 8h of incubation but had no significant impact on the other tested cytokines (Figure 17A). The EVs from activated SCAP induced an increase of inducible nitric oxide synthase (iNOS) gene expression after 24h of incubation (the same tendency was observed after 8h) (Figure 17B).

Then, the effect of SCAP-EVs was assessed on LPS-activated BV2 cells. Our data show that, regardless of the incubation time, neither non-activated nor activated SCAP-EVs were able to significantly impact the gene expression of pro-inflammatory cytokines of BV2 cells (Figures 17C and 17D). Only TNFα was significantly affected by activated SCAP-EVs after 24h of incubation. After 8h of incubation, activated SCAP-EVs tended to reduce pro-inflammatory gene expression but it was not significant.

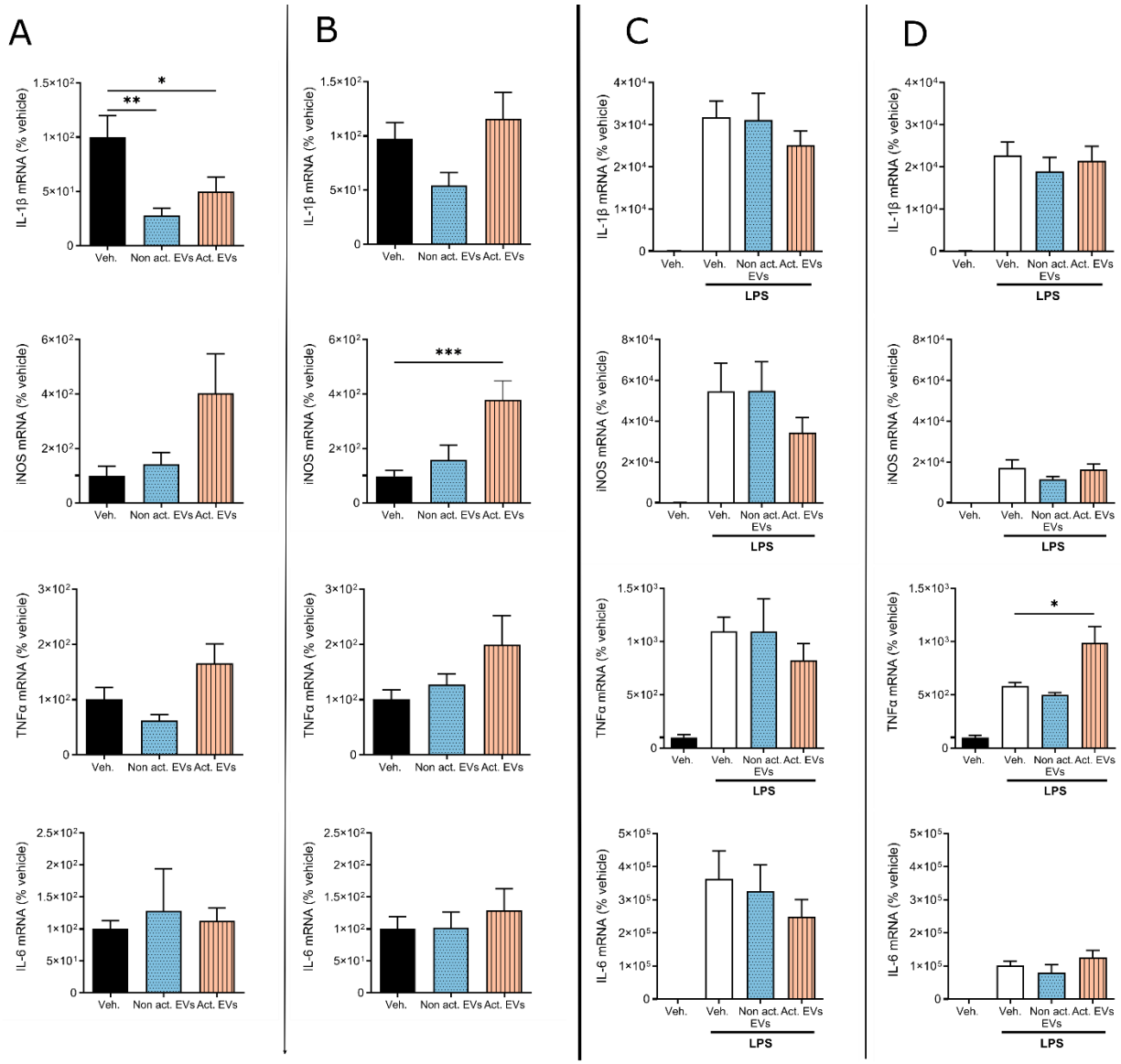



**Figure 17. Impact of SCAP-EVs on the expression of pro-inflammatory cytokines by BV2 cells**. Cells were treated with EVs (5 x $10^9$) isolated from non-activated or activated SCAP for 8h (A) or 24h (B). Alternatively, BV2 were activated by LPS (100 ng/ml) for 1 hour and then treated with EVs (5 x $10^9$) isolated from non-activated or activated SCAP for 8h (C) or 24h (D). The black bars show the impact of vehicle (PBS) on non-activated BV2 cells while the white bars show the influence of vehicle (PBS) on LPS-activated BV2 cells. N = 3, n = 4. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$

## III.5. IMPACT OF SCAP-EVS ON PRO-INFLAMMATORY CYTOKINE EXPRESSION IN SPINAL CORD SECTIONS

To study the impact of non-activated and activated SCAP-EVs on a more complex model of the central nervous system, the same experiment was conducted on mouse spinal cord sections. Spinal cord sections were activated by LPS and then incubated with EV for 8h or 24h. Activated SCAP-EVs induced a decrease in iNOS gene expression after 8h of incubation (Figure 18A) but had no

impact on the expression of cytokines. Non-activated and activated SCAP-EVs were not able to significantly affect the expression of pro-inflammatory cytokines and iNOS after 24h of incubation (Figure 18B). TNFα and IL-6 secretion followed the same pattern (Supplementary data S7).

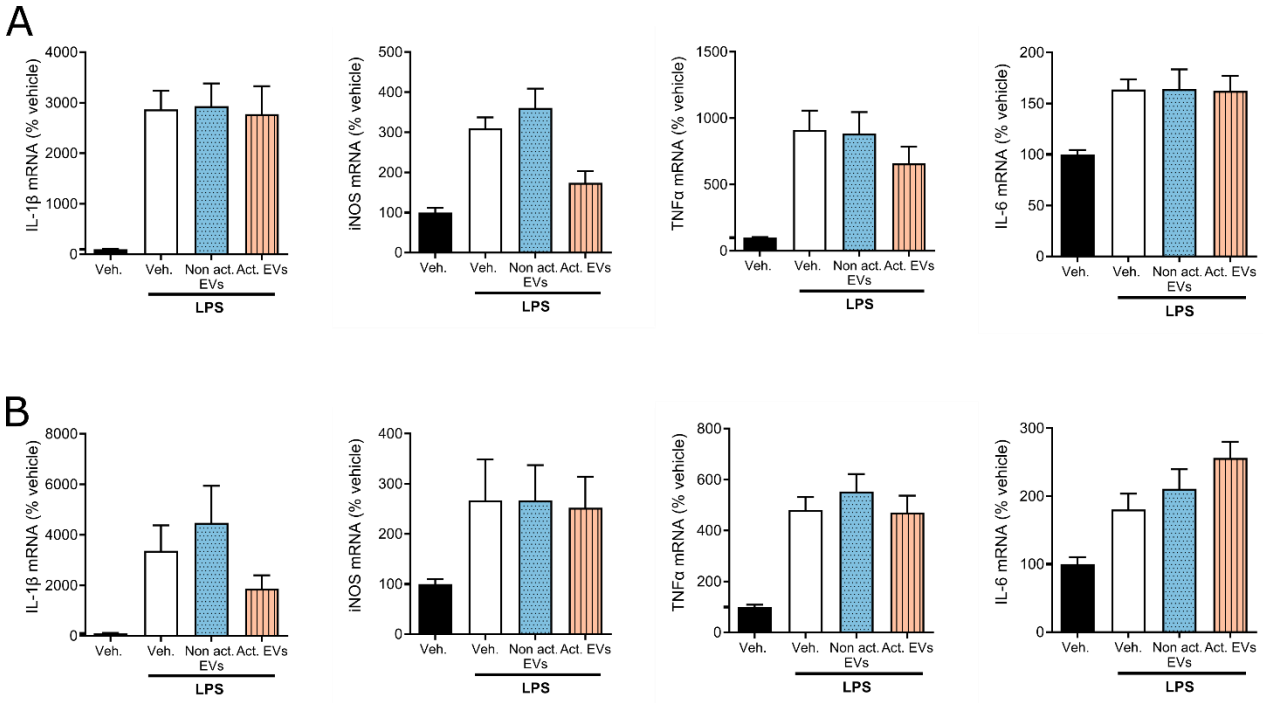



**Figure 18. Impact of SCAP-EVs on the expression of pro-inflammatory cytokines by spinal cord sections**. Mouse spinal cord sections were incubated with LPS (100 ng/ml) for 1h before treatment with EVs (5 x $10^9$) isolated from non-activated or activated SCAP for 8h (A) or 24h (B). The black bars show the impact of vehicle (PBS) on non-activated spinal cord sections while the white bars show the influence of vehicle (PBS) on LPS-activated spinal cord sections. N = 3, n = 4. * $p < 0.05$

## III.6. IMPACT OF THE PROTEIN FRACTION OF ACTIVATED SCAP CONDITIONED MEDIUM ON MICROGLIAL PRO-INFLAMMATORY CYTOKINE EXPRESSION

As it has been shown that the effect of contaminant proteins could be misattributed to the EV [379], we evaluated the impact of pooled protein fractions from activated-SCAP conditioned medium on LPS-activated BV2 cells. BV2 cells were pre-activated with LPS and incubated for 8h with a pool of protein fractions (12-30) or with EVs from activated SCAP. Of note, the protein pool was concentrated to the same fold as the EVs.

Here, activated SCAP-EV significantly decreased the gene expression of IL-1β, iNOS, and IL-6 (Figure 19) with the same fold as observed in Figure 17, confirming the impact of activated SCAP-EVs on BV2 cell pro-inflammatory marker expression. The highest fold decrease was observed

with IL-6 and iNOS (fold decrease of 1.5, Supplementary data S8). However, no effect of the protein fractions was observed, except on IL-6 mRNA expression.

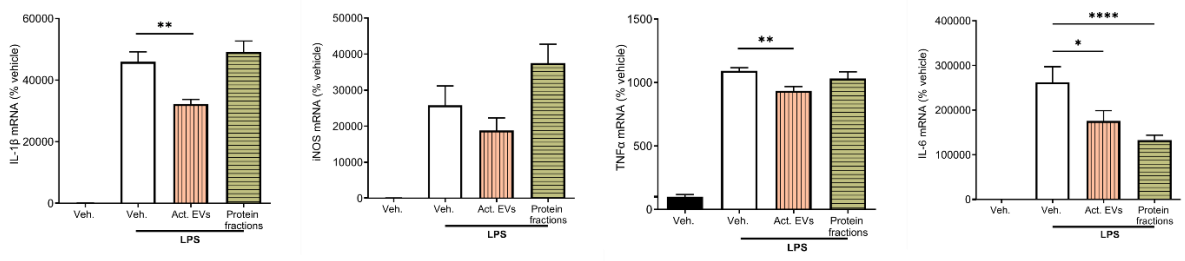



**Figure 19. Impact of activated SCAP-EVs and their pooled protein fractions on the expression of pro-inflammatory cytokines by BV2 cells.** BV2 cells were incubated with LPS (100 ng/ml) for 1 hour and then treated for 8h with EVs (5 x $10^9$) isolated from activated SCAP or a pool of their protein fractions. The black bars show the impact of vehicle (PBS) on non-activated BV2 cells while the white bars show the influence of vehicle (PBS) on LPS-activated BV2 cells. N = 3, n = 4. * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$

## IV. DISCUSSION

The interest in EVs, as biomarkers, therapeutic tools, or drug delivery vehicles, has constantly increased during the last decade [214]. In our previous work, we showed that SCAP secreted immunomodulatory molecules in the presence of pro-inflammatory stimuli [368]. They also decreased the pro-inflammatory cytokine expression of LPS-activated BV2 cells when co-cultured with them [367]. Although EVs from SCAP have previously been reported (28, 29) for their effect on angiogenesis and cisplatin-induced acute kidney injury, the objective of this work was to isolate EVs based on their size and, for the first time, determine whether SCAP exposure to a pro-inflammatory stimulus would impact the composition of their EVs and their effect on activated glial cells. Our hypothesis was that the reduction of neuroinflammation observed with SCAP could be due, at least partially, to the EVs they secrete. For the first time, we demonstrated that it was possible to isolate EVs from SCAP, with limited protein contaminants. Regarding the size and the positive markers of these particles, the term EVs includes exosomes and small microvesicles. We then analyzed SCAP-EV small RNA and lipid content and showed that when SCAP were incubated with pro-inflammatory cytokines, the type but not the number of miRNAs associated with EVs was affected while SCAP activation did not have a strong impact on their lipid composition. Finally, when we compared the impact of SCAP-EVs and the protein fraction of SCAP-conditioned medium on microglial pro-inflammatory markers, a reduction of their expression was observed that was independent of the protein present in the medium. Thus, due to their intrinsic properties, SCAP-EVs seem to be an interesting cell-free nanotechnology.

A protocol was developed and optimized for the collection of EVs from SCAP-conditioned medium with the aim to ally the highest recovery and the highest purity possible. We were able to obtain EVs, as assessed by the presence of positive markers and the absence of negative markers, and to separate EVs quite efficiently from soluble proteins and apolipoproteins by combining ultrafiltration and SEC. This method is quite reproducible and has been recently successfully used in our laboratory to isolate EVs from other sources. Several methods have been reported in the literature to isolate EVs [268]. The specific selection of one method, or combination of methods, strongly depends on the subsequent use of the EVs. While ultracentrifugation is probably the most straightforward and historically the most used, it tends to damage EVs and co-isolate soluble proteins with EVs [268]. As for downstream characterization and functional testing, these two parameters are important, we decided to combine ultrafiltration and SEC to isolate the EVs from SCAP [380]. This combination is becoming more and more popular, but this is the first time, to our knowledge, where the cutoff and the pore size of the ultrafiltration membrane and SEC, respectively, were directly compared and combined to reach the best compromise between yield and purity. We obtained a suspension of EVs with a very low concentration of proteins (5.6 pg /$10^6$ EV) that we hypothesized to be EV-associated proteins. This point is particularly important as therapeutic effects attributed to EVs could be due, at least partially, to co-isolated soluble cytokines or growth factors and not due to EVs themselves [379].

Among the different molecules carried by EVs, miRNA is one of the most studied families as they are involved in the physiopathology of various diseases. As such, they have a high therapeutic potential [381]. Gao et al. showed for instance that bone marrow-derived MSC-EVs were able to transfer miR-21-5p to neurons and improve cognitive functions in a rat model of early brain injury [382]. The most abundant miRNA in non-activated SCAP-EVs were miR-22-3p, miR-181a-5p, miR-100-5p, and miR-127-5p which all seem to play a role in the regulation or suppression of the inflammatory response [383-386]. Moreover, Luo et al. showed that miR-100-5p was enriched in exosomes of stem cells from human exfoliated deciduous teeth and thus were able to reduce inflammation via its action on mTOR signaling pathway [385]. However, miRNA that are known to induce inflammation were also found in non-activated SCAP-EVs such as miR-486-5p, miR-92a-3p, and miR-222-3p [387-389]. Among the 22 most expressed miRNA in non-activated SCAP-EVs, 13 miRNA were also found in Human Wharton's Jelly MSC-EVs [390]. By targeting the Notch and MAPK/ERK signaling cascades, these miRNAs were able to drive oligodendroglial maturation in the central nervous system.

As the SCAP secretome is influenced by cell exposure to pro-inflammatory cytokines [368], we hypothesized that their EV content might also be modulated in these conditions. We compared

the miRNA content of non-activated SCAP-EVs and activated SCAP-EVs. MiR-155 and miR-146 were among the miRNAs with the highest fold increase. This correlates with an increased expression of these two miRNAs in immune cells after exposure to TNFα and IFN-γ [391]. MiR-155 is known to inhibit the immune response mediated by SOCS1 in macrophages while miR-146 targets IRAK1, IRAK2, and TRAF6 which are both involved in innate response via Toll-like receptor and IL-1 receptor signalization [392, 393]. However, these two miRNAs seem also to have anti-inflammatory effects as miR-155 can be a link between adaptive and innate immunity [394]. Contradictory effects for the same miRNA have been then reported in the literature, mostly depending on the scientific question, the models used, and the type of output. This highlights the limitations of the *in silico* prediction of miRNA biological effects. However additional studies are needed to identify miRNA targets and their physiological function [395]. The multiplicity of miRNA target genes also complicates the prediction of their biological impact. Thus, understanding the effect of multiple miRNAs delivered by EVs remains a challenge.

The KEGG pathway analysis highlighted 33 pathways potentially affected by SCAP activation. Among them, pathways in cancer, MAPK signaling pathway, focal adhesion, regulation of actin cytoskeleton, and endocytosis pathways are the five most affected pathways. About 500 genes are involved in pathways in cancer and 21 pathways are connected to them. The presence of miRNAs involved in these pathways in SCAP-EVs could highlight the potential role of EVs in the mediation of some cancer processes, such as metastasis [396]. MAPK signaling pathway is the second most affected pathway and is involved in multiple cellular processes including MSC proliferation and differentiation. As the ability of IFN-γ to increase the differentiation potential of MSC has been previously demonstrated, this could explain why this pathway is affected by SCAP activation with IFN-γ and TNFα [397]. Focal adhesion and regulation of the actin cytoskeleton are two pathways involved in immunomodulatory processes. Indeed, regulation of actin cytoskeleton is a key mediator of communication between MSC and B-cells while focal adhesion regulates cell migration, including migration of cells toward the site of inflammation and homing [396]. TNFα is known to regulate MSC migration in order to lead the cells to the inflamed area. Thus, it is not surprising that these 2 pathways are also affected by the activation of SCAP with TNFα and IFN-γ. The fifth most affected pathway is endocytosis. It could be explained by its involvement in EV generation and a modification of EV production under stress conditions such as a pro-inflammatory environment [247].

As major components of EV envelop and as potent biological mediators, the focus on EV lipid composition increased recently, but only a few studies on the subject have yet been published [398, 399]. When looking at the lipid content of human bone marrow MSC-EVs, cells that can be

considered of the same family as SCAP, Holopainen et al. detected ceramides, diacylglycerol, phospholipids, lysophospholipids and sphingomyelin [398]. We report for the first time the lipid composition of SCAP-EVs that seems quite similar to the one reported by Holopainen et al. However, due to differences in report format, method of data analysis, and standardization, it is difficult to establish quantitative comparisons with other works. Based on the same hypothesis as for the miRNA, we also evaluated whether the SCAP-EV lipid composition was affected by SCAP incubation with pro-inflammatory cytokines. In our setting, we did not see a major impact on EV lipid composition. Only eicosanoid 11-HETE was significantly reduced after SCAP activation. Its role in inflammation has not been studied yet. To the extent of our knowledge, no other work has reported the impact of inflammation on EV lipid composition.

Finally, with the objective of deciphering whether SCAP-EVs would recapitulate SCAP anti-inflammatory effect on microglial cells and spinal cord sections [367], SCAP-EVs isolated from activated and non-activated SCAP were incubated with BV2 cells and mouse spinal cord sections, treated or not with LPS. EVs decreased the gene expression of pro-inflammatory cytokines in LPS-treated macrophages by 1.5-fold. Similar results were observed on spinal cord slices. Our results are consistent with other studies that reported an anti-inflammatory effect of about a 2 fold-decrease of pro-inflammatory cytokines for EVs of different origins [400-402]. We also assessed the effect of the protein fraction isolated from activated SCAP-conditioned medium after EV isolation. To minimize interpretation bias, attention was paid to concentrating the pooled protein fractions to a similar extent to the pooled EV fractions. The effect of activated SCAP-EVs on pro-inflammatory cytokines expression by LPS-activated BV2 cells was similar to what we previously observed, with this time a significant reduction of the tested pro-inflammatory cytokine expression. The protein pool had no effect on LPS-activated BV2 cells when looking at pro-inflammatory cytokine gene expression, with the exception of IL-6.

As we chose to isolate EVs from SCAP under serum starvation (72h) to limit FBS protein content and thus protein contamination of EV suspension, the properties of the SCAP producing the EVs, and thus the EVs, might be different from other protocols reported in the literature. This could have impacted EV content [403], and anti-inflammatory potential, and thus may explain the limited effects observed with SCAP-EVs. In the future, replacing serum starvation with a synthetic serum-free medium might be an alternative worth considering. Other optimizations of EV production with other chemical signals such as Cytochalasin-B [352] or hypoxia [404] may also help to obtain better immunomodulatory effects.

It is also important to keep in mind that the effect of human MSC-EVs could depend on the parent cells (type, source, and culture conditions), the concentration of EVs, and the model used (22).

Thus, some articles reported a pro-inflammatory effect of MSC-EVs. Kang et al. for example highlighted the role of EVs from LPS-preconditioned periodontal ligament stem cells in M1 polarization of macrophages while previous studies reported a M2 polarization of macrophages after treatment with LPS-preconditioned human umbilical cord MSC. This highlighted the impact of the origin of MSC on the effect of their EVs on macrophage polarization [405]. The multitude of different protocols and settings complicates the comparison between studies, especially given that what is considered negative results or lack of effect are unfortunately less reported in the literature.

## V. CONCLUSION

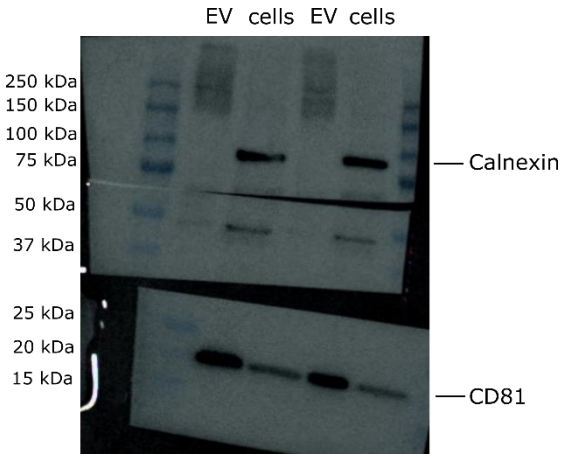
Our aim was to fully characterize EVs produced by SCAP and explore whether they would be affected by a pro-inflammatory stimulus. We also aimed to decipher if SCAP-EVs would be responsible, at least partially, for the immunomodulation observed when SCAP are co-cultured with microglial cells or spinal cord. We thus optimized a protocol that allowed us to collect EVs as free as possible from contaminants and we analyzed their miRNA content and lipid composition. We have shown that SCAP were able to respond to a pro-inflammatory stimulus by modifying the miRNA content of their EVs but not their lipid composition. We observed a slight reduction of the gene expression of pro-inflammatory markers in a microglial cell line, albeit in the range of what was observed by some other studies. We thus conclude that the EVs are likely not the key mediators in the reported effects of SCAP, at least in the model we used.

## Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

The dataset generated and analyzed during the current study is available in the GEO repository under the number GSE208577 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE208577

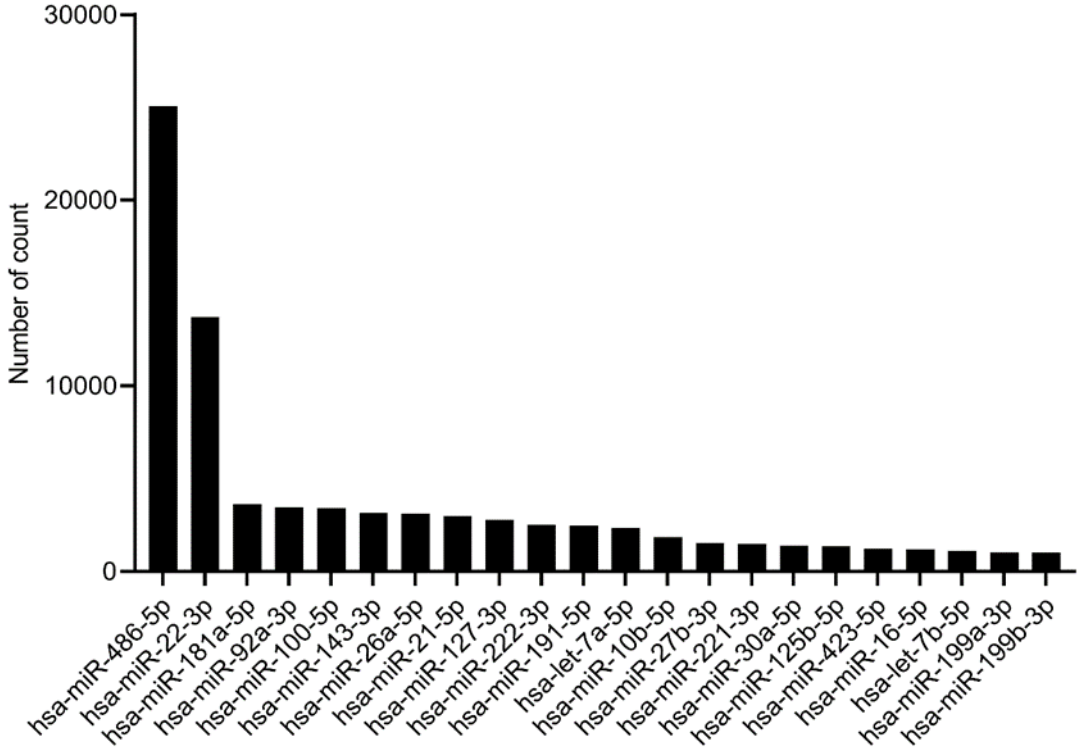
## Supplementary data




**Supplementary data S1. Western Blot on a negative marker of EV (calnexin, 95 kDa) and a positive marker of EV (CD81, 22-26 kDa).**

**Supplementary data S2. List of the 236 miRNAs identified in non-activated SCAP-EV.** The 22 most abundant miRNAs are highlighted in grey.

*Available online: Supplementary data*




**Supplementary data S3. Distribution of the 22 miRNAs with the higher expression in non-activated SCAP-EV**.

**Supplementary data S4. 1868 gene targeted by the 22 miR with the highest expression in EV isolated from non-activated SCAP**

*Available online: Supplementary data*

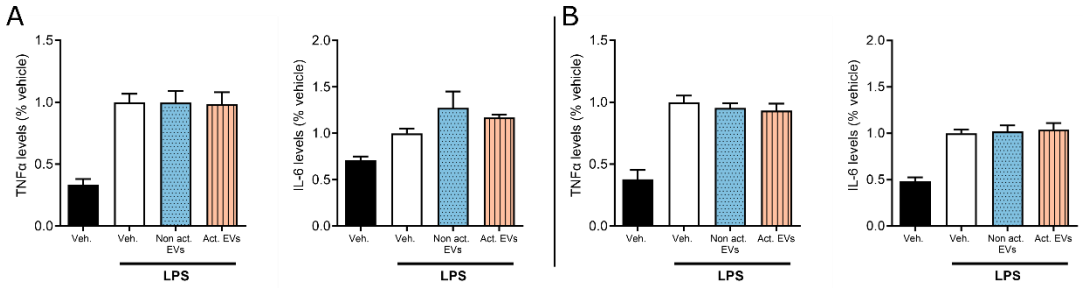
**Supplementary data S5. List of the 248 miRNAs identified in activated SCAP-EV.** The 24 most abundant miRNAs are highlighted in grey.

*Available online: Supplementary data*

**Supplementary data S6. List of differentially expressed miRNAs between non-activated and activated SCAP-EVs**

| miRNA | log2FoldChange | pvalue | padj |
|---|---|---|---|
| hsa-miR-324-5p | 4,898418369 | 0,001401692 | 0,004380286 |
| hsa-miR-155-5p | 4,257031992 | 0 | 0 |
| hsa-miR-146a-5p | 3,704375653 | 3,46E-07 | 1,69545E-06 |
| hsa-miR-218-5p | 3,345806403 | 7,78368E-05 | 0,000286165 |
| hsa-miR-146b-5p | 2,784710116 | 6,48E-32 | 8,53E-31 |
| hsa-miR-22-5p | 2,688108555 | 0,009530085 | 0,024311442 |
| hsa-miR-210-5p | 2,606818006 | 0,010474734 | 0,026415688 |
| hsa-miR-543 | 2,585211859 | 9,11976E-05 | 0,000330426 |
| hsa-miR-210-3p | 2,579076127 | 3,36E-42 | 5,25E-41 |
| hsa-miR-4449 | 2,576610813 | 7,69264E-06 | 3,15272E-05 |
| hsa-miR-431-3p | 2,341043047 | 0,004274292 | 0,01200644 |
| hsa-miR-125a-3p | 2,193270391 | 0,006006813 | 0,015975565 |
| hsa-miR-539-3p | 1,967943299 | 0,013876833 | 0,032728379 |
| hsa-miR-424-3p | 1,914763131 | 3,34E-10 | 2,14E-09 |
| hsa-miR-92b-3p | 1,912794922 | 1,45E-157 | 1,82E-155 |
| hsa-miR-125b-1-3p | 1,899037114 | 3,12E-73 | 1,56E-71 |
| hsa-miR-130a-3p | 1,885512563 | 8,62E-57 | 3,59E-55 |
| hsa-miR-21-3p | 1,857021081 | 5,76E-27 | 6,54E-26 |
| hsa-miR-574-5p | 1,838921411 | 0,000740225 | 0,002372515 |
| hsa-miR-27a-3p | 1,813884914 | 1,49E-50 | 3,10E-49 |
| hsa-miR-324-3p | 1,752045837 | 0,01873916 | 0,041094648 |
| hsa-miR-193b-5p | 1,749552079 | 0,003979737 | 0,011306071 |
| hsa-miR-221-3p | 1,679340358 | 8,05E-144 | 6,71E-142 |
| hsa-miR-34a-5p | 1,533710409 | 0,000255446 | 0,000874814 |
| hsa-miR-887-3p | 1,51896755 | 3,69019E-05 | 0,00013978 |
| hsa-miR-142-3p | -2,796010628 | 5,11568E-06 | 2,20503E-05 |
| hsa-miR-204-5p | -2,732351806 | 2,14124E-05 | 8,23554E-05 |
| hsa-miR-199b-5p | -2,416256656 | 2,69E-19 | 2,32E-18 |
| hsa-miR-150-5p | -2,260108278 | 1,46E-56 | 5,20E-55 |
| hsa-miR-144-3p | -2,160828165 | 8,75E-09 | 4,97E-08 |
| hsa-miR-671-3p | -2,049749666 | 0,000195331 | 0,000678232 |
| hsa-miR-1246 | -1,801764052 | 1,11E-16 | 9,29E-16 |
| hsa-miR-181a-2-3p | -1,789184826 | 4,93E-20 | 4,40E-19 |
| hsa-miR-30c-5p | -1,682880287 | 3,72E-22 | 3,45E-21 |
| hsa-let-7a-5p | -1,681867266 | 6,12E-89 | 3,82E-87 |
| hsa-miR-31-5p | -1,680541967 | 1,27748E-05 | 5,06937E-05 |
| hsa-miR-126-5p | -1,649061164 | 4,08E-31 | 4,85E-30 |

| | | | |
|---|---|---|---|
| hsa-miR-301b-3p | -1,616038851 | 0,017098045 | 0,038204124 |
| hsa-miR-223-3p | -1,557709301 | 8,94929E-06 | 3,60859E-05 |
| hsa-miR-149-5p | -1,513511706 | 1,11913E-06 | 4,99613E-06 |




**Supplementary data S7**. **Impact of SCAP-EV on the level of pro-inflammatory cytokines by spinal cord sections.** Mouse spinal cord sections were incubated with LPS (100 ng/ml) for 1h before treatment with EV (5 x $10^9$) isolated from non-activated or activated SCAP for 8h (A) or 24h (B). Cytokines were quantified by ELISA in the cell supernatant. The black bars show the impact of vehicle (PBS) on non-activated spinal cord sections while the white bars show the influence of vehicle (PBS) on LPS-activated spinal cord sections. N = 3, n = 4.

**Supplementary data S8. Pro-inflammatory cytokines expression fold decrease**

| **Cytokines** | **Fold decrease** |
|---|---|
| IL-1β | 1.4 |
| iNOS | 1.5 |
| TNFα | 1.3 |
| IL-6 | 1.5 |

# CHAPTER II SIPONIMOD-LOADED EXTRACELLULAR VESICLES INCREASED OLIGODENDROCYTE PROGENITOR CELL DIFFERENTIATION

V. Gratpain, S. Razafindrakoto, K. Vanvarenberg, S. Cam, B. Ucakar, S. Y. Chew, F. Gazeau, A. K. Silva, A. des Rieux

**ABSTRACT**

Multiple sclerosis is a common cause of disability in young adults characterized by inflammation and demyelination. Therapies available on the market are focused on reducing inflammation but none enable remyelination, one reason being the difficulty of drugs reaching the central nervous system. Here, we propose extracellular vesicles (EVs) derived from human stem cells from apical papilla (SCAP) as biological nanocarriers for siponimod, an approved treatment for MS that has been associated with protection against demyelination and promotion of remyelination due to a direct action on oligodendrocyte progenitor cells (OPC) and oligodendrocytes. We encapsulated siponimod in EVs with a scalable technique based on mechanical shear stress. Their ability to decrease pro-inflammatory cytokine expression in microglia cells and to increase gene expression of oligodendrocyte markers was assessed *in vitro*. We showed that siponimod-loaded EVs reduced pro-inflammatory cytokine expression and enhanced OPC differentiation induction compared to the free drug. In conclusion, SCAP-EVs loaded with siponimod appear to be a promising system for remyelination.

## I. INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating, and neurodegenerative disorder of the central nervous system, recognized as the most common cause of non-traumatic neurological disabilities in young adults [406]. While the majority of available treatments focus on reducing inflammation, there is currently no curative treatment for promoting remyelination. One of the fundamental mechanisms behind the remyelination process following myelin sheath damage involves the differentiation of oligodendrocyte progenitor cells (OPC) into oligodendrocytes, which are responsible for producing myelin.

Among the recently approved drugs for MS therapy, siponimod has garnered significant interest. This hydrophobic compound is able to block the egress of lymphocytes from secondary lymphoid organs into the systemic circulation. This effect is mediated by the G-protein coupled receptor subtype Sphingosine-1-phosphate receptor 1 ($S1P_1$) on which siponimod acts as a functional antagonist [159]. Beyond its interaction with $S1P_1$, siponimod also targets S1P receptor subtype $S1P_5$. Recently, $S1P_5$ have been associated with the promotion of remyelination in both *in vitro* and *in vivo* studies [160]. These findings are supported by studies demonstrating that siponimod may induce oligodendrocyte progenitor cell differentiation and thus remyelination [155, 187, 209].

Stem cells have also been extensively studied for the treatment of MS. Among the various types of stem cells, mesenchymal stem cells (MSC) have emerged as promising candidates [284]. Our previous works have demonstrated that stem cells from the apical papilla (SCAP) can exert anti-inflammatory and neuroprotective effects [367] and that these effects were mediated, at least partially, by their extracellular vesicles (EVs) [407]. EVs are small vesicles (nanometres scale) consisting of an aqueous core surrounded by a lipid bilayer, containing genetic materials, proteins, and lipids [214]. Recently, their use for drug delivery applications has raised a lot of interest as they can be loaded with a specific bioactive molecule, can cross biological barriers, and are characterized by low immunogenicity and no toxicity.

Here, we hypothesized that combining the anti-inflammatory and neuroprotective properties of SCAP-derived EVs with the pro-remyelination effect of siponimod could resolve inflammation and promote remyelination. We encapsulated siponimod into EVs derived from SCAP, using an innovative turbulence-based method. We then assessed the efficacy of the EV-Siponimod in reducing pro-inflammatory cytokine expression in microglia cells and inducing OPC differentiation.

## II. MATERIALS AND METHODS

### II.1. CELL CULTURE

Previously characterized human SCAP from healthy teeth were used [369]. Cells were cultured at 37°C and 5% $CO_2$ in minimum essential medium Eagle (MEM, Sigma-Aldrich, St Louis, USA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine 200 mM (Thermo Fisher Scientific, Waltham, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific).

### II.2. PRODUCTION OF SCAP-EVS LOADED WITH SIPONIMOD (EV-SIPONIMOD) BY TURBULENCE STIMULATION

EVs were produced by turbulence and siponimod was incorporated in EVs by turbulence-induced mechanical stress (patent WO2020136361A1). SCAP were cultured in three 250 ml spinner flasks bioreactors, each containing 2.5 g/L of 170 µm Cytodex 1 dextran microcarriers (GE Healthcare, Chicago, USA), at a concentration of 7 cells per bead. For the seeding step, cells were submitted to 21 cycles of 45 min of rest interspersed with 5 min of gentle mixing at 34 rpm to ensure homogeneous adhesion of cells on microcarriers. After the cell adhesion period, continuous gentle mixing at 34 rpm was performed until cell confluence was observed on the microcarriers (3 days). Siponimod was then added to the cell medium (20 µM in ethanol) and spinner flasks were subjected to 290 rpm mixing for 4h for the high-throughput production of EV-Siponimod in the serum-free culture medium. Prior to EV isolation, the medium of the 3 spinner flasks were pooled. EV-Siponimod were then isolated by centrifugation at 2000g for 10 min and 0.45 µm filtration to eliminate microcarriers and cell debris. This was followed by tangential-flow filtration (TFF) using a 100 kDa cut-off TFF cartridge, allowing for EV collection in 13 – 15 ml of phosphate-buffered saline (PBS). To produce blank EVs, the same protocol was applied but without the addition of siponimod to the medium during EV production.

### II.3. EV CHARACTERIZATION

#### II.3.1. Nanoparticle tracking analysis (NTA)

Particle concentration and size distribution of EVs were analyzed by NTA using a Nanosight NS3000 (Malvern Panalytical, Malvern, United Kingdom). EVs were diluted in PBS to a concentration between $10^7$ and $10^8$ particles/ml.

### II.3.2. Marker analysis by nanoflow cytometry (NanoFCM) and western blotting

For NanoFCM, a Nano Analyzer (NanoFCM Co., Ltd., Nottingham, UK) equipped with 488 nm laser, was calibrated using 200 nm polystyrene beads (NanoFCM Co.) with a defined concentration of 2.08 x $10^8$ particles/mL. In addition, monodisperse silica beads (NanoFCM Co.) of four different sizes (68 – 155 nm) served as size reference standards to calibrate the size of EVs. Each dot plot was derived from data collected for one minute with a sample pressure of 1.0 kPa. The EV samples were diluted with filtered PBS, resulting in a particle count in the optimal range of 2,000 – 12,000 events. For immunofluorescent staining, the antibodies were purchased from Biolegend: PE-conjugated mouse anti-human CD9 antibody, PE-conjugated mouse anti-human CD81 antibody, and PE-conjugated mouse anti-human CD63 antibody, 2 ng/µl of each antibody in 100 µL of PBS. Antibodies were then added to 2 x $10^8$ EVs, followed by incubation overnight at 4°C under constant shaking and washing with 1 mL PBS by ultracentrifugation at 110,000g for 60 min at 4°C (Beckman Coulter MAX-XP centrifuge, TLA-145 rotor). The pellet was resuspended in 50 µL of PBS for NanoFCM analysis.

For Western blotting, the protocol described in [407] was used.

### II.3.3. Determination of siponimod loading in EVs

Siponimod was released from EVs using Triton 0.1% in acidic conditions (pH = 2). The concentration of siponimod in EVs was determined by a validated liquid chromatography with tandem mass spectrometric (LC-MS/MS) method [408]. The LC-MS/MS system consisted of Shimadzu LC-40DXR pumps, a CTO-40S column oven, a SIL-40CXR autosampler (with rack changer), DGU-405 online degasser and a LCMS-8040 tandem mass spectrometer (Shimadzu, Columbia, USA) with a Waters XSelect HSS T3 column (3.0 x 75 mm, 3.5 µm particle size; Waters corporation, Milford, USA). Chromatographic elution of the analyte was performed using 30% water containing 0.1% formic acid (mobile phase A) and 70% of acetonitrile containing 0.1% formic acid (mobile phase B) at a flow rate of 1.00 ml/min. The eluent was directed to the electrospray ionization source of the MS system between approximately 1.5 and 2.5 minutes. The following MS transition was monitored: $m/z$ 517.2 – 416.1. Blank EVs were used as control.

## II.4. IMPACT OF EV-SIPONIMOD ON BV2 CELL ACTIVATION

Murine microglial cells (BV2 cells) were cultured at 37°C and 5% $CO_2$ in high-glucose DMEM medium with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. BV2 cells were seeded and grown overnight into 24-well plates (2.5 x $10^5$ cells per well) and incubated for 1h with fresh

medium containing 100 ng/ml LPS and 1% EV-depleted FBS (FBS centrifuged for 1h at 100,000 g to remove EVs). Then, siponimod (1μM), blank EVs (6.3 x $10^8$ particles), and EV-Siponimod (6.3 x $10^8$ particles corresponding to 1μM siponimod loaded into the vesicles) were added to the cells and incubated for 8h before proceeding to BV2 cell RNA extraction.

## II.5. IMPACT OF EV-SIPONIMOD ON MYELIN WRAPPING IN VITRO

### II.5.1. Isolation and culture of OPC on electrospun nanofibers

Cortical mixed glial cells (MGC) were prepared from Sprague-Dawley rat pups (postnatal day 1-3) as described previously by Miron et al. [54]. All the experiments involving animals were done in accordance with Directive 2010/63/E.U and were declared to the ethical committee for animal cares (07-OTLADDB-2023-7). Briefly, brains were dissected aseptically, and cerebellum, olfactory bulbs, and meninges were removed. Brains were then digested with papain (40 μg/ml) and slowly mechanically dissociated using a G18 and a G23 syringe. The cell suspension was diluted in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and seeded in T75 flasks for 10 to 14 days [54]. Microglia were then removed by shaking at 230 rpm for 1h at 37°C. To retrieve OPCs, the medium was replaced with fresh medium (10 ml per flask) and flasks were shaken at 220 rpm for 18h at 37°C. Finally, the collected OPCs were grown on poly-D-Lysine coated (1 μg/ml) electrospun nanofibers placed in 12-well plates with a cell density of 35,000 cells per well [409]. The culture medium consisted of 50:50 DMEM:Neurobasal Media, 1% B-27, 5 μg/ml N-acetyl cysteine, 10 ng/ml D-biotin, 1% ITS supplement and 1% modified SATO (100 μg/ml bovine serum albumin fraction V, 60 ng/ml Progesterone, 16 μg/ml Putrescine, 400 ng/ml Tri-iodothyronine, 400 ng/ml L-Thyroxine; reagents from Sigma-Aldrich).

### II.5.2. In vitro myelination

To assess the impact of EV-Siponimod on myelination, OPCs seeded on electrospun nanofibers were treated with free siponimod (1μM), blank EVs (6.3 x $10^8$ particles), and EV-Siponimod (6.3 x $10^8$ particles corresponding to 1μM siponimod loaded into the vesicles) for 48h. The medium was changed and cells were kept for 5 more days of culture before fixation with 4% paraformaldehyde in PBS and immunostaining.

### II.5.3. Immunostaining and confocal microscopy

OPCs were permeabilized with 0.1% TritonX-100/PBS for 5 min before incubation with primary antibody against myelin basic protein (MBP) (rat anti-MBP, 1:250) overnight at 4°C. They were

washed 3 times and then incubated with the secondary antibody (goat anti-rat Alexa 488 nm, 1:1000) and DAPI (1:1000) for 1h. Finally, cells were washed and mounted with Mowiol mounted media (Carl Roth, Karlsruhe, Germany) on microscope slides. Images were obtained with a Zeiss LSM 980 confocal microscope.

### II.6. IMPACT OF EV-SIPONIMOD ON OLIGODENDROCYTE PROGENITOR CELL DIFFERENTIATION IN A PRIMARY MIXED GLIAL CULTURE

As explained in II.5.1, MGC were isolated from Sprague-Dawley rat pups (postnatal day 1-3) before being plated at 5 x $10^5$ cells/ml in poly-D-Lysine coated (1 μg/ml) 24-well plates for 8 days. On day 8, mixed glial cells were treated with free siponimod (1μM), blank EVs ($6.3*10^8$ particles), and EV-Siponimod ($6.3*10^8$ particles corresponding to 1μM siponimod loaded into the vesicles) for 48h. Medium was changed and cells were kept for 5 more days of culture before cell RNA extraction.

### II.7. RNA EXTRACTION, REAL-TIME QPCR, AND RNA SEQUENCING ANALYSIS

Total RNA from BV2 cells and mixed glial cells were extracted using TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse transcription was performed using the GoScript Transcription System (Promega, Madison, USA) from 1 μg of total RNA. qPCR was performed with qPCR Master Mix (Promega) and a STEPone PLUS instrument and software (Applied Biosystems, Foster City, USA) as [377]. Data were analyzed with the ΔΔCt method using the 60S ribosomal protein L19 (RPL19) as a reference gene for murine cells and the 60S ribosomal protein L13 (RPL13) as a reference gene for rat cells. Primer sequences are given in Table 10.

**Table 10. List of primers sequences**

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| Mouse RPL19 | GAAGGTCAAAGGGAATGTGTTCA | CCTTGTCTGCCTTCAGCTTGT |
| Mouse IL-6 | ACAAGTCGGAGGCTTAATTACACAT | TTGCCATTGCACAACTCTTTTC |
| Mouse IL-1β | TCGCTCAGGGTCACAAGAAA | CATCAGAGGCAAGGAGGAAAAC |
| Mouse iNOS | AGGTACTCAGCGTGCTCCAC | GCACCGAAGATATCTTCATG |
| Mouse TNFα | AGCCCCCAGTCTGTATCCTT | GGTCACTGTCCCAGCATCTT |
| Rat RPL13 | GGCTGAAGCCTACCAGAAAG | CTTTGCCTTTTCCTTCCGTT |
| Rat MBP | CACACACAAGAACTACCCACTAC | GGTGTACGAGGTGTCACAATG |
| Rat MAG | GTGTGTAGCTGAGAACCAGTATG | AGAAGGATTATGGGAGCAAACTC |
| Rat PLP | GGCGACTACAAGACCACCAT | AATGACACACCCGCTCCAAA |

## II.8. EV UPTAKE BY MIXED GLIAL CELLS

### II.8.1. Staining of EVs

EVs (1 x $10^{10}$ particles) were stained with CFDA-SE (BioLegend, San Diego, USA). EVs were incubated with 40 µM of CFDA-SE for 1h at 37°C. EVs were separated from free CFDA-SE by size exclusion chromatography (qEV Original® 30 nm, Izon Science, Lyon, France). Fractions from 1 to 30 (500 µL each) were collected. Fractions 7 to 11, containing EVs, were pooled and concentrated 20 times using an ultrafiltration device with a 10 kDa cut-off (Amicon Ultra 4, Merck Millipore, Burlington, USA).

### II.8.2. EV uptake study

Cortical MGC were seeded at 1.68 x $10^5$ cells/ml in poly-D-Lysine coated (1 µg/ml) 96-well plates for 8 days. On day 8, MGC were incubated with 3.8 x $10^7$ CFDA-stained EVs. After 24h, cells were washed once with PBS and fixed with 4% paraformaldehyde/PBS for 15 min. Different cell types were identified by immunofluorescence by incubating MGC with primary antibodies against oligodendrocytes (rat anti-MBP, 1:250), early differentiating OPC (mouse anti-GalC, 1:500), microglial cells (rabbit anti-iba-1, 1/125), OPC (rabbit anti-PDGFRα, 1/125), and astrocytes (mouse anti-GFAP, 1/300) overnight at 4°C. Cells were washed and incubated with the appropriated secondary antibody and DAPI (1:1000) for 1h at room temperature. The cells were washed three times with 0.01% PBS-Tween 20 and stored in PBS. Images of whole wells were acquired and analyzed using an ImageXpress Pico Automated Cell Imaging System (Molecular Devices, San Jose, USA) (4 ×, 10 ×, and 20 × objective). The cells were detected by DAPI staining, and positive cells were detected using a fluorescence intensity classification method and summed. Antibodies and stains used are listed in Table 11.

**Table 11. List of antibodies and stains**

| Primary antibody | Reference | Secondary antibody | Reference |
|---|---|---|---|
| MBP | Abcam, ab7349 | AlexaFluor 647 | ThermoFisher A-21247 |
| GalC | Millipore, MAB342 | AlexaFluor 594 | ThermoFisher A-21155 |
| PDGFRα | ThermoFisher, PA5-16571 | DyLight 650 | ThermoFisher SA5-100041 |
| GFAP-Cy3 | Sigma, C9205 | / | / |
| Iba-1 | Wako, 019-19741 | DyLight 650 | ThermoFisher SA5-100041 |

## II.9. IMPACT OF EV-SIPONIMOD ON REMYELINATION IN A FOCAL DEMYELINATED LESION IN VIVO

Focal demyelination was induced in the corpus callosum of 8 – 10 week-old female C57BL/6 mice by stereotaxic injection (ML: 0.8; AP: -0.8; DV: -1.5 mm) of 2 µl of 1% lysolecithin (v/v) using a

Hamilton syringe [54]. Three days after lysolecithin injection, 10 µL of EV-Siponimod (corresponding to 3 x $10^8$ EVs) or controls (PBS, free siponimod, and blank EVs) were injected into the right lateral ventricle (ML: 0.3; AP: -0.3; DV: -2.5 mm). Five days after treatment (8 days after the induction of the lesion), mice were sacrificed by overdose of isoflurane. Brains were post-fixed for 48h in PFA 4% followed by incubation in 20% sucrose for 24h and 30% sucrose for 24h before storage at -80°C in OCT until further analysis.

### II.10. STATISTICAL ANALYSIS

Statistical analysis was performed using PRISM (GraphPad Software, CA, USA). Error bars represent the standard error of the mean (SEM) in all figures. One-way ANOVA followed by Tukey, or Kruskal-Wallis followed by Dunn's test were performed to compare different conditions ($p < 0.05$). The number of experiments and the number of replicates are indicated in the figure legends.

## III. RESULTS

### III.1. TURBULENCE INDUCED HIGH-YIELD PRODUCTION OF SCAP-DERIVED EVS ENCAPSULATING SIPONIMOD

Siponimod is a hydrophobic small molecule that has been described to induce OPC differentiation [155].

Siponimod was encapsulated in EVs using a turbulent flow as it allows the production of large numbers of EVs (Figure 20A) [410]. The addition of siponimod had no impact on the size distribution of EVs as the average diameter of EV-Siponimod and blank EVs was 108.9 ± 0.6 nm and 111.3 ± 6.2 nm, respectively (Figure 20B). However, cells released more EVs when they were cultured with siponimod compared to the control medium (3.9 x $10^4$ EVs/cell versus 2.36 x $10^4$ EVs/cell, respectively). To confirm that the particles in the pool were indeed EVs, samples were first analysed by Western Blotting for positive and negative markers (Figure 20C) [244]. Isolated particles were positive for CD81 (EV positive marker) while they were negative for calnexin (EV negative marker). These results were confirmed by single-particle analysis using nano-flow cytometry of the three tetraspanin EV positive markers CD81, CD63, and CD9 (Figure 20D). EV-Siponimod were positive for CD81, CD63, and CD9 at 24.4%, 3.5%, and 16.4% while 16.5%, 3%, and 8.9% of blank EVs were positive for these markers. Siponimod loading into EVs was quantified by LC-MS and was about 12 ± 3%.

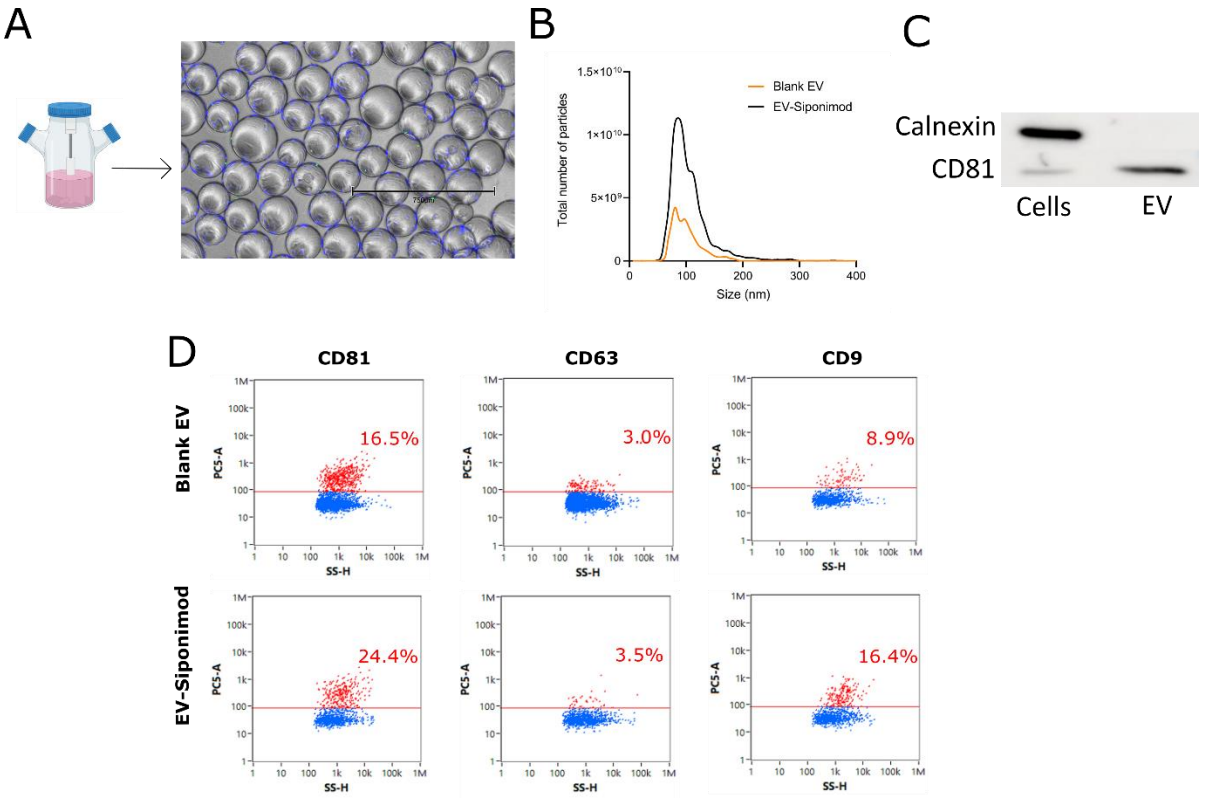



**Figure 20. Turbulences allowed the encapsulation of siponimod into EVs derived from SCAP.** A. Bright-field and epifluorescence (DAPI fluorescence) microscopy of SCAP on microcarriers in a 3D cell culture bioreactor for turbulence EV production. B. Size distribution of isolated EVs for EV-Siponimod and blank EVs. C. Western blot for a negative marker of EVs (calnexin) and a positive marker of EVs (CD81). D. Nano-flow cytometry on EV-siponimod and blank EVs. EVs were fluorescently labeled with PE-conjugated antibodies specific to CD81, CD63, and CD9 and analyzed by NanoFCM. Bivariate dot-plots of fluorescent versus side scatter (SSH) were shown. The percentage of positive particles is provided for each plot.

## III.2. EV-SIPONIMOD REDUCED PRO-INFLAMMATORY CYTOKINE EXPRESSION IN MICROGLIAL CELLS

The impact of EV-Siponimod on the expression of pro-inflammatory cytokines produced by activated microglial cells was studied in LPS-activated BV2 cells. Siponimod concentration was determined in a previous experiment where oligodendrocyte progenitor cells were treated with various concentrations of siponimod (Supplementary data S1). EV-Siponimod significantly reduced interleukin (IL)-6 and iNOS gene expression, while free siponimod decreased interleukin (IL)-1β and IL-6 gene expression, and blank EVs had no effect (Figure 21).

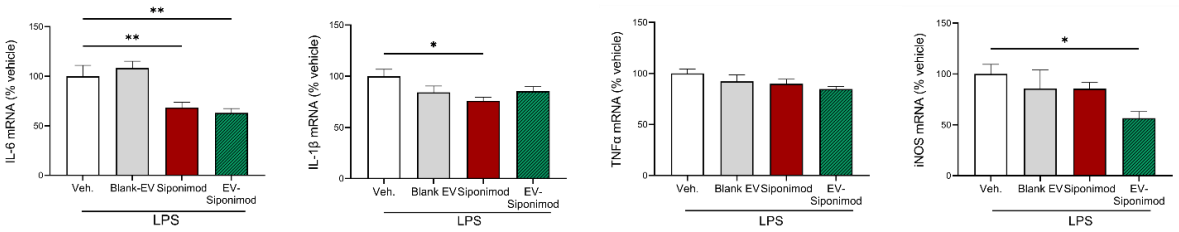



**Figure 21. EV-Siponimod reduced the gene expression of pro-inflammatory cytokines of LPS activated BV2 cells.** BV2 were activated by LPS (100ng/ml) for 1 hour and then treated with either EVs (6.3 x $10^8$ particles) or siponimod (1µM) or EV-Siponimod (6.3 x $10^8$ particles, 1µM). N = 3, n = 4. * $p < 0.05$, ** $p < 0.005$

## III.3. EV-SIPONIMOD SEEMED TO INDUCE MYELINATION

To visualize the efficacy of EV-Siponimod in inducing myelination, we used a microfiber culture system where oligodendrocytes derived from OPC differentiation ensheath inert fibers. OPCs isolated from mixed glial cultures were cultured on Poly-L-lactic acid (PLLA) electrospun fibers. After 2 days, the cells were incubated with EV-Siponimod for 48h followed by 5 days in OPC culture medium without treatment. Confocal microscopy images showed that oligodendrocytes expressed myelin basic protein (MBP) and began to form myelin sheaths on the microfibers (Figure 22). In each condition, no complete remyelination was observed. However, EV-Siponimod and blank EVs resulted in more extensive myelination compared to free siponimod, with EV-siponimod showing a superior myelination compared to blank EVs.

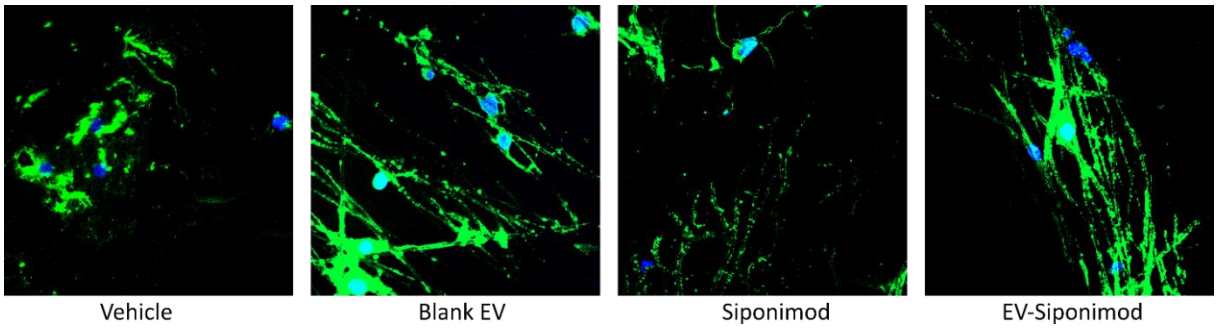



**Figure 22. Confocal images of OPCs on neuron-free fibers.** OPCs were cultured on PLLA electrospun fibers. After 2 days, cells were treated with EVs (6.3 x $10^8$ particles) or siponimod (1µM) or EV-Siponimod (6.3 x $10^8$ particles) for 2 days. Five days after the treatment removal, cells were stained with anti-MBP antibodies and visualized by confocal microscopy.

## III.4. EV-SIPONIMOD INDUCED OPC DIFFERENTIATION IN A PRIMARY MIXED GLIAL CULTURE IN VITRO

The impact of EV-Siponimod on OPC differentiation was also assessed in a primary MGC culture, composed of microglia, astrocytes, oligodendrocytes, and OPCs [54]. This complex culture system has the advantage of assessing the effect on primary OPCs in a more complex cellular environment. EV-Siponimod increased the gene expression of mature oligodendrocyte markers (myelin basic protein MBP, myelin associated glycoprotein MAG, and myelin protein lipid protein PLP) compared to the controls (Figure 23). No significant effect of EVs and siponimod was observed.

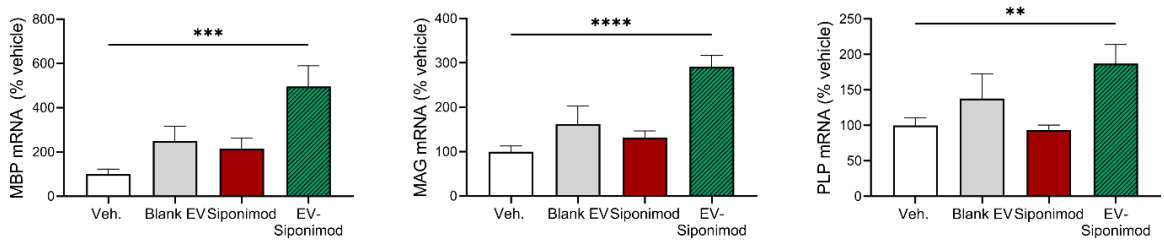



**Figure 23. EV-Siponimod increased the gene expression of mature oligodendrocyte markers in a MGC model.** MGC were treated with EVs (6.3 x $10^8$) or siponimod (1µM) or EV-Siponimod (6.3 x $10^8$, 1µM) for 2 days. Quantification of MBP, MAG, and PLP mRNA by RT-qPCR was performed 5 days after 48h of treatment. N = 3, n = 4. ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

## III.5. EV WERE TAKEN UP BY MIXED GLIAL CELLS OR INTERACTED WITH THEM

To evaluate if the effects observed with EV-Siponimod could be explained by the uptake of EVs by mixed glial cells, EV internalization was assessed on mixed glial cells. EVs were stained with CFDA-SE and then incubated (3 x $10^7$ EV) with cells. After 24 h, cells were stained with anti-MBP (mainly expressed by myelinating oligodendrocytes), anti-GalC (mainly expressed by pre-oligodendrocytes), anti-Iba-1 (expressed by microglial cells), anti-PGFR-α (mainly expressed by oligodendrocyte progenitor cells) and anti-GFAP (expressed by astrocytes). Scanner images showed that all the cell types were able to internalize EVs. More precisely, 86.8% of GalC positive cells were able to uptake EVs as well as 58.6% of GFAP positive cells. Regarding the other cell types, 24.5%, 30.2%, and 26.2% of MBP, Iba-1, and PDGFR-α positive cells, respectively, internalized EVs (Figure 25).

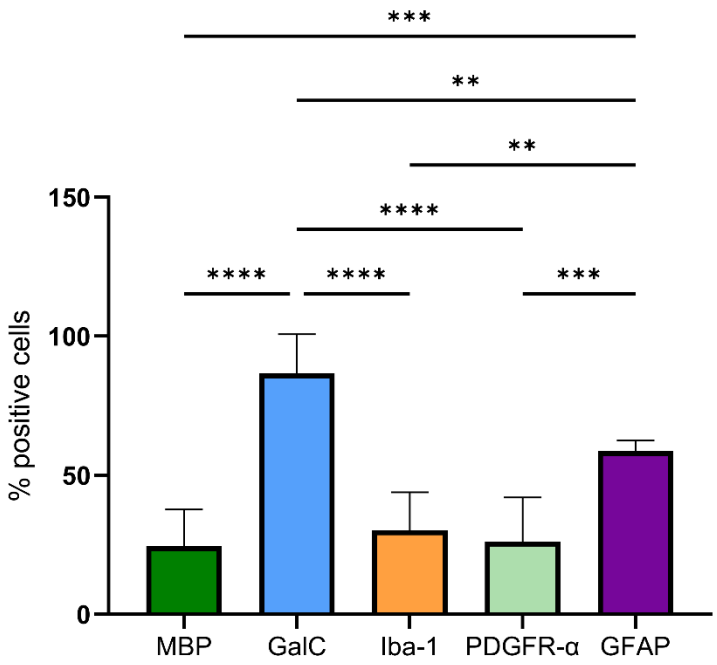



**Figure 25. Mixed glial cells can uptake EVs.** Eight days after isolation, mixed glial cells were incubated with CFDA-SE-EVs for 24 h. Results are expressed as percentage of cells co-stained with CFDA-SE and the marker of interest (Myelin Basic Protein (MBP) for myelinating oligodendrocytes; Galactocerebroside C (GalC) for pre-oligodendrocytes; Ionized calcium-Binding Adapter molecule 1 (iba-1) for microglial cells; Platelet-derived growth Factor Receptor α (PDGFR-α) for OPC and Glial Fibrillary Acidic Protein (GFAP) for astrocytes). Analysis was done on whole wells using IX-PICO software. N = 3, n = 5. ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

## III.6. IMPACT OF EV-SIPONIMOD ON REMYELINATION IN A FOCAL DEMYELINATED LESION IN VIVO

This experiment is currently ongoing.

## IV. DISCUSSION

Currently, no available treatment for MS stimulates the remyelination process, one reason being the difficulty for drugs to reach the central nervous system. This hurdle could be overcome by using nanocarriers. During the last decade, the interest in EVs, as therapeutic tools or drug delivery vehicles has constantly increased, as a large variety of drugs could be encapsulated into EVs, they possess functional activity including immunomodulatory properties, and have a homing to inflamed tissue [214]. In a previous study, we showed that SCAP exposure to a pro-inflammatory stimulus impacted their EV composition and that stimulated SCAP-EVs had a slight effect on the production of pro-inflammatory cytokines in microglial cells [407]. Recently, siponimod, approved immunotherapy for relapsing and progressive MS treatment, has been associated with protection against demyelination and promotion of remyelination due to a direct effect on OPCs and oligodendrocytes [155]. The objective here was to combine the SCAP-derived EVs with the pro-remyelination effect of siponimod to resolve inflammation and promote remyelination. For the first time, we demonstrated that it was possible to encapsulate siponimod into SCAP-EVs using turbuloporation. We then showed that EV-Siponimod decreased pro-inflammatory cytokine expression in microglia cells and increased OPC differentiation *in vitro*.

SCAP-derived EVs loaded with siponimod were produced using a scalable and high-yield approach enhancing the translational potential of our system [410]. Specifically, the turbulence approach used in this study enabled a significant increase in EV production yield, allowing the production of a large amount of EVs (80-fold more) within a short period (4 hours vs 72h) compared to the FBS starvation in flasks [407]. Advantageously, this approach allowed to combine in one step EV production and the encapsulation of siponimod in EVs at a high yield. This encapsulation method is reproducible and has been described in the literature for the encapsulation of other compounds in EVs from different sources [411]. EVs were characterized by the presence of positive EV markers and the absence of negative markers. CD81 was the most prevalent marker on both blank EVs and EV-Siponimod, with CD9 and CD63 being the second and the least presented tetraspanins, respectively. While the presence of CD63 in only 3% of the EVs may seem unexpected, these results are consistent with other results described in the literature when using NanoFCM, although the tetraspanin profile can vary between EV sources [412, 413]. Furthermore, the lack of CD63 in EVs isolated by size or density has been reported in a few papers [414].

As neuroinflammation is a major component of MS, the efficacy of EV-Siponimod in reducing the expression of pro-inflammatory cytokines (i.e. IL-6, IL-1β, TNF-α, and iNOS) was assessed on LPS-treated microglia cells. EV-Siponimod significantly decreased IL-6 and iNOS expression,

whereas blank EVs did not show any effect. While stimulation by pro-inflammatory cytokines and mechanical shear are both stressful for the cells, they impacted differently SCAP-EVs, as EVs produced under a pro-inflammatory stimulus induced a reduction of pro-inflammatory cytokine gene expression, while under turbulence they did not. This is in correlation with various studies demonstrating that EVs produced under biomechanical force possess different cargo according to the shear stress applied [415]. Siponimod alone reduced the expression of IL-6 and IL-1β, presumably through its interaction with $S1P_1$ receptor, as described in a few studies [209, 416].

As S1P receptors have been associated with protection against demyelination and the promotion of remyelination in both *in vitro* and *in vivo* studies [155, 187, 190], the efficacy of EV-Siponimod to induce myelination was first assessed using a microfiber culture system allowing oligodendrocytes to ensheathe poly-L-lactic acid fibers [417]. This model provides biophysical cues to oligodendrocytes mimicking axons, as myelinating cells do not require molecular instruction from axons but need physical support for myelination [409]. Qualitatively, the formation of MBP+ myelin-like extensions around nanofibers appeared to be at a more advanced stage with EV-Siponimod, as well as with blank EVs, compared to the free drug, highlighting the ability of EV-Siponimod to induce myelination. It is important to note that oligodendrocyte differentiation and myelination are two distinct stages of OPC development, each with unique mechanisms [418]. While the efficacy of siponimod in promoting OPC differentiation has been demonstrated in previous studies, its direct effect on myelination has not been assessed, as far as we know.

The efficacy of EV-Siponimod to promote OPC differentiation was evaluated in mixed glial cultures, as interactions between CNS-resident cells are essential during this process [419]. The expression of mature oligodendrocyte markers was significantly increased with EV-Siponimod treatment, whereas free siponimod had no effect. These results validated our hypothesis, that the combination of siponimod and SCAP-EVs could promote remyelination to a greater extent than the free drug. This superior effect of EV-siponimod may be explained by several hypotheses. One involves the impact of siponimod incubation with SCAP during EV production, as it has been described that medium composition may impact EV content [407]. Furthermore, a study conducted on mouse SCAP demonstrated the presence of S1P receptor $S1P_1$ at the plasma membrane of these cells [420], but the involvement of S1P pathways in SCAP activation is still under investigation [421]. Another explanation could be a synergistic or additive effect of siponimod and blank EVs, which results in an improvement of the effects of siponimod and blank EVs, separately, on OPC differentiation.

The uptake of EVs into their target cells or their interaction with the target cells are an essential prerequisite for their therapeutic action. We evaluated EV uptake by mixed glial cells and found

that EVs were internalized by astrocytes, microglia, OPCs, and oligodendrocytes or at least were able to interact with these cells. Almost all GalC-positive cells, which correspond to pre-oligodendrocytes, were able to capture or interact with EVs. The precise mechanism of action of siponimod on OPCs or oligodendrocytes has not been fully described. Evidence suggests that S1P receptor subtype $S1P_5$ may be involved. However, recent studies demonstrated the effects of fingolimod on T-cells [422, 423] and of siponimod on astrocytes [424], independently of S1P pathway, highlighting a direct effect of these two compounds inside the cells. We could thus hypothesize that siponimod may also act on OPCs and oligodendrocytes through direct effect within the cells, rather than solely through its interaction with $S1P_5$. As EV-Siponimod promoted OPC differentiation to a greater extent than siponimod, we could also hypothesize that EV-Siponimod may enable a more effective delivery of siponimod within the cells. EVs were also taken up by more than half of the astrocyte population, which are the most represented cell types in mixed glial culture. Although there is no consensus on EV uptake, a few processes have been described: clathrin-dependent endocytosis, caveolae-dependent endocytosis, lipid-raft mediated endocytosis, macropinocytosis and in some cases, phagocytosis [425]. Various studies reported that astrocytes, as well as microglia, could uptake EVs via micropinocytosis and phagocytosis [426], leading to the release of EV content, including siponimod, into the extracellular space. Siponimod could thus act on S1P receptors present on the cell membrane.

However, several important considerations must be considered before interpretating this work. First, the isolation of EVs using TFF retains large proteins, which could aggregate and be mistakenly quantified as EVs, potentially affecting dosing accuracy [273]. Additionally, these proteins may associate with siponimod, leading to the formation of non-vesicular extracellular particles. Second, siponimod is not water-soluble. Although it was dissolved in ethanol before use, addition of siponimod into the cell medium during EV production might result in its aggregation.

## V. CONCLUSION

Our aim was to use SCAP-derived EVs as biological nanocarriers and combine them with the pro-remyelination effect of siponimod to resolve inflammation and promote remyelination. We thus encapsulated siponimod into SCAP-EVs using a scalable and GMP-compatible method based on mechanical shear stress. Our findings demonstrated that EV-Siponimod decreased pro-inflammatory cytokine expression in microglial cells. We also observed an increase in the gene expression of mature oligodendrocyte markers, indicating the efficacy of EV-Siponimod in inducing OPC differentiation. In addition, EV-Siponimod enhanced the effect of the free drug on OPC differentiation. We thus conclude that EVs are promising nanocarriers for siponimod, in the scope of demyelinating diseases such as multiple sclerosis.

## Supplementary data

### Supplementary data S1. Choice of siponimod dose

To determine which concentration of siponimod and thus which EV concentration will be used for further experiments *in vitro*, different concentrations of siponimod were assessed (10, 100 and 1000 nM) on oligodendrocyte progenitor cells. To do so, cells were treated with siponimod for 48h. After treatment removal, cells were cultured for 5 days in DMEM medium before RNA extraction.

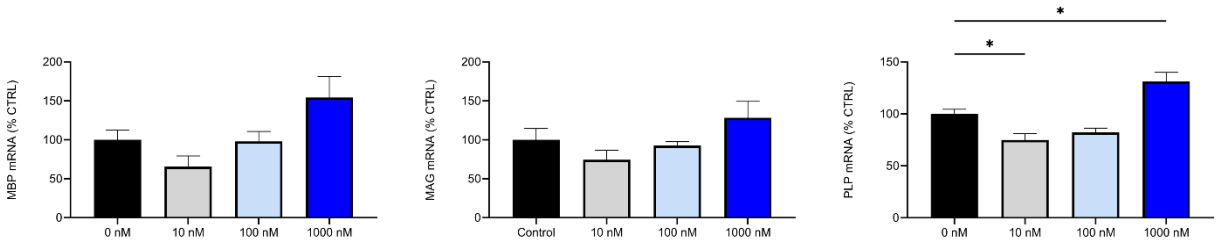



**Figure S1. Siponimod at different concentrations (10nM, 100nM and 1µM) on oligodendrocyte progenitor cells for 48h**. OPCs were treated with siponimod at 10 nM, 100 nM, or 1µM for 48h. Quantification of MBP mRNA by RT-qPCR was performed 5 days after 48h of treatment. N = 3, n = 4.

# PART IV
# GENERAL DISCUSSION

The aim of my thesis was to use EVs as a drug delivery system for the central nervous system, targeting neuroinflammation and enhancing remyelination in the context of multiple sclerosis.

The experimental work was divided into two complementary chapters. First, we isolated EVs from SCAP, evaluated the impact of a pro-inflammatory stimulus on their composition, and assessed the ability of both activated and non-activated SCAP-EVs to reduce pro-inflammatory cytokine expression in microglia cells. Second, we encapsulated siponimod, a pro-remyelinating compound, into EVs and evaluated the efficacy of the resulting nanomedicines in reducing pro-inflammatory cytokines expression in microglia cells and enhancing OPC differentiation *in vitro*.

Although the results have been discussed in previous chapters, further concepts will be discussed in this section. First, a summary of the main achievements of this thesis will be presented. Second, a discussion around some of the questions raised during this PhD will be proposed, followed by my personal opinion on EVs.

# I. MAIN ACHIEVEMENTS

## I.1. PRODUCTION AND ISOLATION OF EVS DERIVED FROM ACTIVATED AND NON-ACTIVATED SCAP

Before exploring whether EVs produced by SCAP were affected by a pro-inflammatory stimulus and contributed, at least partially, to the immunomodulation properties observed with their parent cells, we isolated EVs from SCAP-conditioned medium. To achieve this, we activated cells with TNFα and IFN-γ to mimic a pro-inflammatory environment and produce EVs for 48h in a serum-free medium. As this work was the first in our laboratory dedicated to EVs, we optimized an isolation protocol based on ultrafiltration followed by size exclusion chromatography, allowing us to collect EVs with minimal contaminants. Since it is not possible to separate exosomes from microvesicles based on size alone, the collected EVs represented a mixture of these two types of vesicles.

## I.2. THE COMPOSITION OF SCAP-EV CARGO WAS IMPACTED BY A PRO-INFLAMMATORY STIMULUS

We evaluated the impact of a pro-inflammatory stimulus on the miRNA and lipid content of SCAP-EVs, as both are major components of EVs, and evidence demonstrated the impact of various stimuli on them. For the first time, we showed that miRNA content of SCAP-EVs was modified in response to a pro-inflammatory stimulus, but not their lipid composition.

### I.3. ACTIVATED SCAP-EVS PARTIALLY RECAPITULATED THE IMMUNOMODULATORY PROPERTIES OF THEIR PARENT CELLS

Given the immunomodulatory properties of SCAP, we assessed the capacity of both activated and non-activated EVs to decrease pro-inflammatory cytokine expression in microglia cells and spinal cord slices. Under non-inflammatory conditions, EVs derived from activated and non-activated SCAP did not exhibit pro-inflammatory properties. Additionally, we observed a slight decrease in pro-inflammatory cytokine expression when cells were treated with activated EVs, demonstrating the impact of culture conditions (specifically, a pro-inflammatory environment) on the properties of EVs.

### I.4. ENCAPSULATION OF SIPONIMOD INTO SCAP-EV DURING EV PRODUCTION

In chapter 2 of part III, we used, for the first time, SCAP-EVs as a drug delivery system. Thus, we successfully encapsulated siponimod into SCAP-EVs using an innovative turbulence-based technique. Siponimod was chosen due to its recently highlighted pro-remyelinating properties.

### I.5. SCAP-EV ENCAPSULATING SIPONIMOD COULD DECREASE PRO-INFLAMMATORY CYTOKINE EXPRESSION AND ENHANCE OPC DIFFERENTIATION *IN VITRO*

The capacity of EV-Siponimod to induce myelination and OPC differentiation was assessed using OPC cultured on neuron-free fibers and a mixed glial cells culture. We showed that treatment with EV-Siponimod stimulated the myelination process. While EV-Siponimod were able to significantly increase the expression of MBP, MAG, and PLP in the MGC culture, these effects were not recapitulated with blank EVs or free siponimod, highlighting the potential therapeutic benefit of EV-Siponimod. Similar results were observed on microglia cells, where we evaluated the ability of EV-Siponimod to decrease pro-inflammatory cytokine expression. However, in this model, the effects were mainly mediated by siponimod, although blank-EVs demonstrated a slight effect.

# II. GENERAL DISCUSSION

## II.1. WHAT IS THE INFLUENCE OF OUR EXPERIMENTAL SETTING ON OUR RESULTS?

### II.1.1. How can EV production and isolation methods affect the results?

It is now well established that culture conditions affect EV composition, although the mechanisms behind it have not yet been described [427]. Two production methods have been used in this project. The first involved serum starvation, while the second was based on turbulence.

While it has been demonstrated that MSC retain their characteristics, such as morphology, proliferation kinetic, and differentiation potential after long-term starvation [428], serum starvation has been reported to modify EV composition [429]. However, this method increases the yield of EVs produced by a single cell and avoids contamination with EVs and proteins from fetal bovine serum, which could lead to the misattribution of biological activity to SCAP-EVs [430].

In chapter 2 of part III, we combined mechanical shear stress with serum starvation to increase the yield of EVs produced per cell and to encapsulate siponimod into EVs. This method has also the advantages of being scalable and GMP-compliant [431]. However, mechanical shear stress may impact cells and consequently, their EVs. Researchers have demonstrated that turbulent shear stress on MSC leads to cell quiescence [432]. Comparing our results with these studies is challenging because the impact of shear stress on cells depends on the shear rate. The shear rates reported in the literature for studying the effect of turbulence on MSC were similar to those we used in the cell expansion phase but not during the EV production phase.

We did not assess the impact of turbulence on SCAP either during the cell expansion phase or during the EV production phase. Therefore, we do not know the precise impact of turbulence on our EVs. When comparing the results obtained on BV2 cells with non-activated SCAP-EVs produced under serum starvation to those obtained with SCAP-EVs produced in stirred-tank bioreactors, we observed that EVs produced with turbulence decreased pro-inflammatory cytokine expression, an effect not seen with non-activated SCAP-EVs. This suggests a positive effect of turbulence on EV bioactivity. However, it is important to note that the isolation method differed in the two cases, complicating the comparison, since the isolation method also impacts EV biological activity [433]. To address this, an experiment has been performed to compare the effects of two isolation methods on the biological activity of EVs produced under turbulence (same methods as in chapter 2 of part III). SCAP-EVs were either isolated with ultrafiltration followed by SEC (as in chapter 1) or with TFF (as in chapter 2). No differences in pro-inflammatory cytokine

expression in LPS-activated BV2 cells were observed, suggesting that turbulences positively affect EV biological activity (Figure 26).

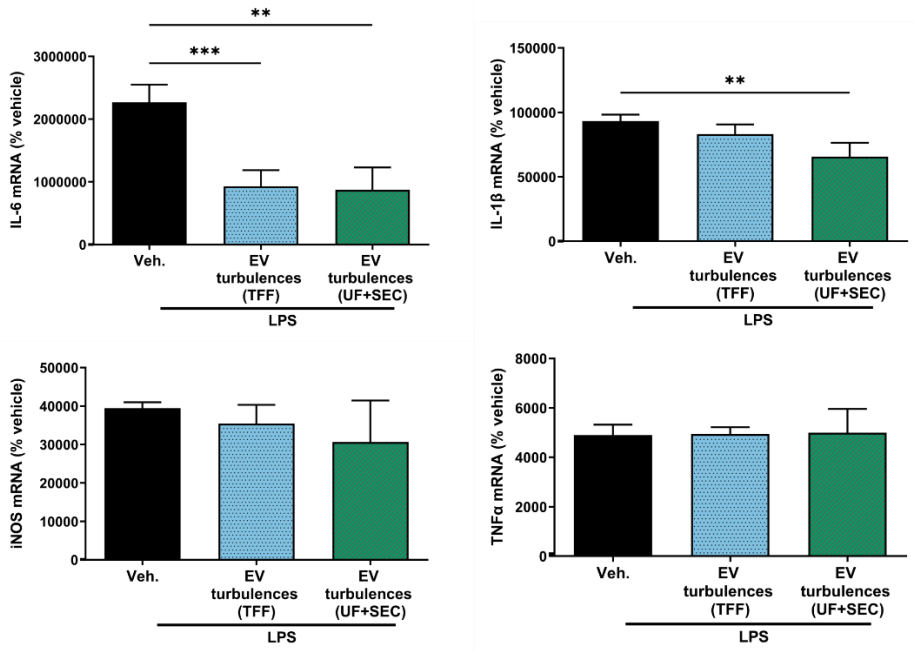



**Figure 26. Impact of isolation methods (TFF or ultrafiltration followed by SEC) on SCAP-EV ability to reduce pro-inflammatory cytokine expression in microglia cells.** BV2 were activated by LPS (100ng/ml) for 1 hour and then treated with either EVs isolated with TFF (6.3 x $10^8$ particles) or EVs isolated with ultrafiltration followed by SEC (6.3 x $10^8$ particles). TFF: tangential flow filtration; UF: ultrafiltration; SEC: size exclusion chromatography. N = 1, n = 4. ** $p < 0.005$, *** $p < 0.001$

As explained in the introduction of this thesis, the common isolation methods described in the literature do not allow the separation of exosomes and microvesicles due to their common markers and overlapping sizes. Therefore, our EVs were a mixture of these two populations. Furthermore, some researchers have inaccurately used the term "exosomes" while their samples also contain microvesicles, leading to the misconception that only exosomes have biological activity [239]. However, it is now well established that exosomes and microvesicles have different biogenesis pathways, and, therefore, likely have different cargo, which may affect their functional activity [250]. Consequently, using a mix of exosomes and microvesicles could impact functional assays, although it is unclear whether this impact is positive or negative.

### II.1.2. Are RP89 the best source of EVs?

The cells used during my thesis were RP89 cells. RP89 is a SCAP cell line derived from the mandibular third molar apical papillae from a single donor, coexpressing mesenchymal stem cell markers CD73, CD90, and CD105 [369]. The expression of these molecular markers is considered

the minimal criterion for identifying true mesenchymal stem cells [434]. Since RP89 cells are from a single donor, we can assume that cell heterogeneity, and consequently the heterogeneity of their EVs is reduced.

Although the maintenance of MSC marker expression through 20 passages has been demonstrated at both gene and protein levels in RP89 cells [369], some studies have shown that EVs isolated from cells with higher cell passages were less bioactive compared to those from cells with lower passages [435]. Unfortunately, it was not possible to isolate EVs from cells from the same passage consistently. Therefore, we decided to collect EVs produced by cells between passages 6 and 9. This range, the lowest that allowed us to expand cells sufficiently to isolate EVs from a large number of cells, was chosen to minimize variability in our results.

### II.1.3. Are EVs and siponimod doses relevant?

Regarding siponimod, we selected a dose based on reports of the effects of fingolimod in vitro. Jung et al. showed that low nanomolar doses of fingolimod (10 nM) promoted rat OPC differentiation, while higher doses (> 1 μM) inhibited the differentiation of OPC into mature oligodendrocytes, highlighting a dose-dependent activity [154]. This effect may be linked to S1P receptor internalization following fingolimod recognition, where fingolimod binding initially induces downregulation of $S1P_5$. To compensate for this mechanism, $S1P_1$ is then upregulated [190]. Such effects have not been reported with siponimod due to a lack of studies, as most studies have assessed the effect of siponimod on remyelination *ex vivo* or *in vivo,* but not *in vitro* [155, 209]. While fingolimod and siponimod have similar mechanisms of action, we can assume a similar dose-dependent response for siponimod. Therefore, we assessed the effect of various siponimod concentrations on OPC differentiation and observed a higher differentiation marker expression at a higher dose (1 μM) compared to a low dose (10 nM). The difference with fingolimod may be explained by the timing of the experiment, as receptor internalization is a dynamic process.

Regarding EVs, analysis of the literature highlights a wide range of EV numbers reported in publications (from $5 \times 10^5$ to $10^{10}$ EVs) [400, 436-438], and a large number of studies reports administered dosages as a protein concentration [287, 439]. Additionally, establishing a correlation between EV dose and effect is challenging, as the relationship appears to be non-linear. Therefore, in chapter 1 of part III, we treated BV2 and spinal cord slices with the highest amount of EVs feasible, corresponding to $5 \times 10^9$ EVs. In chapter 2, the EV dose was based on the siponimod dose and encapsulation efficiency (1 μM, $6.3 \times 10^8$ EVs). To ensure this quantity was not deleterious to the OPCs, we assessed first the effect of different doses of SCAP-EVs on the expression of

mature oligodendrocyte markers (MBP, MAG, and PLP). We observed an increase in marker expression when OPCs were treated with up to 5 x $10^8$ EVs, while oligodendrocyte marker expression drastically dropped when OPCs were treated with more than $10^9$ EVs. These results confirm that the EV dose used in this chapter (6.3 x $10^8$ EVs) had no negative impact on OPC differentiation (Figure 27). Considering these findings, it would have been interesting to assess different EV concentrations on LPS-stimulated BV2 cells. These results are consistent with what has been described by Hagey et al., in which low doses of EVs from various source produce unique transcriptional responses, while high doses induce lysosomal activity [440]. Thus, the underlying mechanism remains unknown but may involve miRNAs [441].

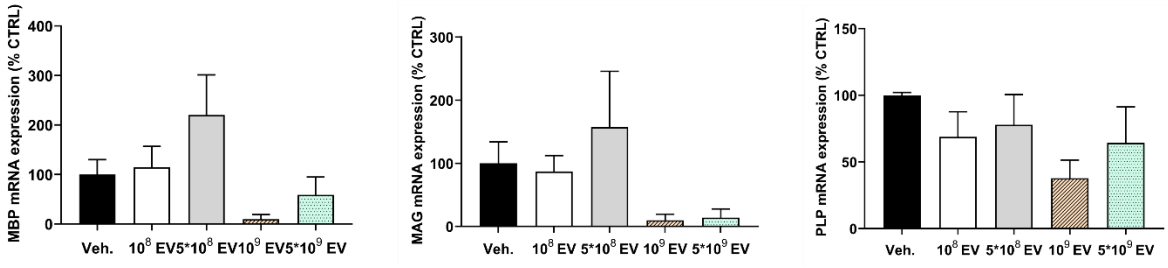



**Figure 27. EVs at different concentrations ($10^8$, 5 x $10^8$, $10^9$, and 5 x $10^9$) on mixed glial cells**. MGC were treated with EVs for 2 days. Quantification of MBP, MAG, and PLP mRNA by RT-qPCR was performed 5 days after 48h of treatment. N = 1, n = 4.

### II.1.4. Can the timing induce different effects?

MSC-EVs are more and more studied *in vitro* for their immunomodulatory effects. However, the timing of these studies varies widely: 2h, 4h, 8h, 24h, 48h, 72h, or 4 days [287, 401, 438]. Sometimes, a lower dose is used for a longer time and vice versa [400, 438]. This variability makes it difficult to choose the appropriate timing based on the selected dose. Furthermore, the precise mechanism of action has not been described, so we cannot rely on this parameter to select a timing for our experiments. Therefore, in our first study, we decided to test two timings: 8h, because it has been used in other LPS-stimulated BV2 cell experiments in our laboratory [376] and 24h to evaluate the impact of a longer experiment duration on pro-inflammatory cytokine expression. Given this arbitrary selection, we could assume that different timings might yield different results. Unfortunately, due to the limited EV yield, it was not possible to assess more timings or to combine different timings with different doses.

In our second study, the timing was based on: 1) previous results obtained on microglia cells and 2) siponimod. Since the internalization of S1P receptors is a dynamic process, the timing used in the experiments may affect the results [154]. In a preliminary experiment, we compared the effect

of 1 µM siponimod on OPC differentiation for 48h and 72h. We observed a higher increase in the expression of oligodendrocyte differentiation marker MBP after 48h compared to 72h (Figure 28). Therefore, we decided to treat OPCs for 48h with our formulation. However, it could have been interesting to treat BV2 and OPCs for the same duration to obtain more translational results.

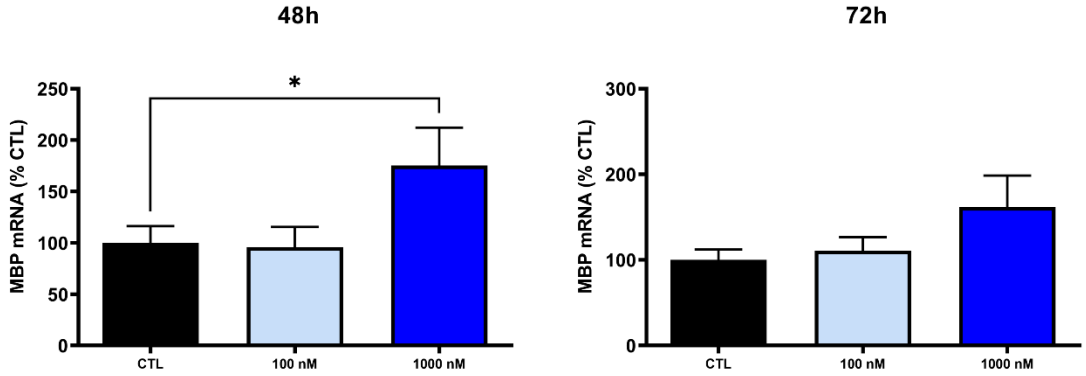



**Figure 28. Siponimod at different concentrations (100nM and 1µM) on oligodendrocyte progenitor cells for 48h or 72h**. OPCs were treated with siponimod for 48h or 72h. Quantification of MBP mRNA by RT-qPCR was performed 5 days after 48h or 72h of treatment. N = 1, n = 4.

### II.1.5. Can the storage conditions have an impact on the results?

Although some studies recommend processing EVs from fresh samples, it is not always practical. Therefore, a key question when working with EVs is how to store the samples. Different storage conditions have been described: +4°C for up to 7 days and freezing at -20°C or -80°C, with or without cryoprotectants such as trehalose for longer storage durations [442]. Evidence suggests that storage significantly impacts EV properties, including concentration, size, integrity, molecular cargo, surface composition, and function [443]. An interesting study comparing various storage strategies demonstrated that albumin-supplemented PBS, in addition to trehalose, improved EV recovery rates (particle concentration, diameter, protein, and RNA amount) compared to PBS alone for long-term storage [444]. Higher recovery rates were also observed in less pure EV preparations. Freeze-drying has also been considered and appears to be a convenient way to store EVs [425].

In our first study, EVs were used directly after their isolation, minimizing the impact of storage conditions. However, in the next study, EVs were frozen in PBS due to technical imperatives (EVs produced in Paris). EVs were aliquoted to avoid successive freeze-thaw cycles. We decided not to add a cryoprotectant as EVs were isolated with tangential-flow filtration, a method known to co-isolate protein contaminants that could have a cryoprotective effect [445].

### II.1.6. Why did we choose the lysolecithin model?

Since we demonstrated the ability of EV-Siponimod to increase the expression of oligodendrocyte differentiation markers, the aim of the preclinical study was to investigate remyelination following treatment with EV-Siponimod. Therefore, the lysolecithin-induced focal lesion model appeared to be more appropriate than experimental autoimmune encephalomyelitis [110]. While the oral cuprizone model is also appropriate for studying remyelination, we opted for intracerebroventricular injection to eliminate the impact of the administration route and obtain a first proof-of-concept. Of course, later on, as this administration is not compatible with the chronic treatment required for models like the cuprizone model, a less invasive administration route will be selected, most likely chosen based on a biodistribution study.

## II.2. ENCAPSULATION OF SIPONIMOD INTO EVS, AS SIMPLE AS IT LOOKS LIKE?

In the last decade, the interest in EVs as drug delivery systems has increased considerably, with various encapsulation methods reported, as presented in Table 6 of the introduction. However, the loading efficiency of lipophilic drugs remains largely unexplored, as very few studies report the amount of lipophilic cargo loaded in EVs [309, 310, 446, 447]. Therefore, we assessed different methods such as incubation, electroporation, sonication, saponin treatment, or fusion with liposomes, but these methods yielded inconclusive results. Other PhD students in the lab encountered similar challenges when trying to encapsulate lipids, hydrophilic drugs, or miRNA into EVs. Given the complexity of loading a drug into EVs, we evaluated a new method developed by collaborators in Paris, using turbulences to stimulate the production of EVs and at the same time encapsulate siponimod. This method also has the advantage of isolating high yields of EVs, an important criterion for assessing their functional activity *in vivo* [411]. The mechanism behind the encapsulation of siponimod into EVs using turbulence has not been described.

Two hypotheses could be stated:

1) Pre-EV production hypothesis: siponimod might be captured by SCAP through an endocytic pathway, leading to the formation of multivesicular bodies that contain vesicles encapsulating siponimod. This mechanism may involve the binding of siponimod to its receptor, as some studies have suggested the involvement of S1P receptor activation in multivesicular body formation [252].
2) Post-EV production hypothesis: turbulence might cause temporary permeabilization of the EV membrane, allowing siponimod to enter. In that case, the risk of losing EV content exists. This hypothesis is supported by the fact that shear stress can induce transient pores in lipid bilayers [448].

As with every loading method, turbulence has its drawbacks. This loading method's main inconvenience is the risk of creating EV-like nanoparticles termed extracellular blebs. These particles are created by inducing cell membrane tension with shear stress, which over time results in elongation of the membrane. When the maximum thermodynamic stability is reached, the membrane tears and fragments into free linear pieces that rapidly and spontaneously self-assemble into spheres to form EV-like nanovesicles [449]. It has been demonstrated that these nanovesicles have similar size, zeta potential, morphology, key protein markers, and lipidomic profiles to natural EVs but are no longer true EVs [450]. Their functional activity has also been reported in some studies [451]. However, regarding the many obstacles encountered with natural EVs as drug delivery systems such as considerable complexity, low isolation yield, and inefficient drug loading, these EV-like nanoparticles may not be a drawback of turbulence but in fact, a promising solution to overcome certain limitations of natural EVs.

In chapter 2 of part III, we observed that EV-Siponimod significantly increased the gene expression of mature oligodendrocyte markers (myelin basic protein MBP, myelin associated glycoprotein MAG, and myelin protein lipid protein PLP) compared to either free siponimod or blank EVs. To decipher whether this effect was due to encapsulation or to an additive effect of siponimod and EVs, independently of encapsulation, we evaluated the impact of co-administration of blank EVs and siponimod on mixed glial cells. The effects observed in chapter 2 of part III were recapitulated with blank EVs, siponimod, and EV-Siponimod. The co-administration of EVs with siponimod also increased the expression of MBP and PLP but had no impact on MAG expression (Figure 29).

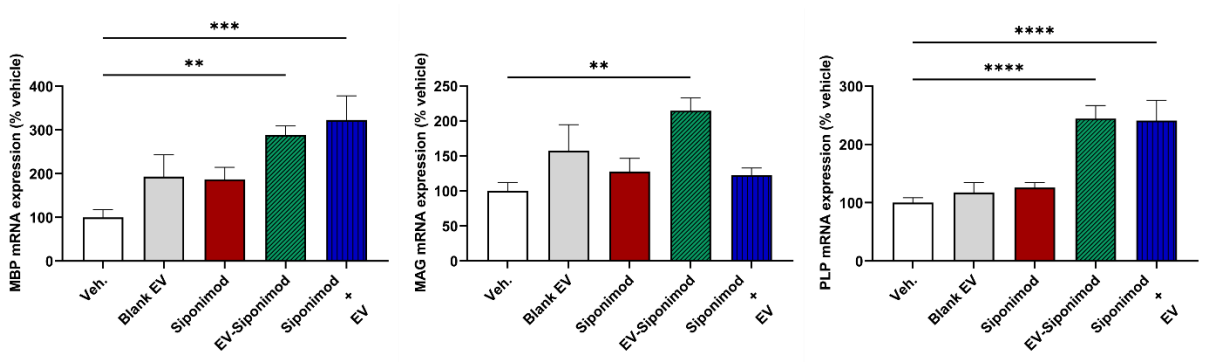



**Figure 29. Impact of blank EVs, siponimod, EV-Siponimod, or EVs co-administrated with siponimod on the expression of oligodendrocyte differentiation markers by mixed glial cells**. Mixed glial cells were treated with blank EVs (6.3 x $10^8$ particles) or siponimod (1µM) or EV-Siponimod (6.3 x $10^8$ particles) or blank EVs + siponimod (6.3 x $10^8$ particles and 1µM, respectively) for 2 days. Quantification of MBP, MAG and PLP mRNA by RT-qPCR were made 5 days after the treatment removal. N = 3, n = 4. ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

Considering these results, an additive or synergistic effect of siponimod and EVs could be responsible for the observed effects with EV-Siponimod, independent of the encapsulation of siponimod into EVs. However, the effects of EV-Siponimod on MAG expression were not replicated by the co-incubation of EVs and siponimod. Furthermore, we cannot rule out the possibility that an encapsulation occurs when siponimod and EVs are co-incubated, as incubation is a reported method for drug encapsulation into EVs.

Additionally, other parameters should also be considered before concluding that encapsulation is not necessary. One such parameter is EV yield. Adding siponimod to the cell culture medium led to an increase in EV yield, with 2.36 x $10^4$ particles/cell for blank EVs and 3.9 x $10^4$ particles/cell for EV-Siponimod. Another parameter, not assessed in this thesis, is biodistribution. Homing of EVs to inflammation sites has been reported, suggesting that EV-Siponimod could target siponimod to demyelinated lesions. Furthermore, the effects of co-incubation of blank EVs and free siponimod on LPS-stimulated microglia cells were not evaluated.

## II.3. EVs AS NANOMEDICINE, A UTOPIA?

### II.3.1. How far are we from clinics?

In this PhD project, we demonstrated the successful encapsulation of siponimod into EVs using a scalable method, achieving higher loading efficiency compared to other techniques such as sonication, extrusion, and freeze-thaw cycles. We also showed that the expression of oligodendrocyte differentiation markers was increased with EV-Siponimod, but not with free siponimod or blank EVs, although it remains uncertain whether encapsulation was solely responsible for these effects. Various studies have also reported the successful encapsulation of nucleic acids, small molecules, or proteins into EVs and demonstrated their therapeutic potential. Therefore, it appears to be reasonable to ask: could we envisage having commercialized EVs as nanomedicines in the near future?

Recently, several companies have been created with the objective of commercializing EVs loaded with antisense oligonucleotides (e.g. Codiak Biosciences), miRNAs (e.g. Avalon Globocare Corp.), siRNAs (e.g. Aruna Bio, Inc.) and mRNAs (Capricor Therapeutics). However, no EV products have been approved by the FDA to date. For a long time, there was no clear regulatory framework, leading to the publication of a public safety notification on exosome products by the FDA in 2019 [452]. Additionally, the EMA has classified EV-based therapeutic products as biological medicinal products, subjecting them to the same regulatory requirements [453].

The commercialization of EV-based products has also been hampered by various limitations, including the low scalability of production and isolation methods used in research, the time-consuming nature of these technologies, and the high level of heterogeneity of EVs, which complicates reproducible large-scale manufacturing [454]. Furthermore, due to these limitations, EV-based therapies may be expensive. As presented in the introduction of this manuscript, various methods have been developed to enhance EV yield, such as production in bioreactors or the use of external signals. While the method used to produce EVs encapsulating siponimod is scalable, the significant loss of siponimod during production renders it unsuitable for large-scale manufacturing.

Therefore, while significant progress has been made in the development of EVs, several challenges remain before their commercialization as nanomedicines, hampering their commercial availability in the coming years.

### II.3.2. Are there real advantages to use EVs compared to synthetic nanocarriers?

While the results were promising, we faced several difficulties, such as low encapsulation efficiency, low EV yield, challenges with reproducibility, and difficulties in purifying EVs. Additionally, one of the main advantages of using EVs as nanomedicines is their functional activity. However, the results observed in chapter 1 of part III did not demonstrate a clear and significant effect of EVs in reducing pro-inflammatory cytokine expression in microglia cells. In chapter 2, although EV-Siponimod were able to increase the expression of oligodendrocyte differentiation markers in mixed glial cells, blank EVs were unable to recapitulate these effects. This raises questions about whether EVs are concretely more advantageous than synthetic nanocarriers.

The closest lipid-based nanomedicine to EVs is liposome. Like EVs, liposomes consist of a lipid bilayer enclosing an aqueous core, allowing hydrophobic drugs to integrate into the bilayer and hydrophilic drugs to be incorporated into the core, with an encapsulation efficiency of up to 80% [455]. Extensive research since their discovery in the 1960s has led to the commercialization of FDA-approved liposome-based therapeutics [456]. However, liposomes also have drawbacks such as rapid clearance from the bloodstream, fast release of the drug, off-target accumulation, and the potential to trigger an innate immune response [457]. Compared to liposomes, EVs have several advantages. Their biological origins make them biocompatible, and they can target specific tissues due to their homing properties. Moreover, EVs are more complex, containing nucleic acid and proteins inside the vesicle or on the membrane. However, EVs also have significant drawbacks, including low loading efficiency and limited control over their composition, leading to issues with

reproducibility [214]. Despite theoretical comparisons between EVs and liposomes, the number of studies providing head-to-head comparisons is still limited [456]. During my PhD, no direct comparison between these two nanocarriers was performed, as it is more challenging than it appears due to differences in drug loading and particle concentration.

Recently, a promising idea has emerged: combining the best of both worlds to create EV-like nanoparticles. This could be achieved by generating: 1) hybrid vesicles composed of both EV and liposomal components, 2) bioinspired synthetic vesicles that incorporate characteristics of EVs, or 3) EV-like particles using a cell shearing procedure such as extrusion [456].

Hybrids vesicles are produced through the PEG-mediated fusion of EVs and liposomes. This approach allows for surface modification and drug loading, while preserving the integrity and biological activity of EVs. Although liposomes can be easily produced and customized for specific drug-delivery challenges, the separation process tends to be slow and complex. Given the potential immunogenicity of liposomes, which is influenced by their phospholipid composition, it is important to mimic the natural lipid-composition of EVs to minimize immunogenic responses [458].

Bioinspired synthetic vesicles consist of the creation of fully artificial EV-biomimetics using lipids and proteins found in natural EVs. This strategy requires an in-depth understanding of the nature and function of most EV components to identify the fundamental ones. However, the complexity of artificial vesicles will inevitably be lower than their biological counterparts. The optimal formulation should balance the amount of different lipid categories, excluding the less abundant ones, while maintaining appropriate ratios among various types of fatty acids (saturated, mono- or polyunsaturated). Regarding protein composition, it is essential to identify proteins involved in homing, such as integrins, as well as those involved in cargo transfer, including tetraspanins, lectins, proteoglycans, and fibronectin. The development of artificial EV-biomimetics should proceed incrementally, with increasing complexity [459].

Finally, EV-like particles produced via a cell shearing procedure offer a viable and cost-effective alternative to natural EVs, requiring only a reasonable number of cells and avoiding time-consuming purification procedures. Recent studies have shown that these EV-mimetics exhibit similar physicochemical properties compared to natural EVs, with no significant differences in size, morphology or classical markers [460]. Furthermore, they hold great potential for cargo packaging such as for lipophilic drugs like paclitaxel [461]. However, despite a 71% similarity in membrane proteins between the two groups, the similarity drops to 21% for total protein cargo [460].

### II.3.3. My personal view on the question

Interest in EVs as drug delivery systems has considerably increased over the past decade. Numerous papers have been published, highlighting the potential of EVs as promising nanomedicines and demonstrating interesting results for drug encapsulation within EVs. While the theoretical foundations and published results were encouraging, the practical reality appears to be more complicated. Our results, along with discussions with other researchers from the EV field, suggest that the results frequently do not match the reported successes in the literature. Caution is warranted when interpreting reported effects as every detail, such as EV production, EV isolation, and EV storage can impact EV functional activity. Therefore, identifying the most suitable conditions for working with EVs remains a challenge.

Furthermore, despite extensive research on EVs, many unknowns remain regarding their biology, including the mechanisms of cargo loading, the mechanisms of EV secretion, their capture, and the release of their cargo. Answering these questions is fundamental for optimizing EV production, isolation, and encapsulation.

In my opinion, incorporating a drug into EVs after their isolation is not a good idea. The risks associated with membrane destabilization, leading to the loss of EV content and the potential failure of EV reformation are too high. Thus, among post-EV isolation encapsulation methods, incubation is the only suitable option. However, this method is not effective for hydrophilic molecules. For lipophilic drugs, incubation results in a low amount of drug encapsulated. In the case of EV-Siponimod, encapsulation using turbulence yielded an encapsulation efficiency of 12%. While this is higher than the efficiencies reported with other methods, it also means that 88% of siponimod was lost. This loss is acceptable in this instance because siponimod is relatively not expensive ($150 for 5 mg). However, this level of loss is not compatible with the encapsulation of more expensive drugs.

On the other hand, encapsulation before EV isolation remains, in my opinion, promising, as EVs can be modified via engineered parent cells to incorporate nucleic acids or proteins [214]. Furthermore, companies aiming to commercialize EV-based therapies are predominantly focused on engineered EVs. However, even if this encapsulation method allows for the incorporation of molecules of interest into EVs, several significant hurdles remain, including ensuring reproducibility, producing and purifying EVs using scalable methods that minimize protein contamination, and determining optimal storage conditions for EVs. Additionally, while the impact of EV production and isolation methods on EV functional activity has been reported, its precise

influence is still not fully understood. Therefore, technical advances are crucial to overcoming these EV-related issues. Given these complexities and the current state of the field, I do not believe that EV-based nanomedicines will be approved in the near future, or even in the more distant future.

To be clear, I am not dismissing the extensive work done by other research teams or our own efforts regarding EVs as drug delivery systems. I believe that some of this work has been essential for advancing our understanding of EV biology, EV cargo release, and the functional activity of EVs. Throughout my journey in the EV field, the first part of my research allowed me to gain a better comprehension of SCAP-EVs and the impact of a pro-inflammatory environment on EV content. The second part exposed me to the numerous challenges of encapsulating siponimod into EVs but also yielded promising results on OPC differentiation. Although I remain sceptical about EVs being the future of nanomedicines, I believe that insights from the EV field could be highly beneficial for developing more complex drug delivery systems than those currently reported in the literature. Therefore, in my opinion, EV-like nanoparticles may emerge as promising nanomedicines.

## III. PERSPECTIVES

Based on the main results obtained during this thesis and on the related discussions and conclusions elaborated, several short-term perspectives could be proposed to i) optimize the encapsulation, ii) understand better the mechanisms behind EV-Siponimod effects and iii) consider a realistic use of EVs:

i) Optimization of the encapsulation method:
As shown in chapter 2 of part III, we achieved an encapsulation efficiency of 12% with the turbulence technique. While this is relatively high for the encapsulation of a drug into EVs, it may seem low when compared with synthetic nanocarriers. However, due to time limitations (the encapsulation was performed during my stay at Université de Paris), no optimization was conducted. It could thus be interesting to modify various parameters such as siponimod concentrations and shear stress and evaluate their impact on encapsulation efficiency. Additionally, exploring the underlying mechanism of encapsulation could provide valuable insights that may help improve the method.

ii) Several assessments could be performed to gain a better understanding of EV-Siponimod

- Exploring the mechanisms of action of EV-siponimod to determine if the EV-Siponimod effect is mediated by S1P receptors on the plasma membrane and/or within the cells. Since siponimod acts on S1P receptors $S1P_1$ and $S1P_5$, we could inhibit siponimod binding to

these receptors using W146 (selective antagonist for $S1P_1$) or suramin (dual antagonist for $S1P_3$/$S1P_5$) before treating the cells with EV-Siponimod. This could be performed on microglial cells and OPCs, as the results might be more complicated to interpret with mixed glial cells.

- Exploring the internalization of EV-Siponimod by cells. Different internalization pathways have been described in the literature [260]. We could block these pathways using cytochalasin (blocks endocytosis), chlorpromazine (clathrin-mediated endocytosis), genistein (caveolae-mediated endocytosis), and methyl-β-cyclodextrin (lipid raft-mediated endocytosis) for example [425]. This could be performed on microglial cells, OPCs, or immortalized astrocyte cell lines separately, as the mechanism depends on the cell type. Understanding the internalization of EV-Siponimod by the cells could help us better comprehend the mechanism of action of EV-Siponimod. However, caution is necessary as none of the inhibitors reported in the literature exhibit absolute selectivity [462]. Labelled-EVs with fluorescent lipid membrane dye, such as PKH26 and DID, could also give information on the fate of EVs, as well as the knockdown of certain genes involved in the endocytic processes [260, 462].
- Exploring the impact of turbulences on EVs. Since it is known that the EV production method impacts EV composition, it could be interesting to evaluate this impact by comparing the lipids, proteins, and miRNA content of EVs produced by turbulence with those produced in a flask. A comparison between blank-EVs and EV-Siponimod could also be interesting, as siponimod may impact EV composition.

iii) Several assessments could be performed to consider a realistic use of EVs as nanomedicine:

- Finding the optimal sort and long-term storage conditions. Evidence demonstrated that EVs could be degraded if the storage conditions are not appropriate. As explained in the discussion of this thesis, EV-Siponimod were stored at -80°C, but their stability at this temperature was not assessed. It would thus be interesting to compare different storage conditions (-20°C vs -80°C, with or without cryoprotectants) and evaluate the EV degradation and the release of siponimod over time.
- Finding the optimal route of administration. As explained in this chapter, intracerebroventricular administration was chosen to ensure that the efficiency would not be impacted by a poor accumulation in the CNS, but it is not suitable for chronic treatment. Therefore, a biodistribution study should be performed to compare different routes of administration including intravenous, intranasal, and oral administration. Intranasal administration offers a promising non-invasive option to deliver drugs to the brain, and

oral administration is currently used for siponimod. This could be conducted in both healthy mice and mice treated with lysolecithin to consider the attraction of EVs to inflamed sites.

With the objective of proposing an alternative drug delivery system to synthetic ones currently commercialized, several long-term perspectives could be elaborated:

i) Comparing EV-Siponimod with siponimod-loaded liposomes in terms of encapsulation, efficacy, and stability since liposome structure is similar to EVs. Synthetic standard liposomes could be used for this purpose. However, as discussed earlier, a few hurdles must be overcome to perform this comparison including differences in drug loading and particle concentrations.

ii) Designing EV-like nanoparticles encapsulating siponimod. These could be liposomes with a lipid composition similar to the one described in our first study. We could start by assessing a basic composition that includes the most abundant lipids found in our EVs such as phosphatidylcholine 16:0/16:0, phosphatidylethanolamine 16:1/16:0, and cholesterol, and then progressively enhance the complexity by adding lipids such as sphingomyelin 16:0 and ceramide 18:1/16:1. These EV-like nanoparticles could be further decorated with common EV proteins such as tetraspanins, integrins, and CD47. A quality by design approach should be employed to evaluate the impact of the modification in the composition on critical parameters, including particle size, stability, ability to interact with cells and to deliver their cargo. Additionally, promising miRNAs found in activated SCAP-EVs such as miR-100-5p could be incorporated to reduce inflammation, in addition to the encapsulation of siponimod.

## IV. CONCLUDING REMARKS

Currently, no available treatment for multiple sclerosis stimulates the remyelination process, one reason being the difficulty drugs face in reaching the central nervous system. This challenge could be addressed by using nanocarriers. During the last decade, the interest in EVs, as therapeutic tools or drug delivery vehicles has constantly increased. EVs can encapsulate a large variety of drugs, possess functional activity including immunomodulatory properties, and have a homing to inflamed tissue. Experiments conducted during this thesis investigated the potential of EVs from stem cells from apical papilla as biological nanocarriers with functional activity for siponimod, a compound that can promote the differentiation of oligodendrocyte progenitor cells, a fundamental mechanism preceding remyelination. In a first study, we isolated and characterized EVs from non-activated SCAP and SCAP activated with TNFα and IFNγ, and determined whether SCAP-EVs

could replicate the therapeutic properties of their parent cells. Furthermore, in a second study, we encapsulated siponimod in EVs, using an innovative technique named turbuloporation, allowing us to increase EV yield and load the drug into the vesicles. We then assessed the ability of EV-siponimod *in vitro* to reduce pro-inflammatory cytokine expression in microglia cells and to induce OPC differentiation.

Although some experiments could be performed to optimize our formulation and to improve our comprehension of EV-siponimod, this work made a few contributions to the EV field:

- We showed that the miRNA content of SCAP-EVs, but not their lipid composition, varied depending on whether the SCAP were stimulated with a pro-inflammatory stimulus.
- We observed a slight reduction of the gene expression of pro-inflammatory markers in a microglial cell line following their treatment with activated SCAP-EVs.
- We showed that SCAP-EVs could be use as drug delivery system for siponimod, improving the effect of the free drug on OPC and more broadly on mixed glial cells, although the mechanism of action has not been studied.

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