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Monocyte-derived miRNA and Extracellular Vesicles in patients with Multiple Sclerosis: evaluation as biomarkers

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ABBREVIATIONS

APCs = Antigen-Presenting Cells

A-SMase = Acid Sphingomyelinase

BBB = Blood-Brain Barrier

BCSFB = Blood-Cerebrospinal Fluid Barrier

CIS = Clinically Isolated Syndrome

CNS = Central Nervous System

CPE = Epithelium Of The Choroid Plexus

CSF = Cerebrospinal Fluid

DCs = Dendritic Cells

DIS = Dissemination In Space

DIT = Dissemination In Time

DMF = Dimethylfumarate

EAE = Experimental Autoimmune Encephalomyelitis

EDSS = Expanded Disability Status Scale

EVs = Extracellular Vesicles

EXOs = Exosomes

GA = Glatiramer Acetate

HCs= Healthy Controls

HDs = Healthy Donors

IFN = Interferon

Igs = Immunoglobulins

mAB = Monoclonal Antibody

MBP = Myelin Basic Protein

MHC = Major Human Histocompatibility System

miRNAs = microRNA

MoAs = Mechanism Of Actions

MOG = Myelin Oligodendrocyte Glycoprotein

MS = Multiple Sclerosis

MVs = Microvesicles

NK = Natural Killer

NTZ = Natalizumab

OCBs = Oligoclonal Bands

OND = Other Neurological Disorders

PLP = Proteolipid Protein

PML = Progressive Multifocal Leukoencephalopathy

PP-MS = Primary Progressive Multiple Sclerosis

PRMS = Progressive Relapsing Multiple Sclerosis

RA = Rheumatoid Arthritis

RIS = Radiologically Isolated Syndrome

ROS = Reactive Oxygen Species

RR-MS = Relapsing Remitting Multiple Sclerosis

Sema3A = Semaphorin 3A

S1PR = Sphingosin-1 Phosphate Receptors

SP-MS = Secondary Progressive Multiple Sclerosis

TF = Teriflunomide

Tregs = Regulatory T-Cell

ABSTRACT

Multiple Sclerosis (MS) is a chronic autoimmune inflammatory demyelinating disease of the central nervous system (CNS). Recently, growing attention has been given to extracellular vesicles (EVs), microvesicles (MVs) and exosomes (EXOs), as important mediators of intercellular communication, in both physiological and pathological conditions such as MS.

In immune cells, especially of the myeloid lineage, MV shedding is induced by the stimulation of the ATP receptor P2X7 through activation of acid sphingomyelinase (A-SMase) and mediates release of the inflammatory cytokine IL-1β. Accumulating evidence indicates that MVs may contain and transfer between cells small non-coding RNAs (miRNAs), which are deregulated in the immune and CNS of MS patients and are emerging as diseases biomarkers.

In this study project we firstly evaluated how Fingolimod, a second line treatment for MS, may affect MVs production by the monocytes of the affected patients, as well as P2X7R, IL-1β expression and A-SMase activity.

Thirty-seven MS patients were enrolled, nineteen of which started assuming Fingolimod. Purified monocytes from PBMCs were isolated from venous blood samples after 12 months of treatment. Eighteen healthy donors (HDs) were also recruited and similarly investigated. NBD C_6-Sphingomyelin-labelled MVs were quantified by fluorimetry. A-SMase activity was determined using Amplex Red sphingomyelinase assay. P2X7R, IL-1β, A-SMase expression in monocytes were quantified by qRT-PCR.

We found that basal production of MVs was higher in monocytes from untreated MS patients than Fingolimod treated patients or HDs. Upon BzATP stimulation, MVs production significantly increased in HDs and in patients treated with Fingolimod but not in untreated MS patients. Fingolimod was able to decrease such production compared to MS patients. Treatment, instead, increases P2X7R expression in Fingolimod treated patients compared to HDs in both conditions (KRH and BzATP stimulation). However, the drug reduced IL-1β expression and A-SMase activity in BzATP-stimulated monocytes from MS patients. This evidence reveals that

treatment with Fingolimod reduces MVs production in MS patients by inhibiting A-SMase activity and suggests that monocyte MVs can be considered as a possible diseasebiomarker.

As a second aim, we evaluated EV-miRNA cargo and the relative expression of the same miRNAs in parental cells of MS patients and HDs. For this purpose, 35 MS patients (21 RRMS and 14 PPMS) were enrolled and 16 HDs were similarly investigated. A set of specific miRNAs, important in the immune system and CNS as well as in the crosstalk between monocytes/macrophages and oligodendrocytes or neurons, was evaluated. In preliminary results we found detectable levels of a set of miRNAs, known to be altered in MS, in monocyte-derived EVs from both MS and HDs, i.e.miR-146a, miR-181a, miR-223, miR-23a, miR-30c, and miR125a. Differential expression analysis of these miRNAs in monocytes from HDs and MS patients was then evaluated. Taken together, our results suggest that the study of cellular miRNAs provides interesting information about their role in inflammatory response. Moreover their possible involvement in the synaptic function, in MS as well as in other neuroinflammatory disorders (OND), may certainly deserve consideration for future investigation.

The challenge facing future research will be the optimization and standardization of methods to isolate and characterize EVs content in order to consider them as a possible diagnostic biomarker.

RIASSUNTO

La sclerosi multipla (SM) è una patologia infiammatoria autoimmune cronica, demielinizzante e invalidante, del sistema nervoso centrale (SNC). Recentemente, un crescente interesse è stato rivolto alle vescicole extracellulari (EVs), in particolare microvescicole (MVs) ed esosomi (EXOs), risultati essere importanti mediatori della comunicazione tra cellule, in condizioni sia fisiologiche che patologiche.

Diverse evidenze indicano che il rilascio delle MVs aumenta nelle cellule immunitarie, in particolare quelle della linea mieloide, dopo stimolazione con APT del recettore purinergico P2X7 (un recettore di segnale dell'ATP), attraverso l'attivazione della sfingomielinasi acida (A-SMase), che media anche il rilascio della citochina infiammatoria IL-1β. Il contenuto delle MVs comprende acidi nucleici, proteine cellulari e lipidi che possono essere trasferiti alle cellule target. In particolare, le MVssono in grado di trasferire piccole sequenze di RNA non codificanti (miRNA), che sono deregolati nel sistema immunitario e nel SNC di pazienti affetti da SM e che stanno emergendo come nuovi biomarcatori di malattia.

Il principale obiettivo di questo studio è indagare l'effetto del Fingolimod, un trattamento di seconda linea per la SM, sulla produzione delle MVs e dei pathways cellulari coinvolti in monociti di pazienti affetti, utilizzando un approccio sperimentale che sfrutta la quantificazione spettrofotometrica e la valutazione dell'espressione genica mediante Real-time PCR.

Trentasette pazienti con SM sono stati arruolati, diciannove dei quali in trattamento con il Fingolimod e valutati dopo 12 mesi di trattamento. Diciotto donatori sani (HDs) sono stati inoltre reclutati e analizzati nello stesso modo. Le MVs, marcate con NBD C6-Spingomielina, sono state quantificate mediante metodo spettrofotometrico. L'attività di A-SMase è stata determinata utilizzando il dosaggio fluorimetrico del reagente Amplex Red, mentre l'espressione del recettore P2X7, IL-1β e A-SMase nei monociti è stata quantificata mediante qRT-PCR.

Abbiamo riscontrato che la produzione basale di MVs è più alta nei monociti di pazienti con SM non trattati rispetto ai pazienti trattati con Fingolimod o HDs. La stimolazione con il Benzoil-

ATP, un analogo sintetico dell'ATP, incrementa significativamente la produzione di MVsnegli HDs e nei pazienti trattati con Fingolimod, ma non nei pazienti con SM. Abbiamo osservato che il trattamentoè in grado di ridurre la produzione delle MVs nei monociti rispetto ai pazienti SM non trattati, sia in condizioni di stimolo che di non stimolo. L'espressione del recettore P2X7 aumenta, invece, nei pazienti trattati con Fingolimod rispetto ai HDs in entrambe le condizioni. Tuttavia, il farmaco riduce l'espressione di IL-1β e l'attività di A-SMase nei monociti stimolati con BzATP in pazienti con SM. Complessivamente, i nostri risultati suggeriscono che le MVs aumentano in corso di malattia e che il trattamento con Fingolimod è in grado di ridurre la produzione delle stesse inibendo l'attività di A-SMase. I nostri dati suggeriscono, inoltre, che le MVs monocitarie possono essere considerate come un possibile biomarker di malattia.

In secondo luogo, abbiamo valutato l'espressione di alcuni microRNA nelle EVs e la relativa espressione degli stessi miRNA nei monociti di pazienti affetti da SM e HDs. A tale scopo, sono stati arruolati 35 pazienti affetti da SM (21 RRMS e 14 PPMS) e 16 HDs, analizzati in maniera analoga. Sono stati valutati dei miRNA specifici, noti per essere importanti nella regolazione del sistema immunitario e del SNC, così come nel crosstalk tra monociti/macrofagi e oligodendrociti o neuroni. Risultati preliminari rivelano livelli detectabili di un set di miRNAs, per esempio il miR-146a, miR-181a, miR-223, miR-23a, miR- 30c e il miR125a noti per essere alterati nella SM, in EVs isolate da monociti di pazienti e HDs. Successivamente abbiamo valutato l'espressione differenziale di questi miRNAs in monociti da pazienti e HDs. Complessivamente, i nostri dati suggeriscono che lo studio dei miRNAs cellulari può fornire informazioni sul loro ruolo nella risposta infiammatoria. Inoltre, il loro possibile coinvolgimento nelle funzioni sinaptiche, nella SM e in altre malattie neuroinfiammatorie, è sicuramente meritevole di ulteriori considerazioni per studi futuri.

L'ottimizzazione e la standardizzazione dei protocolli per isolare e caratterizzare il contenuto di EVs rappresenta la sfida che la ricerca futura dovrà affrontare al fine di identificare nuovi biomarcatori.

INTRODUCTION

MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is considered a chronic, inflammatory, demyelinating disease of the central nervous system (CNS) that affects young adults between 20 and 40 years of age, and women three times more often than men [*Sospedra and Martin, 2005; Noseworthy, 2000*]. Affecting over 2,5 million people worldwide, it is the most common cause of neurologic disability among young adults. Typical clinical signs include temporary loss of vision, sensory and motor problems, and neurocognitive dysfunction. There are two major forms of MS. Relapsing-remitting (RR)-MS is the most frequent (85-90%). This form of MS is characterized by episodes of neurologic dysfunction that usually recover. Most RR-MS patients (60%) later develop secondary progressive (SP)-MS, during which disease worsening may still occur with neurodegeneration and progressive intensification of disability. In 10-15% of patients a progressive course is seen from the beginning, which is referred to as primary progressive (PP)-MS [*Sospedra and Martin, 2005*]. It is not clear at present which factors are responsible for the different clinical presentations and disease courses [*Compston and Coles, 2008*].

Based on studies on experimental autoimmune encephalomyelitis (EAE), MS is considered a CD4+T-cell mediated autoimmune disease with a complex genetic background. In both EAE and MS, activation CD4+ autoreactive T cells and their differentiation into Th1 and Th17 cell subsets are pivotal events in the first steps of MS and these cells are probably also important players in the long-term development of the disease. The site of activation of these autoreactive CD4+T cells and their differentiation to specific Th subsets is still unknown in MS.

More recently it was also shown that in addition to T cell activation, other components of the immune system, such as B cells, antibodies, complement, CD8+ T cells, monocyte/macrophages and factors produced by innate immune cells are also involved in MS [*Hirotani, 2010; Frohman, 2006*].

Perturbations in immunomodulatory networks that include Th2 cells, regulatory CD4+ T cells, NK cells, and others may in part be responsible for the relapsing-remitting or chronic progressive course of the disease. However, in some forms of MS, as in many diseases, the monocytes are thought to be the primary cell type responsible for cellular pathology and tissue damage, while T lymphocytes are thought to orchestrate the process by secreting cytokines and other factors to promote leukocyte homing and activation in the lesion [*Noseworthy, 2000; Davidson and Diamond, 2001; Raine, 1994; Lucchinetti, 2000*].

Etiology of Multiple Sclerosis

Etiologically, MS is a complex disease in which both genetic and environmental factors play a role. The most important genetic risk factor in MS is the major human histocompatibility system (MHC), a region on chromosome 6, responsible for the HLA-DR and -DQ genes [*Sospedra and Martin, 2005*]. This area produces human leukocyte antigens that present to T cells and may trigger an immune response. Less information is available about genetic risk conferred by HLA class I alleles [*Sospedra and Martin, 2005*]. Among the evidence are genetic studies demonstrating a protection by the HLA-A*02:01 allele and risk conferred by HLA-A*03:01. Other susceptibility loci such as IL2RA, IL7RA, CD58, EVIS, CLEC16A, TYK2, CD226, TNFRSF1A, IRF8, or CD6, which have all been involved in T-cell activation and proliferation, cytokine pathways, costimulation, and signal transduction, might be associated with dysregulation of the adaptive immune system in MS [*Sospedra and Martin, 2016*].
Additional non-genetic factors that are associated with MS have been identified, including latitude, vitamin D level, Epstein-Barr virus, and smoking [*Files, 2015*].

Immune cells and mechanisms involved in the pathogenesis of Multiple Sclerosis

Anatomically the CNS is protected by the cerebrospinal fluid (CSF), produced by the secretory epithelium of the choroid plexus (CPE), and the meninges that surround the brain and

the spinal cord. The meninges comprised the innermost pial membrane that is intimately in contact with the parenchyma, the intermediate non-vascularized arachnoid membrane, and the outermost vascularized dural membrane, that is attached to the skull (**Fig.1)** [*Louveau, 2015*]. The meninges are an essential immunological site that make possible CNS immune surveillance [*Raper, 2016*].

Experiments in the mid 20th century gave rise to the concept of the CNS as immune-privileged site, to which access of circulating immune cells is firmly controlled by an endothelial blood-brain barrier (BBB) and the epithelial blood-cerebrospinal fluid barrier (BCSFB) within the choroid plexus [*Engelhardt and Ransohoff, 2012*]. This property allows to protect post-mitotic neural cells from potential immune response-mediated injury and death. Immune privilege is based on multiple factors, including: 1) as mentioned above, the presence of the BBB; 2) a relative lack of classical lymphatic drainage of the parenchyma; 3) a poorness of professional antigen-presenting cells (APCs), such as dendritic cells (DCs); 4) low expression levels of MHC molecules; and 5) many anti-inflammatory soluble modulators [*Harris, 2014*].

Under physiological conditions, there is a minimal entry of immune cells, mainly memory T cells, into the CNS through the choroid plexus for the purpose of immune surveillance. The CSF drains into the deep cervical lymph nodes via the cribriform plate into the lymphatic system of the nasal mucosa and then to the deep cervical lymph nodes [*Louveau, 2015*] (**Fig.2A**). Recent findings by Louveau and Aspelund have revealed the presence of a conventional and functional lymphatic system, which is placedin the dura matter and allows fluid, macromolecules and immune cells to drain from the CNS into the deep cervical lymph nodes (**Fig.2B**). The drainage in the deep cervical lymph nodes was dependent on the presence of meningeal lymphatic vessels in which immune cells (T cells, B cells, DCs) were present under physiological conditions, suggesting that the meningeal lymphatics play a role in the trafficking of immune cells out of the CNS in the stationary state [*Louveau, 2015*]. These data have dramatically altered classical viewpoint of immune privilege of CNS by revealing that the interactions between the CNS and

the immune system occur and are not limited to pathology, but also extend to homeostatic functions (**Fig.2**). Therefore, the CNS is still considered immunologically privileged site but our knowledge of the complex neuroimmune interactions occurring suggest that it is a mostly controlled system with an unique immunological environment.

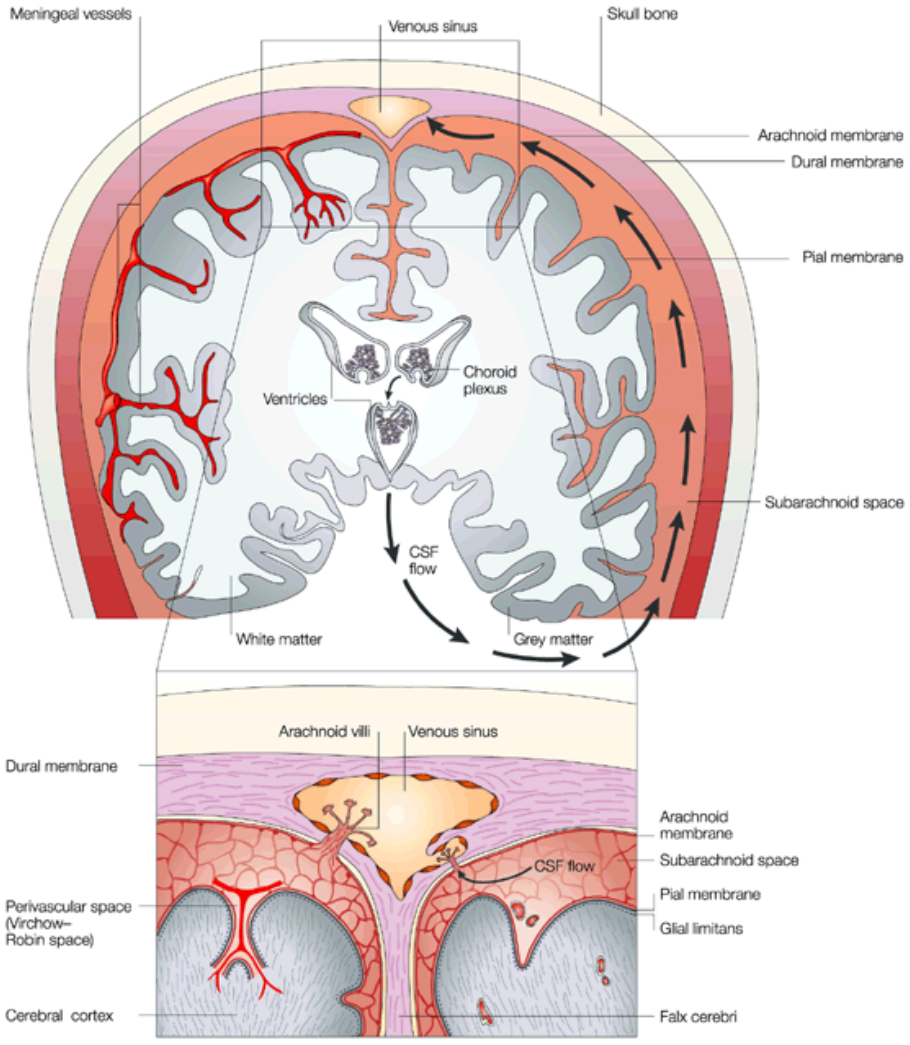


Figure 1. CNS anatomy and the CSF circulation. The CNS is protected by the meninges (arachnoid, dural, and pial membrane). The CSF is produced by the secretory epithelium of the choroid plexus that is placed in the ventricular system of the brain. CSF circulates from the ventricles to the subarachnoid space and is reabsorbed to the systemic circulation through the arachnoid villi that extend into the venous sinuses of the cerebral hemispheres [*Louveau, 2015*].

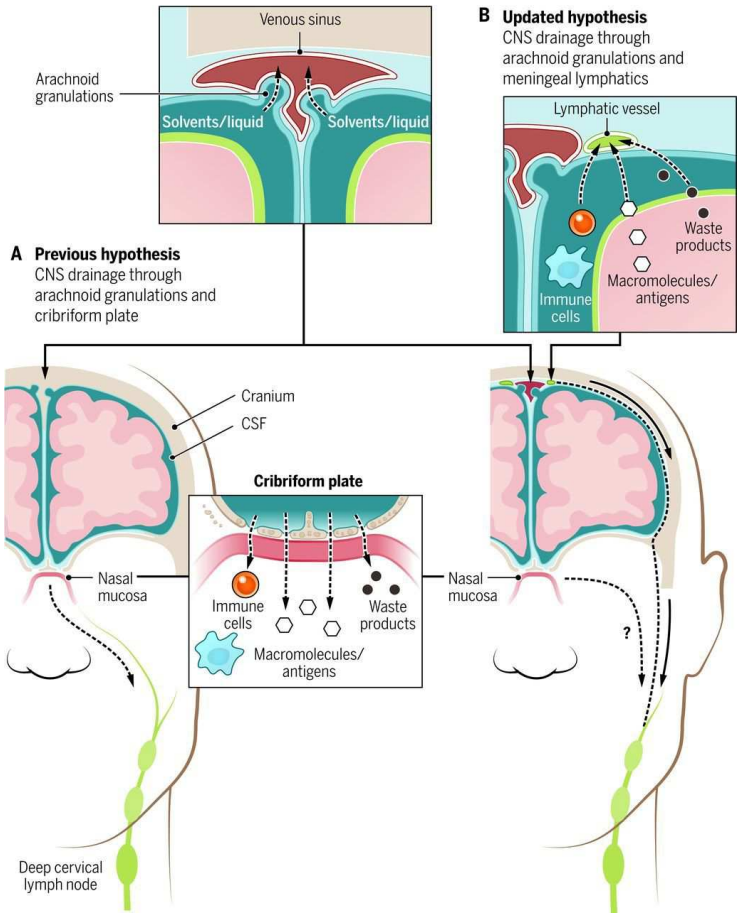


Figure 2. CNS drainage: New concepts for old.(**A**) Previous hypothesis suggested CSF drainage into deep cervical lymph nodes via the cribriform plate. (**B**) New concept of CSF drainage into the deep cervical lymph nodes via meningeal lymphatics [*Kipnis, 2016*].

The triggering inflammatory event occurring in MS is not yet clear, thus generating contradictory hypotheses on the etiopathogenesis of the disease.

The most approved hypothesis for an inflammatory autoimmune pathogenesis of MS is the activation of CNS antigen-specific CD4+T cells in the periphery. This hypothesis origins mainly from animal models, in which disease can be induced by immunization with CNS-derived proteins and is largely driven by CNS-specific CD4+T cells [*Sospedra and Martin, 2016*]. This phenomenon occurs when peptide from pathogens share sequence or structural similarities with self-antigen. In this case an immune event outside the CNS starts the disease process in MS, in which autoreactive CD4+T cells are activated in the periphery by cross-reactivity of peptides derived from a foreign antigen, for example, during a viral infection. This concept, called *molecular mimicry*, has been introduced by Fujinami and Oldstone by demonstrating that

immunization of rabbits with a peptide from hepatitis B virus polymerase with similarity with myelin basic protein (MBP) can induce EAE, and that T cells with cross reactivity against the two peptides are implicated [*Sospedra and Martin, 2016*]. Cross reactivity of these T cells with non-self antigen can lead to activation, migration across BBB, CNS infiltration, and, if they recognize antigens expressed in the brain, tissue damage.

In any case, molecular mimicry alone may not be able to induce disease; in fact other evidences indicate that a focal inflammatory reaction at CNS level and triggered by a still unknown stimulus to a local injury could occur in MS patients [*Martino, 2000*]. In a typical MS brain, it is possible to observe lymphocytes and monocytes infiltrates within demyelinating areas, but also in normal white and grey matter as well as in meningeal spaces. The recruitment of leukocytes within the CNS induces the activation of the resident immune cells, such as astrocytes and microglia, determining a CNS-specific immune reaction [*Martino, 2000*].

Martino et al. proposed a theory, called *the dual signal hypothesis*, offering an integrated and more comprehensive view of the MS pathogenic process, in which two phenomena are merged [*Martino, 2000*]. They suggest that local CNS inflammatory process along with a concomitant, but possibly unrelated, systemic inflammatory event may trigger a CNS-specific autoimmune reaction cascade setting the stage for the MS pathogenesis.

To sum up, in this scenario, peripheral activated T cells cross the BBB and enter the CSF. They have access to the subarachnoid space where they can be restimulated by local myeloid APCs, particularly meningeal and perivascular macrophages. The re-activation of naive and effector T cells in the CNS induces cytokine and chemokine production that amplify the inflammatory response with activation of resident immune cells, upregulation of MHC class II molecules, the recruitment of monocytes, CD8+T cells, B cells from peripheral blood; and the formation of the inflammatory lesion [*Sospedra and Martin, 2005*]. Immune cells infiltrating the CNS can lead to neurodegeneration and tissue damage by secretion of different neurotoxic products, such as reactive oxygen species (ROS) [*Sospedra and Martin, 2016*]. During early stages of MS, new

lesions form frequently, in which the above mentioned adaptive immune mechanisms are the most important drivers. In contrast, during later phases of the disease, inflammation decreases, but the susceptibility of the target tissue for neurodegeneration increases [*Sospedra and Martin, 2016*]. All these events are summarizedin **Fig.3.**

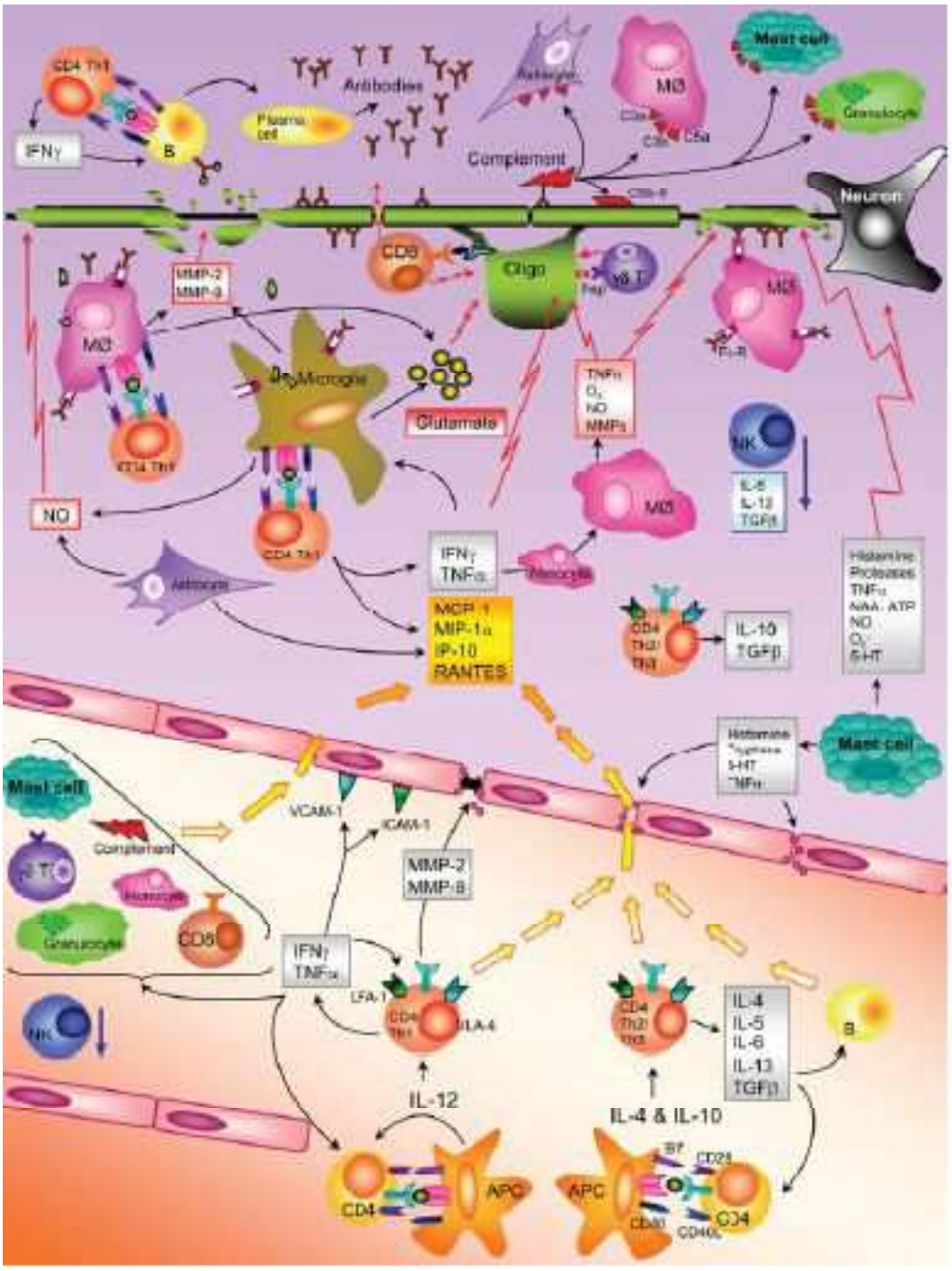


Figure 3. Immune mechanisms in the MS disease process. The figure summarizes the most important events in the pathogenesis of the MS [*Sospedra and Martin, 2005*].

Regarding the functional characteristics of autoimmune T cells in MS, T helper 1 cells, phenotype characterized by the production of interferon-γ (IFN-γ) were thought originally to be the main pathogenic T cells in EAE and MS. It was postulated that during EAE, in the peripheral lymphoid organs, T precursor myelin-reactive T cells are induced to differentiate into myelin-reactive Th1cells when an antigen that crossreacts with a myelin antigen is presented to a T cell by an APC. Th1 cells that react with myelin antigens, such as myelin proteolipid protein (PLP), MBP and myelin oligodendrocyte glycoprotein (MOG), cross the BBB where the myelin antigen are represented to T cell by APCs in the brain (microglia cells), and an inflammatory cascade is triggered with the release of inflammatory mediators that cause damage to the myelin sheath and ultimately the underlying axon. This conclusion was based partly on the observation that IFN-γ was detected in MS lesions and in the CNS of EAE mice [*Traugott and Lebon, 1988a; Traugott and Lebon, 1988b*].The concept of Th1 driven organ-specific autoimmunity was challenged when Th17 cells central role in several EAE models was discovered. These cells produce IL-17 that was found highly expressed in tissue sites of many autoimmune diseases including MS lesions [*Lock, 2002*].

Although most evidence supports an important role of CNS antigen-specific CD4+T cells in sustaining MS, recent data suggest that also CNS antigen-specific CD8+T cells are involved in MS. These cells are activated in periphery by professional APCs presenting CNS-derived or cross-reactive peptides as previously described for CD4+T cells. CD8+T cells are detected in MS lesions and often clonally expanded, suggesting that these cells are involved in CNS tissue damage. Furthermore, because axons and neurons express MHC class I molecules, CD8+ T cells can recognize their cognate antigen on these cells and directly damage them [*Sospedra and Martin, 2016*]

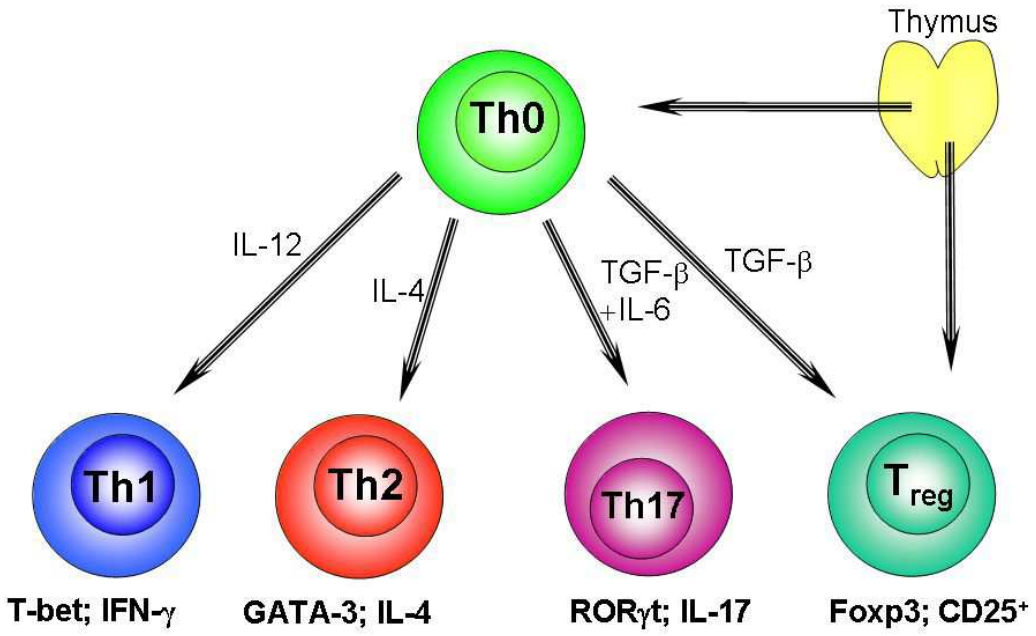


Figure 4. Different T helper cell subsets. Upon activation, Th0 precursor cell differentiates into effector T cell subsets, producing different cytokines and mediating distinct effector functions (image from Wikipedia).

B cells are APCs, implicated in cytokines and antibodies production, and they participate in the formation of ectopic lymphoid tissues (**Fig.5**) [*von Büdingen, 2015*]. The increased levels of immunoglobulins (Igs) in the CSF of MS patients is the earliest evidence that suggests a role for B cells and antibodies in the pathology of MS [*Sospedra and Martin, 2005*]. To date, the strongest evidence for B cells involvement in MS pathology arise from studies evaluating the effect and the efficacy of anti-CD20 B cell depleting therapy such as rituximab, ocrelizumab, and ofatumumab [*von Büdingen, 2015*]. Others evidences are the presence of oligoclonal bands (OCBs) in the CSF, detection of B cells, plasma cells and myelin-specific antibodies in MS plaques and in the areas of demyelination, the presence of B cell follicle-like structures, and a B cell-fostering milieu [*Sospedra and Martin, 2005, Pröbstel, 2015*].

B cells, once inflammation has started, can cross BBB and enter the CNS. In loco, they contribute to MS disease pathogenesis with costimulation of autoreactive T cells, elevated

production ofthe Igs in the CSF, myelin-specific antibodies secretion [*Sospedra and Martin, 2005,*]. It is necessary to control B cell tolerance to reduce autoimmunity risk that can randomly develop, nevertheless, peripheral B cell tolerance mechanisms seems to be impaired [*von Büdingen, 2015*].

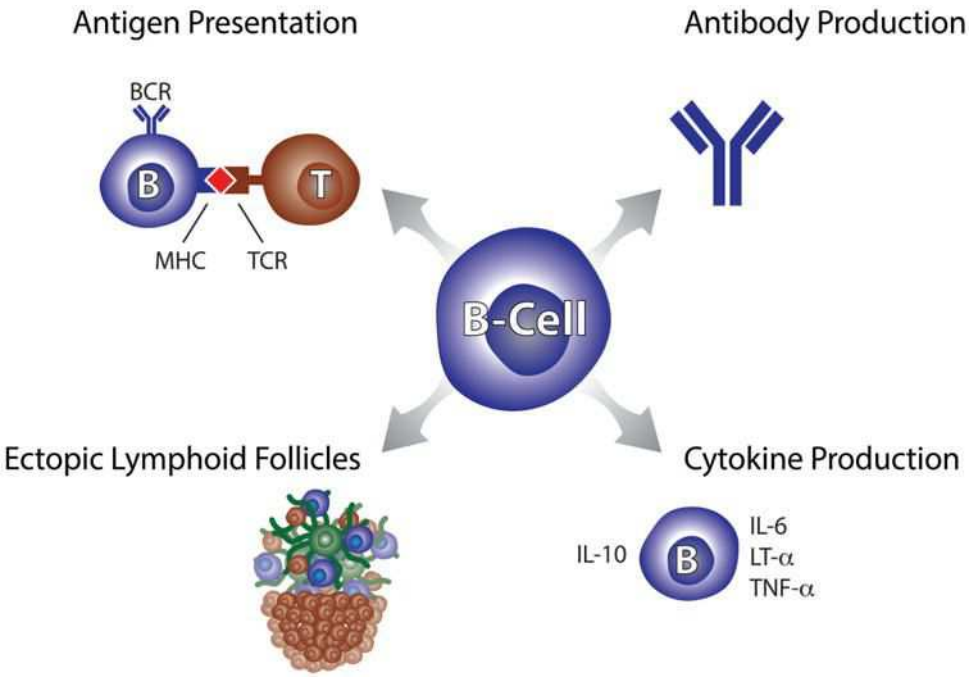


Figure5. B cell functions. Antigen presentation, antibody production, establishment of ectopic lymphoid follicles at sites of inflammation and cytokine production are the most important functions relevant to MS pathogenesis [*von Büdingen, 2015*].

As a matter of fact, myelin-reactive memory B cells can be found in the peripheral blood of MS patients where they may act as APCs. These cells, expressing high levels of CD20, could be depleted following treatment with anti-CD20 targeting monoclonal antibodies reducing MS severity [*von Büdingen, 2015*].

To date, it is difficult to identify the antigen specificity of CSF antibodies in MS, because OCBs could target both myelin proteins and foreign agents. However, known pathogenic nature of autoantibodies, the research for autoantigens has focused on myelin and other CNS components [*Sospedra and Martin, 2005*]. MOG appeared as a very promising candidate B cell autoantigen in MS. Numerous other potential targets for autoantibodies in MS have been described. In the

table below (**Table 1**), are summarized antibody specificities against CNS components other than MOG and MBP, adapted from Sospedra et al. [*Sospedra and Martin, 2005*].

Target antigen	Remarks
myelin-associate glycoprotein (MAG)	Probablyinvolvement in progression
oligodendrocyte-specificprotein (OSP)	Minormyelin component
Transaldolase-H	oligodendrocyte component
glyco-sphingolipids	lipid component of myelin
sulfatides	lipid component of myelin
GD1a and GM3 (ganglioside)	lipid component of myelin
Galactocerebroside (Gal-C)	Majormyelinlipid
α-B crystallin	detected in MS sera
neurofilament-L	indicator of axonaldamage
AN2 (oligodendrocytesurfaceglycoprotein)	expression on oligodendrocyte precursor
Nogo-A	anti-Nogo antibodies are frequent in MS, but also in controls
Proteasome	anti-proteasome antibodies found in serum and CSF
DNA	probably anti-DNA antibodies bind to neurons and oligodendrocytes

Table 1. **Antibody specifities against CNS components**. Adapted from [*Sospedra and Martin, 2005*].

To date, the most prominent are those directed against the potassium channel KIR4.1 and antibodies against neurofascin and contactin-2 [*Sospedra and Martin, 2016*].

In parallel with the activation of damaging inflammatory events, in MS patients there is wide evidence that regulatory mechanisms of inflammation are activated in order to restrain tissue damage and initiate regeneration. These mechanisms represented an intrinsic capability of the immune system to limit its overactivation. Regulatory T-cell (Tregs) populations have been described as the main cell population in controlling pro-inflammatory events. Tregs are generally defined as being CD4+ and CD25high and characterized by their transcription factor, Foxp3.

Their function is to inhibit effector T cells both by direct cell–cell contact and by secreting inhibitory cytokines, such as TGF-ß, IL-10, and IL-35 [*Naegele and Martin, 2014*]. However, in MS, a reduced number and activity of CD4+CD25hi Tregs are observed [*Sospedra and Martin, 2016*]. Apart from Tregs, other immune cells may also be involved in neuroprotection and lesion resolution via relative production of Th2/Th3 cytokines, such as IL-10 and TGF-β, and secretion of growth factors [*Sospedra and Martin, 2016*]. However, it should not be forgotten that resident inhibitory mechanisms of the immunoprivilegied CNS also contribute to the repression of inflammatory events. As a matter of fact, oligodendrocyte precursors that are still present in the adult CNS are also activated, and surviving oligodendrocytes begin to regenerate myelin sheaths, although they are structurally abnormal [*Sospedra and Martin, 2005*]. Despite this, a large number of lesions exhibit no remyelination.

Based on studies of MS pathology, possible explanations for differences in remyelination potential include disease heterogeneity or differences in disease chronicity. Lucchinetti et al have identified four pathologic MS subtypes on the basis of the relative contribution of different immune cells, antibody and complement deposition, myelin loss, and oligodendrocyte death [*Lucchinetti, 2000*]. The following pathologic subtypes are described:

Pattern I. In this pattern, inflammation is dominated by T cells and macrophages, and the demyelinating process is induced mainly by TNF-α, IFN-γ , and ROS.

Pattern II. This pattern is very similar to pattern I, but in this case antibody and complement deposition predominate, and both MOG- and MBP-specific antibodies are involved.

Pattern III. Lesions present inflammatory infiltrates, composed mainly by T cells, macrophages and activated microglia. Igs and complement deposition are absent. Plaques are characterized by loss of oligodendrocytes without remyelination process.

Pattern IV. This pattern occurs primarily in PP-MS and show similarities to the classical pattern I and II lesions. Demyelination was associated with oligodendrocytes death [*Lucchinetti, 2000*].

Clinical course of Multiple Sclerosis

In 1996, the US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in MS defined the clinical subtypes of MS [*Lublin and Reingold, 1996*]. The characterizations were rapidly adopted into practice and served to better communicate a patient's clinical course and to define clinical trial populations. At the time, no biomarkers or magnetic resonance imaging (MRI) signals were available to distinguish between the different MS clinical courses.

The Committee provided standardized definitions for 4 MS clinical courses: RR, SP, PP, and progressive relapsing (PR) (**Fig.6**). RRMSis the most common clinical form. Approximately 85% of patients present this form of pathology [*Sospedra and Martin, 2005*], which is characterized by acute episodes of disease (called relapses) followed by partial or complete recovery. Periods between disease relapses are characterized by lack of disease progression [*Lublin and Reingold, 1996*]. RRMS onset typically occurs in early adulthood, and, within around two decades, approximately half will go on to develop SPMS. SPMS is defined as a clinical form with initial RR course, followed by progression, with or without relapses [*Lublin and Reingold, 1996*]. PPMS affects only 10–15% of the MS population [*Sospedra and Martin, 2005*] and is associated with a rapid disease progression from onset without remission or with occasional plateaus and temporary minor improvements [*Lublin and Reingold, 1996*]. PRMS shows progression at the beginning of the disease with clear acute relapses with or without full recovery; and periods between relapses are characterized by sustaining progression.

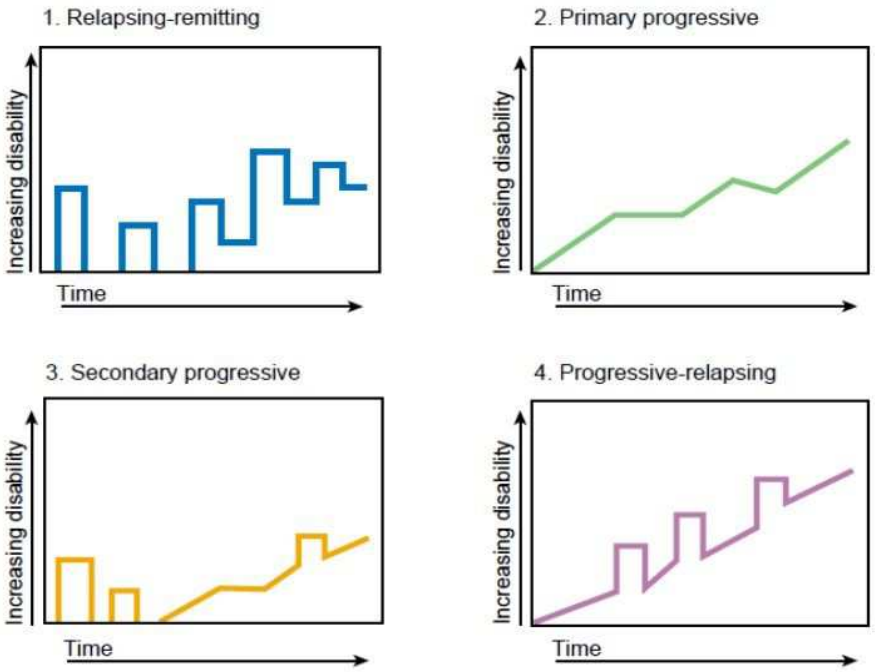


Figura 6. Clinical course of MS.

In 2013, the International Advisory Committee on Clinical Trials of MS has introduced a revision with new insights and clarifications in order to better define MS disease phenotypes, in the light of the more recently identified clinical aspects of the disease.

First, it was introduced Clinically Isolated Syndrome (CIS) as a distinct MS phenotype. CIS was recognized as the first clinical presentation of a disease that shows characteristics of inflammatory demyelination that could be MS, but has yet to fulfill criteria of dissemination in time. Second, a more complicated phenotype was descripted as Radiologically Isolated Syndrome (RIS), in which inflammatory demyelination areas are observed in the absence of clinical signs or symptoms. RIS is not considered to be part of MS subtypes as patients lack clinical evidence of demyelinating disease (a current criterion for MS diagnosis) and MRI findings alone are insufficient to establish a diagnosis of MS [*Lublin, 2014*].

Actually, MS patients may be described as having 1) relapsing MS that is active or not active, with or without worsening; or 2) primary or secondary progressive disease that is active or not active, with or without progression. [*Lublin, 2014*] (**Fig.7**).

Disease activity can be assessed at least annually by clinical and brain imaging criteria, whereas disease course can be determined by clinical evidence of progression.

Clinical evaluation is usually made referring to the Expanded Disability Status Scale (EDSS), which is a method of quantifying disability in MS and monitoring changes in the level of disability over time. The EDSS rating scale ranges in half-point increments from 0 (normal neurologic examination) to 10 (death owing to MS).

As regarding diagnosis, the original diagnostic criteria for MS were previously based on clinical features of demyelination alone. Over time, several criteria have been developed and replaced. McDonald criteria for MS are usually used for MS diagnosis. Diagnostic criteria based on analysis of dissemination of lesions in space (DIS) and time (DIT) with other clinical and paraclinical evaluations in order to exclude alternative diagnosis. The McDonald criteria have been resulted very specific in earlier diagnosis of MS, allowing an earlier treatment [*Polman, 2011*].

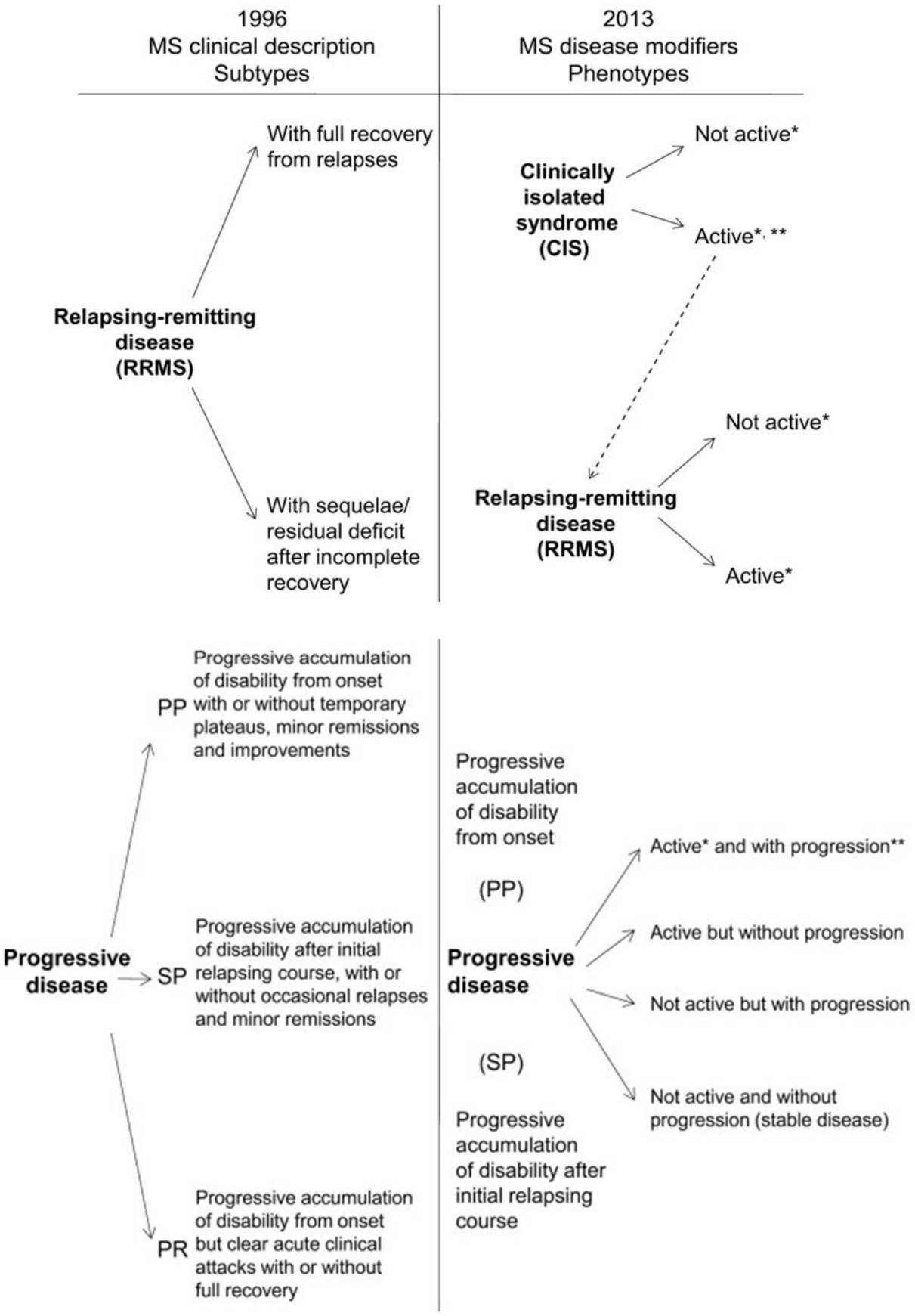


Figure 7. The 1996 vs 2013 MS phenotype descriprions for relapsing and progressive disease [*Lublin, 2014***].**

Multiple Sclerosis Therapies

Treatment of MS is difficult, because the disease process itself is very complex. Much has changed in the management over the last 20 years, and it will likely continue to evolve in the near future. Currently the therapies are not able to determine a regression or an arrest of the disease, but they can modify the course of the disease in the long term evolution, reducing the accumulation of disability.While relapse treatment has not changed, there are now 10 drugs that have been approved for the various form of MS from the first clinical manifestation (CIS) over RRMS to SPMS, and, for the first time also for PPMS. The success of these several and varied treatments with different mechanism of actions (MoAs) has not only positive for people living with MS, but also enhanced our knowledge about immune mechanisms of MS.

The drugs are generally divided into two groups: first and second-line drugs. The first line drugs represent the initial therapeutic approach for MS patients, where the clinical benefits overcome the possible side effects. If patients do not respond to first-line treatment or in the case of a very active disease with a high risk of progression to disability, second-line therapy is applied.

Interferon-β. Interferon-β (IFN-β) is a first line therapy for which we have clinical experience over two decades. There are a number of potential MoAs by which IFN-β may inhibit several aspects of the pathophysiology of MS. They include stabilization of the BBB by blocking matrix metalloproteases, reduced activation of T lymphocytes via the downregulation of HLA class II molecules and antigen presentation on glial cells and B cells, and Th1-Th2 shift. Whether IFN-β antiviral activity is relevant for the treatment of MS is not yet clear, but it was observed that therapy does not compromise immune system with respect to protection against viral, bacterial, or fungal infections. Major side effects include flu-like symptoms, as well as depression, local skin reactions, allergic reactions, and liver abnormalities [*Martin, 2016; Cross and Naismith, 2014*].

Glatiramer Acetate. Glatiramer acetate (GA), another first line drugs, is composed of a synthetic four amino acid copolymer developed in order to simulate MBP. GA was first

approved in 1996 after a complex clinical development. GA needs to be injected subcutaneously, inhibits relapse rates by approximately 30%, and is very well tolerated [*Johnson, 1995*]. Among immunomodulatory effects of GA, it affects antigen presentation, shifts the Th1 response in Th2 response, induces regulatory CD8+T cells, and induces the production of neurotrophic factors. Which of these GA immunomodulatory effects are most important in reducing the disease activity in MS is difficult to assess, but it is very well tolerated without any impairment of protective immune responses [*Martin, 2016*].

Teriflunomide. Teriflunomide (TF) is the active metabolite of leflunomide, which has been approved in the USA since 1998 to treat rheumatoid arthritis (RA). TF was approved in 2012 as first-line oral drug for treating RRMS. TF reduces the relapse rates similar to IFN-β and GA treatments. The main MoA is the inhibition of dihydro-orotate-synthetase, a key enzyme in the *de novo* pyrimidine synthesis. As a result TF blocks high levels of activated T lymphocytes proliferation. Side effects include abnormal liver function tests, hair loss, gastrointestinal issues, and immunosuppression [*Martin, 2016*].

Dimethylfumarate. DMF (also known as BG-12) and other derivatives of fumaric acid ester compounds have been used initially to treat psoriasis prior to discover its efficacy in MS [*Gold, 2012*]. DMF was approved as a first-line oral treatment for RR-MS in 2013. DMF is an oral prodrug that is converted into monomethylfumarate and fumarate inside cells. DMF has been shown to induce Th1-Th2 shift by modulating DC function [*Martin, 2016*]; to enhance Nrf2 activity, a transcription factor that controls the expression of a wide range of antioxidant proteins [*di Nuzzo, 2014*]. Further activities include the induction of regulatory B-cell subsets [*Martin, 2016*] and inhibition of NFkB-induced genes. Its efficacy in MS is moderate with some important side effects such as gastrointestinal problems, flush; in some patients profound and long-lasting lymphopenia was observed. So far, no specific immunocompromise with respect to viral or bacterial infections has been reported, but a few progressive multifocal leukoencephalopathy (PML) cases have been observed [*Martin, 2016*].

Natalizumab. Natalizumab (NTZ) was the first monoclonal antibody (mAb) approved for the treatment of RR-MS. NTZ targets the α-4 chain of α4β1 integrin (very-late-antigen-4, VLA-4) that also binds to α4β7 integrin. VLA-4 receptoris expressed on all leucocytes except neutrophils [*Lutterotti and Martin, 2008*]. NTZ is an antagonist of VLA-4 that blocks it from binding to vascular cell adhesion molecule (VCAM), which is upregulated on the endothelium during inflammation. The rationale behind blocking of VLA-4 is to prevent inflammatory cell migration from the vasculature and within the CNS. Furthermore, inhibition of VLA-4/VCAM-1 interactions by NTZ also induces the release of certain B cell maturation precursors from bone marrow and lymphoid organs into the peripheral blood [*Martin, 2016*]. NTZ is efficacious in blocking relapse rates to a much higher extent than IFN-β and GA [*Miller, 2003*]. NTZ is very well tolerated except for one adverse event: PML, a progressive demyelinating disease of the CNS that is caused by the infection of oligodendrocytes with the polyomavirus (JCV). Therefore, the patient must be monitored for the JC virus before starting therapy and can only be used with a restricted indication in the case of very active or breakthrough relapsing MS.

The Sphingosin-1 Phosphate Receptor Agonist Fingolimod. Fingolimod (FTY720), a derivative of the fungal antibiotic myriocin, is a functional antagonist of four of the five sphingosin-1 phosphate receptors (S1PRs), which are expressed by many cells including immune cells [*Martin, 2016*]. FTY720 was the first oral drug approved for the treatment of RRMS in 2011 as second line treatment. Fingolimod is an analog of sphingosine, which is a component of the lipid bilayer of the plasma membrane, and its phosphorylated metabolite S1P. Both sphingosine and fingolimodare phosphorylated inside the cells into their active forms, which bind to specific S1P receptors [*Mehling, 2011*]. In humans, 5 subtypes of S1PRs have been described. FTY720 binds to S1PRs and leads to their internalization and degradation, except for S1P2R (**Fig.8**). The main action of Fingolimod is to control the trafficking of lymphocytes between lymphoid organs and blood, in order to block the egress of lymphocytes from lymph nodes [*di Nuzzo, 2014*]. The result is a relative lymphopenia and change in composition of

peripheral lymphocytes, predominantly in naive and memory T cells [*Mehling, 2011*]. This effect of FTY720 is considered the main MoA in MS; however, effectson immune cells and CNS including the BBB have been observed [*Martin, 2016*].

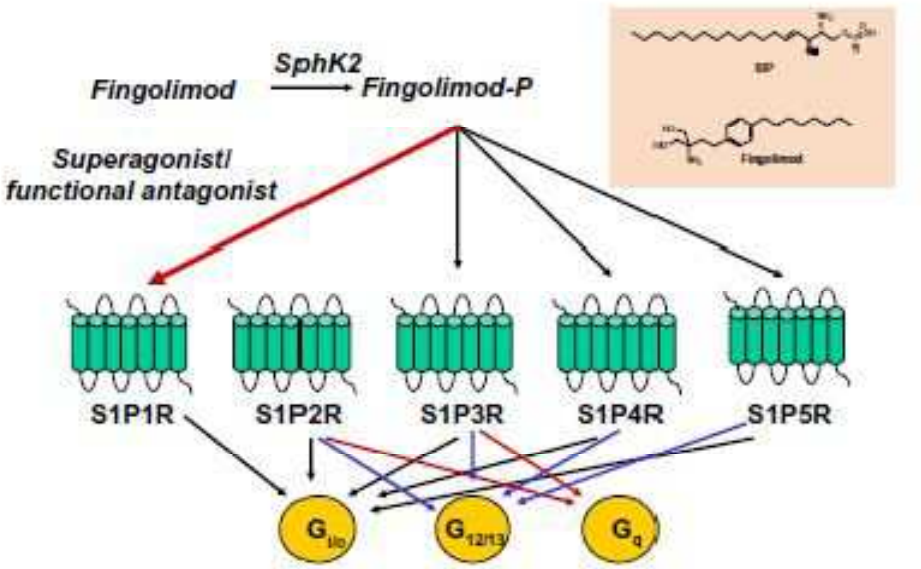


Figure 8. Structure of Fingolimod and its mechanism of action. Fingolimod phosphate activates S1P1R, S1P3R, S1P4R, and S1P5R, but not S1P2R [*di Nuzzo, 2014*].

Anti-CD52 (Alemtuzumab). Alemtuzumab is a humanized IgG1 mAb approved for RRMS treatment in 2014. It targets CD52, a cell-surface glycoprotein abundantly expressed on T and B cells, natural killer (NK) cells, monocytes and some subsets of DCs. Alemtuzumab lyses cells expressing CD52 by antibody-dependent cellular cytolysis, thereby rapidly producing a profound leucopenia [*Lutterotti and Martin, 2008*]. The physiological function of the CD52 molecule is still not known, but it has an elevated efficacy in MS with only few therapy cycles needed. Alemtuzumab significantly reduces clinical and MRI disease activity. However, some patients require additional treatment courses, and surprisingly, alemtuzumab is associated with a considerable risk of secondary autoimmune diseases [*Martin, 2016*].

Anti-CD20 (Rituximab, Ocrelizumab, Ofatumumab). The clinical success of B-cell targeting therapies with anti-CD20 antibodies have provided experimental evidence for an involvement of

B cells in MS pathogenesis. Rituximab, ocrelizumab, and ofatumumab are different anti-CD20 depleting agents.

Rituximab, a chimeric mouse-human anti-CD20 mAb, was first approached with the idea that B cells and their autoantibodies contribute to MS pathogenesis [*Hauser, 2008*]. Rituximab had been developed and approved for treating B-cell malignancies, such as non-Hodgkin's lymphoma, and RA. CD20 is exclusively expressed on all B-cell in different stages of maturation; however, plasma cells and stem cells do not express CD20 on their surface. Thus, Rituximab selectively depletes CD20-expressing B cells, preserving the capacity for B-cell reconstitution and antibody production and secretion, via complement-mediated lysis [*Martin, 2016*, *Lutterotti and Martin, 2008*]. The most prominent adverse effects were related to the infusion.

Ocrelizumab is a fully humanized mAb that deplets CD20+ B cells via cytolysis mechanism in a non-complement manner. Ocrelizumab appears to reduce relapse and disability in MS but quality of evidence is moderate [*Filippini, 2017*]. Ocrelizumabis generally well tolerated, with the most common side events associated to the infusion reactions and infections. Sometimes, in ocrelizumab MS trials the risk of malignancies was observed [*Gelfand, 2017*].

Ofatumumab is a human mAb that also targets CD20 molecule binding to a different epitope than rituximab (and ocrelizumab) resulting in a pronounced complement-mediated cytotoxicity *in vitro.* It is currently approved for the treatment of chronic lymphatic leukemia [*Bittner, 2017*].

Anti-CD25 (Daclizumab). Daclizumab is a humanized mAb against CD25, the alpha chain of the IL-2 receptor (IL2Rα). It was originally approved for the prevention of allograft rejection of kidney in order to block the expansion of alloreactive T cells [*Vincenti, 1998*]. As a matter of fact, the IL2Rα is poorly expressed on resting T cells but is upregulated on activated or abnormal T cells [*Lutterotti and Martin, 2008*]. Therefore, the rationale for the use of anti-CD25 therapy in autoimmune disease is to inhibit autoreactive T cells proliferation. Despite the knowledge that daclizumab is a mAb with a singular target, its MoA in MS is not fully elucidated. Effectiveness

in MS appears to be related to the ability of daclizumab in expansion of a subset of NK cells that express the NK cell marker CD56 at high levels ($CD56^{bright}$NK cells). $CD56^{bright}$NK cells have immunoregulatory effects in addition to their antiviral and antitumor properties [*Lutterotti and Martin, 2008*]. The expansion of $CD56^{bright}$NK cells strongly correlates with the reduction of inflammatory lesions in the brain in multiple phases II and III studies [*Martin, 2016*,]. Several other interesting MoAS have been described for daclizumab, including inhibition of T cell activation via blocking CD25 on DCs [*Wuest, 2011*].

Fig.9 Summarizes the putative cellular and molecular therapeutic targets used in the MS treatment [*Martin, 2016*].

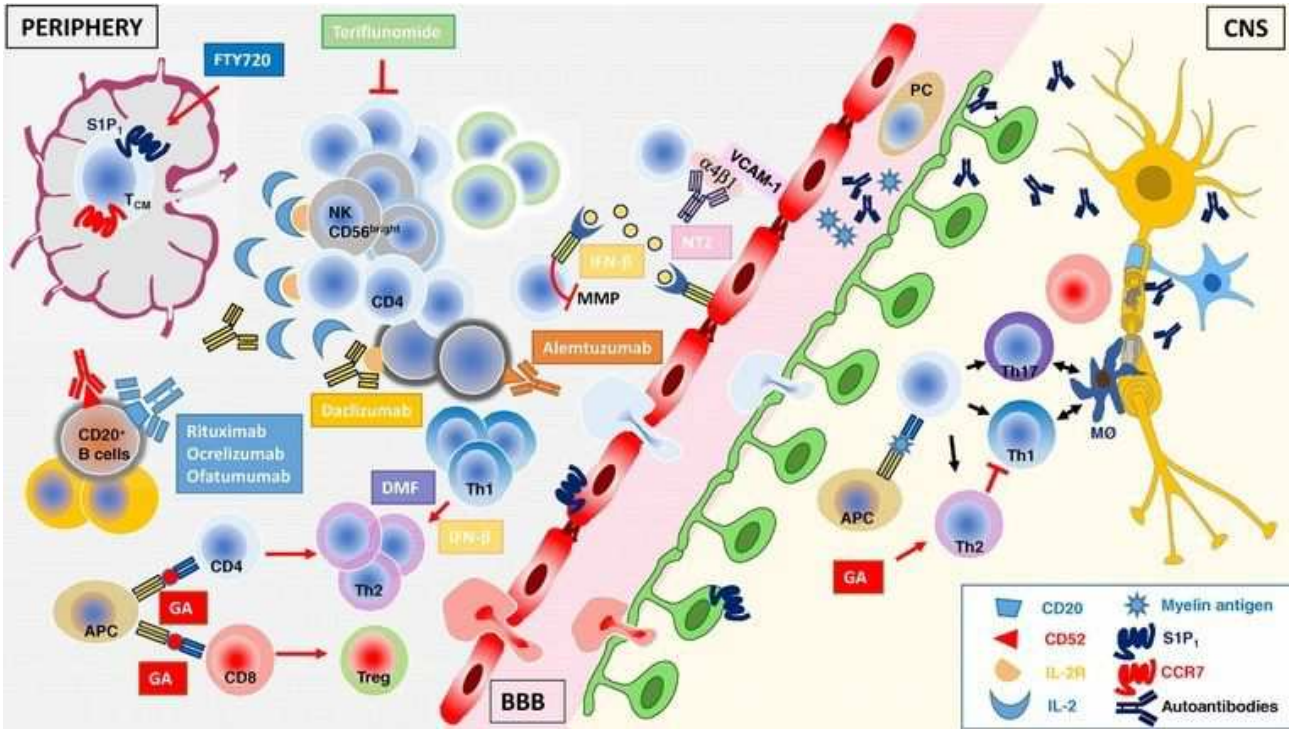


Figure 9. Schematic representation of the putative cellular and molecular therapeutic targets used in the MS treatment. On the left, cellular and molecular targets in the periphery; in the center, the endothelial BBB; on the right, inflamed CNS parenchyma with a neuron (yellow) and its myelinated axon and the oligodendrocyte (blue) is shown [*Martin, 2016*].

EXTRACELLULAR VESICLES

Cell-to-cell communication is an essential hallmark of multicellular organisms required to assure proper coordination among several cell types. Communication can be mediated through

direct cell-cell contact or transfer of secreted molecules. Recent studies have suggested that cells may also communicate by the release of membrane vesicles [*Camussi, 2010*]. For a long time, microvesicles (MVs) were considered to be an *in vitro* artefact or inert cellular debris until De Broe et al. [*De Broe, 1977*] suggested that MVs released from human cells result from a specific process. Because of their small size, upon release, EVs can move from the site of discharge by diffusion and be retrieved in several biological fluids, for example, CSF, blood, urine .

Two classes of membrane vesicles have been described on the basis of size, content and mechanism of formation [*Morel, 2011; Bartneva, 2013*]: exosomes (EXOs) and, shed vesicles (MVs).

EXOs are a population of small membrane vesicles (30-120 nm in diameter) released by an endocytic pathway (**Fig. 10**) [*Camussi, 2010*]. EXOs formed by a series of processes involving the endosomal sorting complex required for transport (ESCRT) and multivesicular bodies (MVBs) [*Février and Raposo, 2004; Schneider and Simons, 2013*]. The first step is the invagination of the plasma membrane forming a vacuole. Once that process is completed, the ESCRT facilitates the development of the vacuoles into early endosomes. The early endosomes can fuse with endocytic vesicles, where they accumulate and mature into MVBs. These MVBs can either fuse with the lysosome if the content is destined for degradation or they can fuse with the plasma membrane releasing EXOs into the extracellular space [*van der Pol, 2010; Frühbeis, 2012; Abels and Breakefield, 2016*]. EXOs are enriched in several proteins and RNAs, such as heat shock proteins, integrins and tetraspanins (CD63, CD9, CD81, CD82) [*Frühbeis, 2012, Théry, 2002; Chivet, 2012*] . They are also characterized by the presence of high levels of cholesterol, sphingolipids, ceramide and glycerophospolipids in their membrane [*Simons and Raposo, 2009*].

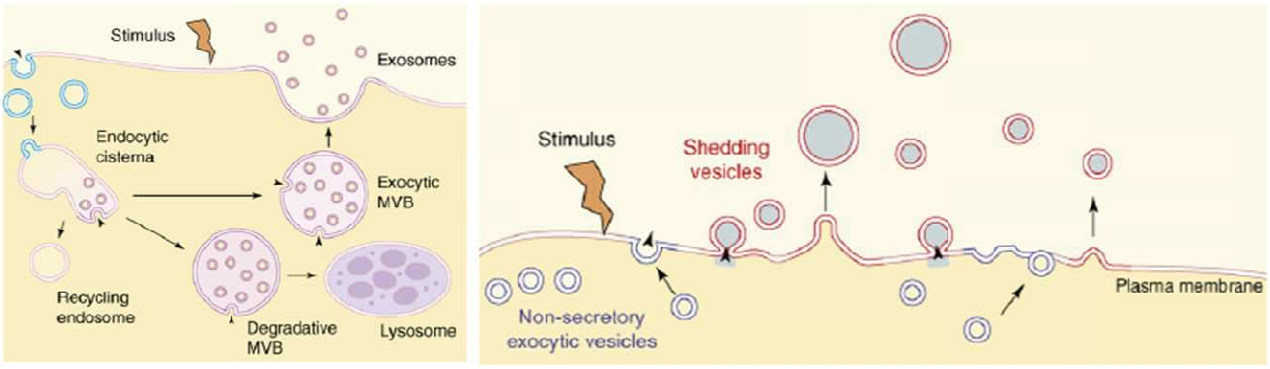


Figure 10. Generation of EXOs and MVs (adapted from Cocucci et al., 2009) [*Sadallah, 2011*].

MVs, also known as shed vesicles or ectosomes [*Sadallah, 2011*], are a population of small vesicles that are more heterogeneous in size (100nm-1μm) and shape as compared to EXOs [*Cocucci, 2009*]. They bud directly from the plasma membrane and are released into the extracellular space in physiological conditions, but their number is often increased upon cellular activation (**Fig. 10**). Several mechanisms are responsible for the shedding of MVs. In general, MVs appear to be formed though the outward budding and fission of the plasma membrane following a cytoskeletal reorganization and plasma remodeling [*Schindler, 2014*]. In terms of composition, MVs contain a variety of cell surface receptors, intracellular signalling proteins and nucleic acids (mRNA, microRNA, DNA) derived from the cell of origin. Therefore, MVs reflect the differential composition of proteins of various donor cells, but their content depend also on the state (e.g. resting, stimulated) of the parental cells and on the stimulus employed for shedding [*Bernimoulin, 2009*]. However, shed vesicles are generally characterized by the presence of high levels of phosphatidylserine (PS) on their surface.

A specialized type of MVs release exists for cells that express the ATP receptor P2X7, which release MVs from the cell surface when exposed to ATP. P2X7 receptor is an ATP-gated ion channel, highly expressed in immune cells, particularly monocytes/macrophages, mastcells and microglia [*Caragnano, 2012; Steinberg and Silverstein, 1987; Cockcroft and Gomperts, 1979; Visentin and Levi, 1997*]. It can act as a selective ion channel or as a nonselective pore

depending on the ATP concentration. Activation of P2X7 receptor can induce efficient inflammosome assembly and rapid secretion of the inflammatory cytokines IL-18 and IL-1β [*Qu, 2007*]. P2X7 receptor differs from other members of the P2X family in its relatively low affinity for ATP and the presence of a long cytoplasmic C-terminus that contains several protein–protein interaction motifs. The activation of P2X7R induces several intra-signalling events, with the involvement of protein kinases and other effector enzymes [*Duan and Neary, 2006*]. In particular, it was known that P2X7-dependent blebbing is preceded by loss of plasma membrane asymmetry and exposure of PS at the outer leaflet of the plasma membrane, commonly accepted as a marker of a cell undergoing apoptosis. Moreover, many studies have shown that blebbing induced byP2X7 receptor requires P38 MAP kinase and ROCK activation, with following disassembly of the cytoskeletal elements, similarly to apoptotic blebbing [*Turola, 2012*]. However, it has been showed by Surprenant and colleagues that P2X7-dependent blebbing occurs within few minutes after receptor activation and may be a reversible event [*MacKenzie, 2001*]. Therefore, ATP stimulation time determines cell fate. In the model proposed by Surprenant, upon P2X7R-mediated macrophage activation, IL-1β is packaged into small plasma membrane blebs that are released into the extracellular space as MVs [*MacKenzie, 2001*]. These results contributed to the first evidence that P2X7R may act as a key player in IL-1β release and may represent a general mechanism for protein secretion from P2X7-expressing myeloid cells. Bianco et al. [*Bianco, 2009*] found that MV shedding from microglia is controlled by acid sphigomyelinase (A-SMase), a key enzyme that controls hydrolysis of sphigomyelin to ceramide. Following P2X7 receptor activation, p38 MAP kinase is phosphorilated; this in turn induces the translocation of A-SMase to plasma membrane outer leaflet, generating ceramide, and thereby inducing budding of MVs (**Fig.11**) [*Bianco, 2009*].

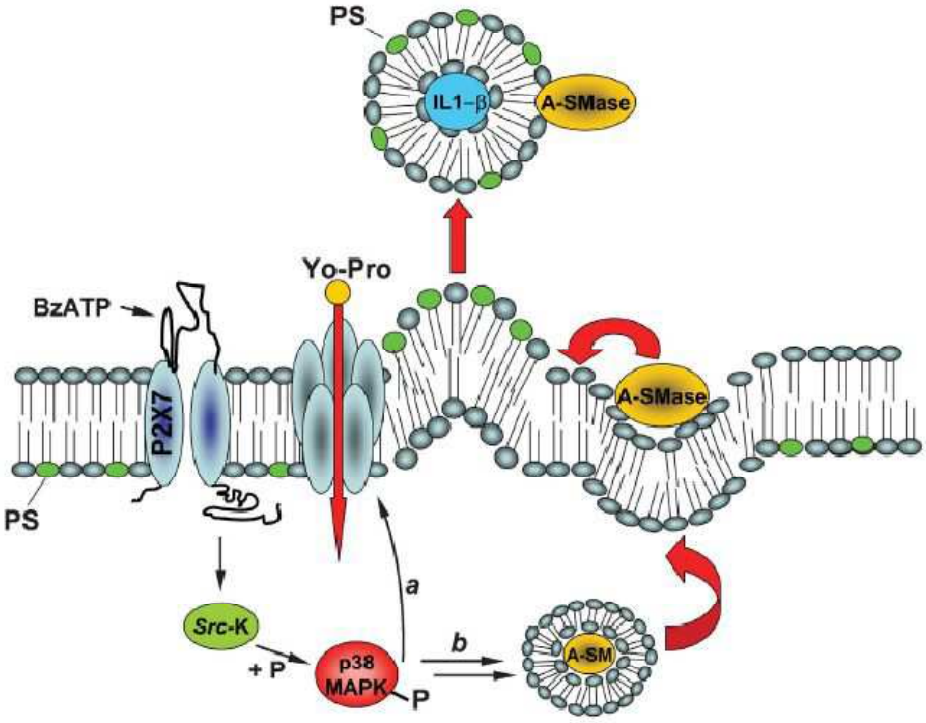


Figure 11. Model for P2X7 receptor-induced signaling pathway involved in MV shedding proposed by Bianco et al. [*Bianco, 2009*]**.** Stimulation of P2X7 receptor with BzATP, a synthetic agonist of ATP, leads to Src-k phosphorylation that in turn activates p38 MAP kinase. This process induces A-SMase translocation from luminal lysosomal compartment to plasma membrane outer leaflet, altering membrane fluidity with IL-1β-MVs release.

Based on the key role of A-SMase in MVs formation, it is possible to inhibit P2X7-induced release using pharmacological or genetic tools for the inactivation of the enzyme. Bianco et colleagues have showed that both approaches strongly abolished MV shedding and of IL-1β release from reactive glial cells [*Bianco, 2009*].

EVs from different cell types reflect composition and activation status of parental cells, thus interacting with target cells through specific receptors and leading to several responses in the target cells. EVs may act in multiple ways (**Fig.12**), for example, as a "signaling complex" by direct stimulation of target cells, a transfer of membrane receptors, a delivery system of proteins or genetic materials [*Camussi, 2010*].

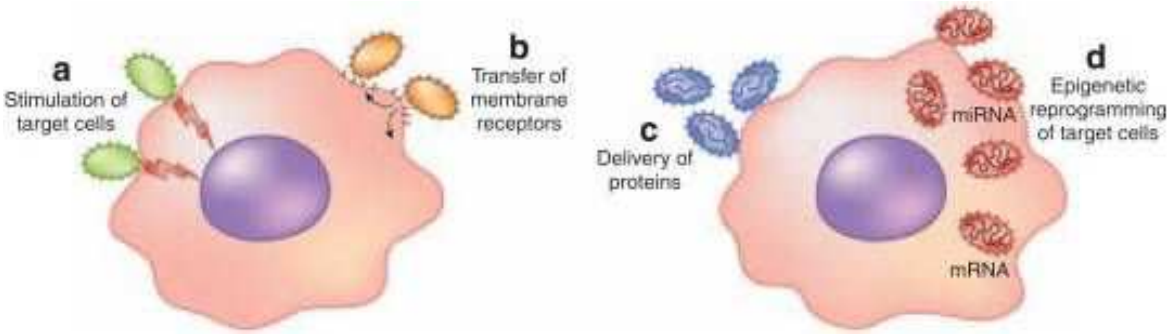


Figure 12. EVs biological activities. Schematic representation of activities induced by EVs in the target cells [*Camussi, 2010*].

It is now recognized that EVs are an integral part of the intercellular communication and the role of EVs has been reported inseveral physiological and pathological process such as cell proliferation, coagulation, vascular function, apoptosis, inflammation and tumor progression.The most well characterized membrane vesicles are those released from blood cells, i.e. platelets, leukocytes, erythrocytes, and endothelial cells. However, accumulating evidences demonstrate that MVs and EXOs can also be released by brain cells and play an important function in the CNS both in pathologic or physiological conditions. A number of studies have demonstrated the involvement of EVs in different scenarios, such as in neuronal development, synaptic activity; and nerve regeneration [*Schindler, 2014*]. It has been observed that EVs are released by neural cells, oligodendrocytes, neurons, microglia, astrocytes in the brain, and Schwann cells in the peripheral nervous system [*Sáenz-Cuesta, 2014b*].

Several studies demonstrate that EVs play an active role during pathogenesis of MS and EAE. Today researchers attention has been focused on the study of EVs as potential biomarkers and therapeutic targets that could have a possible involvement in MS pathogenesis. Increased numbers of MVs have been observed in the blood and in the CSF of MS patients as compared to healthy controls (HCs) and a role for EVs has been proposed in inflammatory progression and lesion repair [*Sáenz-Cuesta, 2014b*]. Endothelial cells, in addition to platelets, leukocytes, myeloid cells, and astrocytes, shed EVs containing metalloproteases and caspase 1 that promote BBB disruption and lymphocytes and myeloid cell transmigration into CNS [*Carandini, 2015*].

Also activated T cells release EVs containing the chemokine CCL5 and arachidonic acid, which is responsible for recruitment of monocytes and upregulation of ICAM-1 on endothelial cells and LFA1 and Mac-1 on monocytes [*Sáenz-Cuesta, 2014b; Barry, 1998*]. Taken together, these data suggest that EVs may promote the migration of lymphocytes and myeloid cells through the BBB promoting critical pathogenic step in the development of disease in MS. Concerning studies on CSF, the first evidence of the EVs presence in CSF of MS patients is given by Scolding et al. [*Scolding, 1989*]. Recently, Verderio et al. demonstrated that the numbers of myeloid cell-derived EVs in human CSF was higher in patients than controls. In particular, higher number of CSF EVs has been associated with the acute phase of MS, suggesting their role in the disease severity and microglia activation [*Verderio, 2012*]. In line with this, in EAE mice the concentration of CSF EVs reflects the course and severity of EAE. Unfortunately, the evaluation of CSF-derived EVs in the human samples are difficult to perform both due to shortage of material usually available, and for impracticality to perform serial lumbar punctures to assess EVs' trend over time. For these reasons, today many studies are directed to find a correlation between EV levels in the peripheral blood and some clinical parameters. A relationship between EVs number in plasma and MS disease progression was first proposed by Minagar et al. [*Minagar, 2001*]. They showed that a high plasma level of endothelial CD31+ EVs were associated to acute phase of the disease, whereas, endothelial CD51+ EVs were present during relapse and remission [*Minagar, 2001*]. Subsequently, the same group demonstrated that most endothelial EVs form complexes with other cells, especially monocytes [*Jy, 2004*]. Interestingly, differences in MVs amount from several cell types were observed. As a matter of fact, Sáenz-Cuesta et collaborators [*Sáenz-Cuesta, 2014a*] reported a significant difference in CD61+ (platelet marker), CD45+ (lymohocyte marker), and CD14+ (monocyte marker) MVs counts in sample from MS patients compared to healthy donors (HDs). MVs were especially high in RRMS patients, while SPMS patients were similar to HDs.

Since it has been postulated that EVs may have an active role in the immunopathogenesis of MS and they could be used as biomarkers of BBB damage, it is plausible to consider them as a marker of drug efficacy. In some cases, including IFN-β, Natalizumab, and Fingolimod, the effect of the therapy on EVs was evaluated. The effect of IFN-β 1b was first explored by Jimenez et al [*Jimenez, 2005*] who reported an inhibitory effect on endothelial-EVs production in plasma from MS patients and on monocyte-endothelial EVs complex formation. Sheremata et al. [*Sheremata, 2006*] showed that IFN-β 1a was able to reduce the number of CD31+ endothelial MVs in plasma of RRMS patients after 12 weeks of treatment. Also Lowery-Nordberg et colleagues [*Lowery-Nordberg, 2011*] tested the effect of IFN-β on endothelial EVs release. They found that CD31+ and CD54+ endothelial MVs were significantly reduced by treatment with IFN-β (Rebif 44) [*Lowery-Nordberg, 2011*]. On the contrary, in a recent study performed by Sáenz-Cuesta et al. [*Sáenz-Cuesta, 2014a*], higher numbers of plasma platelet MVs, lymphocyte MVs, and monocyte MVs, were observed in IFN-β and NTZ-treated patients. These results are in contrast also with results obtained in our laboratory [*Blonda, 2017*] in which we have observed that MVs production was reduced after IFN-β treatment.

Dawson et al. demonstrated that Fingolimod inhibits A-SMase [*Dawson and Qin, 2011*], the enzyme that controls MVs production [*Bianco, 2005*]. Experiments in an EAE model performed by Verderio et al. [*Verderio, 2012*] confirmed that Fingolimod treatment reduced myeloid EVs in the CSF. Despite these conclusions, there have so far been no reports evaluating the effect of Fingolimod on EVs in humans.

In conclusion, there is still incomplete information on the role of EVs in MS, but their role could be relevant in the pathogenesis of the disease opening a new scenario in MS biomarkers evaluation, in therapy choice, and in therapy effectiveness.

MicroRNA

MicroRNAs (miRNAs) are small single stranded non-coding RNAs of 21-23 nucleotides which have recently been discovered to be post-transcriptional modulators of gene expression, either by targeting mRNA degradation or by stopping protein translation. In the most cases, they are transcribed by RNA polymerase II as long primary miRNAs (pri-miRNAs) in the nucleus. The pri-miRNAs is cleaved by Drosha RNAse III endonuclease forming a pre-miRNA, which is exported into the cytosol by exportin-5 and further processed by another RNAse III endonuclease Dicer into mature miRNAs, which is double stranded. This miRNA is unwound and a strand is incorporated into the RNA-induced silencing complex (RISC), which mediates mRNA translational inhibition or mRNA degradation (**Fig.13**) [*Bartel, 2004*].

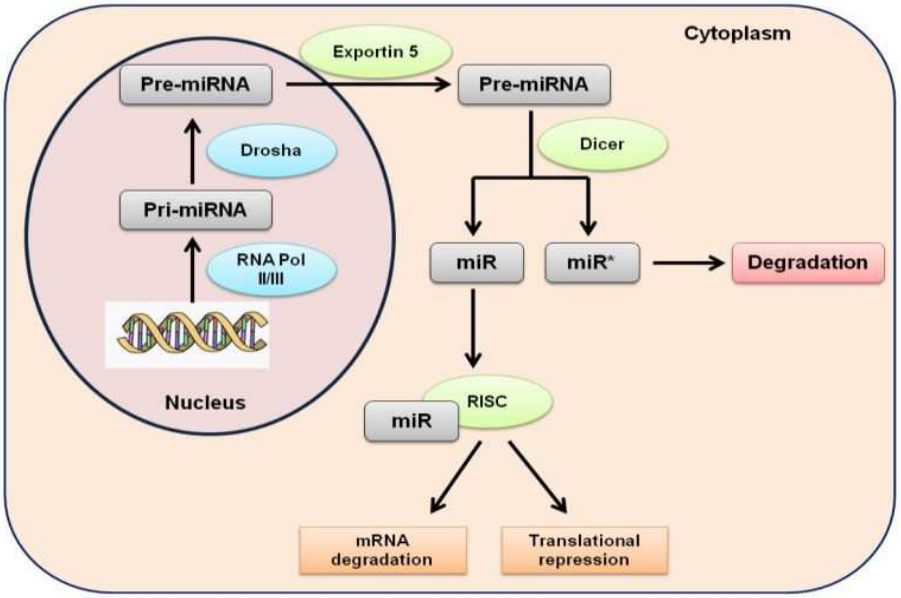


Figure 13. Biogenesis of human miRNAs [*Fenoglio, 2012*].

It has been shown that an individual miRNA is able to control the expression of more than one target mRNA and that each mRNA may be regulated by multiple miRNAs.

miRNAs are important in several biological processes, such as cell proliferation, differentiation, metabolism, apoptosis, inflammation and immunity. Their expression could also affect a number of important human diseases, including cancer, neurodegeneration and autoimmunity [*Fenoglio, 2012*].

miRNAs can be secreted into extracellular environment and delivered to distant target tissues with remarkable stability [*Ge, 2014*]. These miRNAs can be included in EVs acting as messengers mediating cell-cell communication [*Patton, 2015*]. Transport of miRNA by EVs significantly increases their biological relevance since they can modulate genetic behaviour of the target cell.

In recent years, there has been a significant focus on understanding the role of miRNA in the pathogenesis of the MS. Based on their high expression in immune cells as well as in CNS cells, miRNAs could play an important role in the disease mediating pathogenetic mechanisms. The expression of miRNAs in MS was first assessed both in blood-derived cells and in active lesions of MS patients. Recent studies have focused their attention on the miRNA expression in biological fluids, such as serum and CSF [*Jagot and Davoust, 2016*]. The main approach of miRNA profiling studies is based on a microarray analysis in combination with quantitative PCR. Recently also Next Generation Sequencing (NGS) technique was used to assess miRNA expression in MS patients [*Jagot and Davoust, 2016*]. Three groups of miRNAcould be distinguished. First, specific miRNA within CNS lesions, secondly, specific miRNA controlling several aspects of immunity, and thirdly, circulating miRNA found in biological fluids of MS patients.

In the first report of a comprehensive miRNA expression analysis in MS lesions, Junker et al. observed that the most strongly upregulated miRNAs in active MS lesions were expressed by astrocytes. Among these, miR-155, miR-326 and miR-34a were found to be upregulated in active MS lesions in comparison with inactive lesions or normal brain white matter. Interestingly, these miRNAs all target CD47, a regulatory membrane protein found to be downregulated in phagocitically active MS brain lesions [*Junker, 2009*].

miRNAs, which control several aspects of immunity are also involved in MS pathogenesis. The first study about the role of miRNAs and T cells in MS was described by Du et al. [*Du, 2009*]. They identified miR-326 to be increased in Th17 cells isolated from RRMS patients and

correlated with disease severity. They also found that miR-326 is associated with Th-17 cell development by inhibiting Ets-1, a negative regulator of Th-17 differentiation [*Du, 2009*]. Lindberg et al. [*Lindberg, 2010*] observed differentially expression ofmiR-17-5p, miR-92, miR-193a and miR-497 dysregulatedin T and B cells of MS patients. The target prediction of these miRNAs have revealed target genes involved in lymphocyte development, activation and survival [*Lindberg, 2010*]. De Santis et al. [*De Santis, 2010*] found miR-106, miR-25, miR-19a and miR-19b significantly overexpressed in Treg cells of MS patients versus HCs. These miRNAs modulate the TGF-β signalling pathway altering Treg cell functions in MS [*De Santis, 2010*]. Another study by Guerau-De-Arellano et al. [*Guerau-De-Arellano, 2011*] suggested increased expression of miR-128 and miR-27b in naive CD4+ T cells and miR-340 in memory CD4+ T cells of patients with MS. Guerau-de-Arellano et al. demonstrated that these miRNAs are involved in suppressing Th2 differentiation via BMI1 and IL-4 repression and in predisposition to the development of an autoimmune Th1 response [*Guerau-De-Arellano, 2011*].

Several studies performed miRNA profiling in MS patients using peripheral blood mononuclear cells (PBMCs) or whole blood. All reports showed dysregulatedmiRNA expression levels in MS patients compared to HCs. Otaegui et al. [*Otaegui, 2009*] analyzed the expression patterns of 364 miRNAs in PBMCs from MS patients in relapse phase, in remission phase, and in HCs using real time (RT)-qPCR. Results revealed increase of miR-18b, miR-493, and miR-599 in relapsing patients compared to the HCs, whereas miR-96 was dysregulated in remission phase. Interestingly, miR-96 has putative gene targets involved in immunological processes such as the interleukin signaling pathway. In another study, miRNA screening was performed by microarray analysis in peripheral blood samples of RRMS patients compared to HCs. They identified miR-145 as the best miRNA significantly associated with MS patients [*Keller, 2009*]. Fenoglio et al. [*Fenoglio, 2011*] found increased levels of immunologically relevant miRNAs, such as miR-21, miR-146a and -b in PBMCs of RRMS patients compared with controls. Martinelli-Boneschi and colleagues [*Martinelli-Boneschi, 2012*] analyzed 104 dysregulated miRNAs in MS patients

compared with controls, finding only 2 miRNAs, let-7g and miR-150, differentially expressed. Altered expression of let-7g was also observed by Cox et al. [*Cox, 2010*] in peripheral blood samples of MS population. In addition, the same study established a reduced expression of miR-17 and miR-20a.These miRNAsregulate T cell activation genes that are upregulated in MS whole blood mRNA, suggesting new approaches for therapy. At last, Waschbisch et al. analyzed a set of miRNAs in PBMCs of RRMS subjects involved in Th17 cell differentiation, regulation of immune tolerance, and innate immunity. They showed upregulation of miR-142-3p, miR-146a, miR-155, and miR-326 in RRMS patients compared to HCs [*Waschbisch, 2011*].

miRNAs can be also detected in several human biological fluids including plasma, serum, urine, and saliva as circulating molecules [*Fenoglio, 2012*]. The stable expression of miRNAs in biological fluids allows to consider them as potential candidate for a biomarker study. Moreover, their stability suggested that miRNAs released from cells in EVs, protecting them from RNase degradation [*Mitchell, 2008*]. Several studies have analyzed circulating miRNAs and their involvement in MS. One of the first studies has been performed by Siegel et colleagues [*Siegel, 2012*] in which they conducted a microarray analysis on plasma samples of MS patients and HCs. The analysis of over 900 miRNAs revealed that only 7 of them are significantly deregulated. Interestingly, they found miR-422 and miR-22 altered, whose involvement in MS was previously demonstrated [*Fenoglio, 2012*]. In another study performed by Fenoglio et al., decreased expression levels of miR-15b, miR-223 and miR-23a in PPMS serum sample were found compared to HCs [*Fenoglio, 2013*]. Target prediction revealed FGF-2 gene as putative gene target of miR-15b and miR-23a. Some evidences have proposed FGF-2, a member of the fibroblast growth factor family, as a marker of inflammation in MS lesions. Elevated levels of FGF-2 were found in the CSF of MS patients, particularly those with the active disease, and differential expression gene was found in active and chronic MS lesions in post-mortem tissue [*Fenoglio, 2012*]. MiR-223 targets the transcription factor mef-2c (myocyte enhancer factor 2C), which plays a role in the regulation of granulopoiesis and modulates the NF-κb pathway

influencing immune inflammatory responses [*Fenoglio, 2012*]. Recently, Selmaj and colleagues have investigated the total circulating exosome transcriptome in RRMS patients and HCs, revealing a distinct RNA profile in RRMS patients. Interestingly, their results suggest that miRNAs might be used as biomarker to define RRMS activity and indicate a disturbed intercellular communication [*Selmaj, 2017*].

Regarding miRNA analysis in CSF of MS patients, some studies have demonstrated the relevance of miRNAs as potential useful biomarkers of the disease. In the first report of miRNA expression analysis, Haghikia et al. showed differentially expressed levels of miR-922, miR-181c, and miR-633 in MS patients as compared with other neurological diseases (OND) [*Haghikia, 2012*]. A more recent report by Bergman and collaborators [*Bergman, 2016*] showed higher levels of miR-150 in CSF of MS patients, suggesting that this miRNA might be considered a putative biomarker of inflammatory active disease for early diagnosis of MS.

Although these studies highlighted miRNAs involvement in MS pathogenesis, there is a growing interest in studying miRNAs levels in order to define new potential biomarkers of the disease. It is known that the role of other miRNAs has been investigated. Some of these may be interesting because their involvement is not only exclusive in the regulation of the immune response but also they could be involved in the regulation of synaptic function, myelin production and oligodendrocytes differentiation. Moreover, it has demonstrated that some miRNAs could be found in MVs and EXOs [*Prada, 2018*]. Consistently, miR-146a, miR-181a and miR-223 have been previously observed to be deregulated in CNS, plasma and/or immune cells of MS patients [*Jagot and Davoust, 2016*] (**Fig.14**), but also involved in neurodegenerative diseases [*Prada, 2018*]. Others miRNAs, such as mir-23a, miR-125a, miR-30c, miR-155 and miR-124 could be implicated in neurodegenerative disease and immune response. As a matter of fact, these miRNAs share the characteristic to have as putative gene targets both proteins or cells of immunity system and CNS. At the same time, these miRNAs could be transferred by EVs and modulate the molecular and genetic behavior of target cells [*Cocucci and Meldolesi, 2015*].

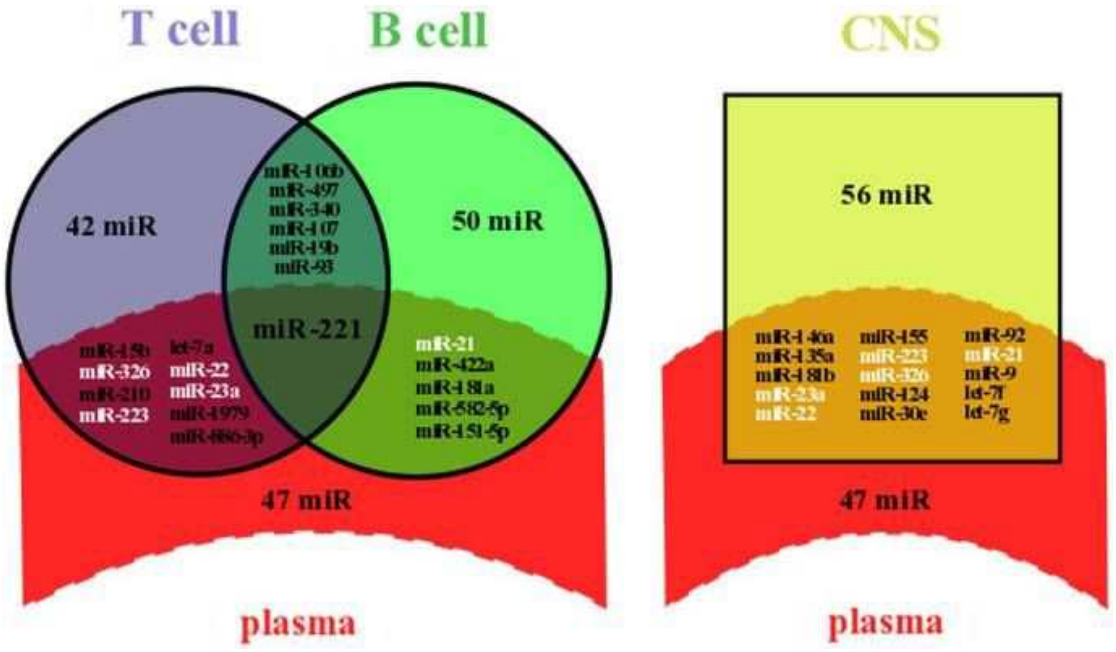


Figure 14. Panel of dysregulated miRNAs that overlap in plasma and lymphocytes or in plasma and CNS of MS patients. miRNAs written in white color are dysregulated in at least three compartments [*Jagot and Davoust, 2016*].

In particular, among predicted targets of miR-146a there are three genes well characterized in neuronal cells that are important for synaptic functionality: neuroligin 1 (Nlgn1), neuro-oncological ventral antigen 1 (Nova 1), and synaptotagmin 1 (Syt 1) [*Jovičic, 2013*].

An interesting miRNA that could be have a role in the MS disease is lymphocyte specific miR-181a. MiR-181a is mainly known for its role in T cell development [*Neilson, 2007*], but has also been shown to be important for neuron development [*Liu, 2013*] and synaptic function [*Saba, 2012*].

MiR-223 could modulate immune inflammatory responses in several ways (induction of M2 polarization [*Essandoh, 2016*], upregulation of CD4+ T cells [*Hosseini, 2016*]), but it could inhibit neuronal differentiation targeting Mefc2 [*Jovičic, 2013*].

Another cited miRNA implicated in inflammatory response is miR-23a that seems also to be involved in regulation of myelin maintenance and oligodendrocytes differentiation targeting Lamin B1 [*Lin, 2013*].

MiR-125a has a role in the macrophage polarization and regulation of inflammatory response [*Essandoh, 2016*], but it targets the post synaptic density protein PSD-95, which is essential for glutamate receptor localization and synapse function [*Stefanovic, 2015*].

MiR-30c has some predicted gene targets critical for T cell activation and signaling, e.g., Kras and Vav3 [*Zeitelhofer, 2017*], moreover it seems to have implications in axon and dendritic growth and in neuronal migration, targeting Semaphorin 3A. This miRNA is more abundant in the brain but there is no evidence of its involvement in MS [*Sun, 2016*].

miR-155 has a pathogenic role in promoting Th1 and Th17 differentiation, as discussed above, and microglia activation [*Jagot and Davoust, 2016*].; but it could modulate the stability and translation of mRNAs implicated in axon growth targeting BDNF [*Varendi, 2014*].

The neuronal miR-124 is involved in demyelination processes [*Dutta, 2013*] but it also seems to be involved in promoting anti-inflammatory responses and M2 polarization in macrophages [*Essandoh, 2016*].

To sum up, the recent discovery of an involvement of miRNAs in MS should greatly improve the diagnosis and management of the disease. MiRNAs could be an advantageous biomarker compared to mRNA or proteins, since they are very stable molecules. Their proven biological relevance in several cellular contexts and diseases opens a new scenario in the research of the new therapeutic targets. In a very near future, the broad spectrum of miRNA profiling methods available should help achieve this lofty goal.

AIMS OF THE STUDY

EVs, a recently described way for intercellular communication, are membranous vesicles that bud directly from the plasma membrane (MVs) or following to exocytosis of multivesicular bodies (EXOs). EVs are released by most cells types both in physiological and pathological conditions, such as MS. Evidence indicates that the rate of MVs shedding is strongly increased in immune cells, especially of the myeloid lineage, upon stimulation with ATP on its cognate receptor such as P2X7 and it is associated with activation of A-SMase. Because of their small size, MVs can move from the discharge site by diffusion and be released in several biological fluids, such as plasma, serum, urine, and CSF. It has been shown that the amount of microglial MVs in human CSF increased in MS patients compared to HDs. Unfortunately, it is very difficult to carry out studies of EVs in the CSF of MS patients. For this purpose, several studies focused on the EVs analysis in more accessible biological fluids, such as blood. Data from an our previous research project showed that MVs production could be influenced by immunomodulatory treatments for MS, such as IFN-β and TF, as well as P2X7R and IL-1β expression.

In this study project we firstly evaluated how Fingolimod, a second line treatment for MS, may affect MVs production by the monocytes of the affected patients, as well as P2X7R, IL-1β expression and A-SMase activity.

Moreover, accumulating evidence indicates that EVs may contain and transfer between cells small non-coding RNAs (miRNAs), which are dysregulated in the immune system and CNS of MS patients and are emerging as diseases biomarkers.

Therefore, as a second aim, we evaluated EV miRNA cargo and the relative expression of the same miRNAs in parental cells of MS patients and HDs. A set of specific miRNAs involved both in immune response and synaptic functions was evaluated. Despite of these miRNAs are not exclusive biomarkers of MS pathogenesis, their expression may provide interesting information about inflammatory response.

MATERIAL AND METHODS

Patients and Controls

Thirty-seven relapsing-remitting MS patients (12M/25F, mean age 35±9, EDSS 2.8±1.3) were selected from the MS Center, Neurology Unit, Department of Medical and Surgical Sciences, University of Foggia. All the patients were clinically inactive at the time of blood withdrawal, therefore totally steroid free. Eighteen healthy donors (HDs) (11M/7F, mean age 37±9) were also included in the study and similarly investigated. Venous blood sample (20 ml each) were collected in EDTA tubes in order to obtain purified monocytes from total peripheral blood mononuclear cells (PBMCs). Patients had received no treatment during the past 3 months. A subgroup of 19 RRMS patients (8M/11F, mean age 35±10, EDSS 2.7±1.4) started assuming Fingolimod treatment and monocytes were obtained from blood sample after 12 months of treatment. For miRNAs analysis, 21 RRMS patients (6M/15F, mean age 38±9, EDSS 2.9±1.4) and 14 PPMS patients (2M/12F, mean age 47±11, EDSS 5.9±1.5) were selected from MS Center of the San Raffaele Hospital of Milan and MS Center, Neurology Unit, Department of Medical and Surgical Sciences, University of Foggia. Sixteen HDs (10M/6F, mean age 45±11) were similarly investigated. Criteria for volunteer selection consist of no recent illness or treatment for a chronic medical condition. No medical history was obtained from donors.

Ethics Statement

The study has been reviewed and approved by the Ethical Committe of Ospedali Riuniti Foggia/University of Foggia and San Raffaele Hospital of Milan. Patients and HDs were given an informed consent to sign before blood samples were collected.

Preparation of monocytes

PBMCs from patients with MS and HDs were isolated from freshly drawn venous blood by density centrifugation, using a Lymphosep®, Lymphocyte separation Media (d=1,077 g/ml)

gradient (Biowest) as described by the manufacturer. Cells were resuspended at a concentration of $1x10^8$ cells/ml in Recommended Medium (PBS 1X, 2%FBS, 1mM EDTA) adapt to realize monocyte positive selection by monoclonal CD14 antibody-conjugated microbeads using EasySep® Human CD14 Selection kit (Stemcell Technologies) according to the manufacturer's protocol. Briefly, 20 µl of EasySep® Positive Selection Cocktail were added to the resuspended cells and, after mixing well, cells were incubated at room temperature for 15 min. 20 µl of EasySep® Magnetic Nanoparticles were added subsequently and incubated for 10 min. The cell suspension was brought up to a total volume of 2.5 ml by adding recommended medium and incubated at room temperature for 5 min into the magnet. The EasySep® magnet was picked up, and in one continuous motion the magnet and tube were inverted, pouring off the undesired fraction. After removing the tube containing monocytes from the magnet, the cells were resuspended in RPMI 1640 medium (Sigma) enriched with FBS at 10% (Gibco Invitrogen). 10 µl of the cell suspension was mixed with 10 µl of 4% trypan blue solution and live/dead cells were counted in a Bio-Rad TC-10 Automated Cell Counter in order to plate $1x10^6$ cells into 35 mm dishes and they were then incubated at 37 °C and 5% CO_2 for 2 hours, the time required to let the monocytes adhere to the plate.

MVIsolation and Quantification

Monocyte-derived MVs were isolated by differential centrifugation following the method described by Bianco et al. [*Bianco, 2009*]. Briefly, monocyte cultures were washed with PBS and then labelled with the fluorophore-conjugated phosphocholine compound NBD C_6-Sphingomyelin dye (5µM) for 5 min. After extensive washing, monocytes were exposed for 30 min to 200 µM BzATP dissolved in 1 mL of Krebs-Ringer solution (KRH, 125mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO, 2mM $CaCl_2$, 6mM D-glucose, and 25mM HEPES/NaOH, pH 7.4) at 37 ^oC or to KRH without BzATP as control. Collected supernatants containing shed MVs were centrifuged at 300 x *g* for 10 min at 4 ^oC two times in order to discard cells and

debris. Finally, total green fluorescence of shed vesicles present in the 200 µl of supernatant was assayed at 485/535 nm with a spectrophotometric system (F5 Filter Max – Molecular Devices). Arbitrary fluorescence units (AU) of BzATP-stimulated monocytes were compared with arbitrary fluorescence units of unstimulated monocytes.

Extracellular Vesicles Purification

In order to study the release of EVs, monocyte cultures from MS patients and HDs were stimulated with BzATP as described previously. MVs and EXOs were isolated by differential centrifugation in the following way: collected supernatants were centrifuged at 300 x *g* for 10 min at 4 ^{o}C for two times. The supernatant (SN) was carefully removed and centrifuged at 10,000 x *g* for 45 min at 4 ^{o}C for collecting MVs. The SN was again collected, EXOs were pelleted from SN by ultracentrifugation at 100,000 x *g* for 60 min at 4 ^{o}C. All the EV-enriched pellets were re-suspended and lysed in 800 µl Trizol, and stored at -80^{o}C .

RNAPurification and QuantitativeReal-Time PCR

Total RNA was isolated from monocytes using the PureLink® RNA Minikit (Ambion By Life Technologies), according to the manufacturer's instructions. On-column digestion of DNA during RNA purification was also performed using PureLink^{TM}DNase to exclude possible genomic contamination. The concentration of RNA was determined by measuring the absorbance at 260 nm with NanoDrop 1000 Spectrophotometer.

RNA (100 ng) was reverse transcribed into cDNA using High Capacity cDNA Reverse Trascription Kits (Applied Biosystem). The resulting cDNA transcript were used for PCR amplification using TaqMan® Universal Master Mix II (Applied Biosystems) and gene specific Taqman® assays (Applied Biosystems) for P2X7R (Hs00175721_m1), IL-1β (Hs00174097_m1) and SMPD1 (Hs03679347_g1).Quantitative real-time PCR was performed with a Step One Real

time PCR system (Applied Biosystems). The relative mRNA levels were calculated using the comparative Ct method, using beta-actin (Hs01060665_g1) as endogenous control.

Total RNA Isolation and Quantitative Real-Time PCR miRNAs Analysis

In order to perform miRNAs analysis, total RNA was isolated from EVs and monocytes using Direct-zol™ RNA MiniPrep kit (Zymo Research), according to the manufacturer's instructions. Concentration of RNA was determined by measuring the absorbance at 260 nm with a NanoDrop 1000 Spectrophotometer. RNA (10 ng) was reverse transcribed into cDNA using Universal cDNA synthesis kit II (Exiqon). The resulting cDNA transcript were used for PCR amplification using ExiLENT SYBR® Green Master Mix II (Exiqon) and miRNA specific primer set (miRCURY LNA™ Universal RT microRNA PCR, Exiqon) for miR-181a-5p, miR-146a-5p, miR-223, miR-23a-3p, miR-30c-5p, miR- 125a-5p, miR-155 and miR-124 (**Table 2**). Quantitative real-time PCR was performed with a QuantStudio™5 Real time PCR system (ThermoFisher Scientific). The relative mRNA levels were calculated using the comparative Ct method, using RNU1A1 reference gene as endogenous control.

Exiqon primers	Cat.numbers
hsa-miR-146a-5p	Ref# 204688
hsa-miR-181a-5p	Ref# 206081
hsa-miR-223-3p	Ref# 205986
hsa-miR-23a-3p	Ref# 204772
hsa-miR-30c-5p	Ref# 204783
hsa-miR-125a-5p	Ref# 204339
hsa-miR-155-5p	Ref# 204308
hsa-miR-124a-3p	Ref# 206026
hsa-RNU1A1	Ref# 203909

Table 2. Exiqon primers used for miRNAs analysis.

Assay for Acid Sphingomyelinase

A-SMase activity was determined using the Amplex Red Sphingomyelinase assay kit according to the manufacturer's protocol (Molecular Probes).

Cells were lysed in RIPA buffer (Sigma-Aldrich) with freshly added protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 13000 x *g* for 10 min, and the supernatant, 50 µg of protein determined by Bradford assay according to the manufacturer's protocol (Bio-Rad), was used for the sphingomyelinase assay.

For A-SMase activity, the reaction was carried out in an acidic buffer (50mM sodium acetate, ph 5.0) containing 5mM sphingomyelin, and the reaction was detected with Amplex reagents in 100 mM Tris-HCl (ph 8.0, optimal pH for Amplex Red detection). First, to generate phosphocholine and ceramide, 5mM sphingomyelin (SM) was added to the supernatant fraction and incubated for 30 min at 37 ^{o}C. Then, the fluorogenic probe Amplex Red reagent, which is sensitive to H_2O_2, was added and further incubated at 37^{o}C for 60 min to generate H_2O_2 (through alkaline phosphatase hydrolysis of phosphocholine and choline oxidation by choline oxidase to generate betaine and H_2O_2). H_2O_2 in the presence of horseradish peroxidase reacts with Amplex Red to generate the fluorescent resorufin.

The fluorescence was measured in a fluorescence microplate reader at the excitation and emission wavelengths of 535 and 595 nm, respectively. Sphingomyelinase activity of BzATP-stimulated monocytes was calculated as arbitrary fluorescence units and compared with A.U. of unstimulated monocytes.

Statistical Analysis

Statistical comparison between fluorescence of MVs, P2X7R, IL-1β, A-SMase, miRNAs expression betweendifferent groups was performed using the multiple comparison non-parametric Kruskal-Wallis followed by the non-parametric Mann Whitney U test as post-hoc

test, whereas to compare differently *in vitro* stimulated monocytes the non-parametric Wilcoxon test was used. P value <0.05 was considered significant.

RESULTS

1. *Fingolimod reduced MV production*

The mean fluorescence value, which indicates amount of shed vesicles, was higher in MS patients compared to HDs ($p<0.001$) only in unstimulated monocytes (**Fig.15A**), whereas upon BzATP stimulation, only monocytes of HD showed a significant increase of fluorescence ($p<0.001$) (**Fig.15B**). Fingolimod was able to reduce the ATP-induced MV shedding after 12months of treatment in unstimulated monocytes ($p<0.001$) compared to untreated MS patients (**Fig.15A**). It has the same effect also in BzATP stimulated monocytes compared to stimulated monocytes from MS patients ($p<0.05$) (**Fig.15A**). In both conditions, the mean fluorescence value of monocytes of Fingolimod treated patients are similar to the values of HD monocytes.

2. *Fingolimod effect on P2X7R expression*

Since Fingolimod treatment reduced MVs production, we would expect to see the same effect on P2X7R expression in monocytes of treated patients compared to HDs and MS patients. Treatment, instead, increases P2X7R expression in Fingolimod treated patients compared to HDs in both conditions (KRH and BzATP stimulation) (**Fig.16**).

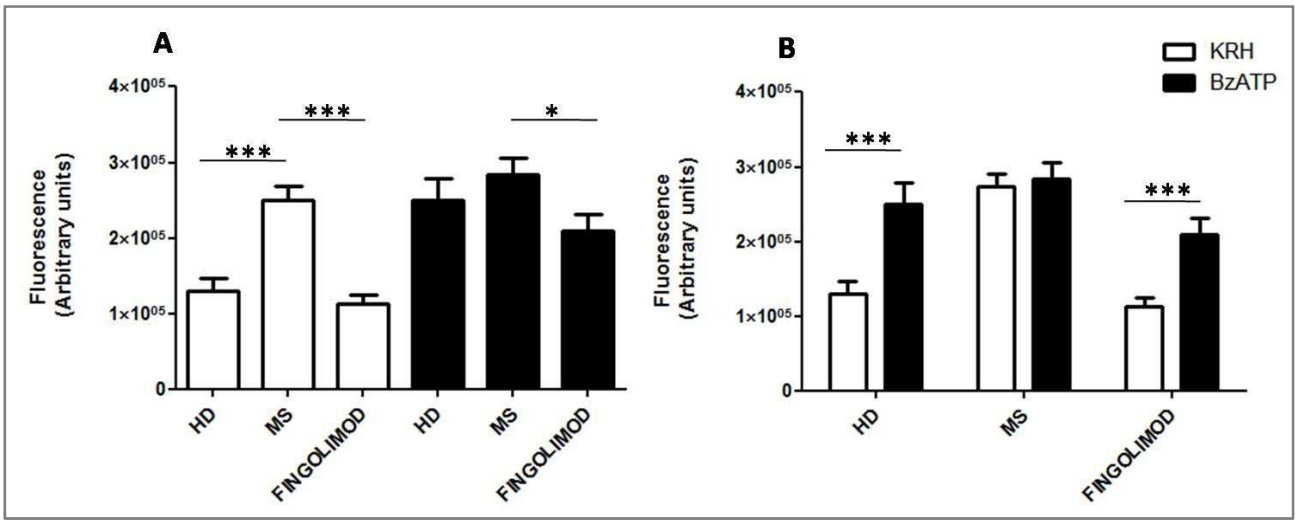


Figure 15. MVs production. Comparison of microvesicles (MV) production between HD, untreated MS patients and Fingolimod treated patients: (A) monocyte-derived MVs released in unstimulated and BzATP-stimulated conditions. (B) Comparison before and after BzATP stimulation of mean fluorescence values of monocyte-derived MVs from HD, MS and Fingolimod treated patients. Data obtained by spectrophotometric analysis are expressed as mean ± SEM of fluorescence arbitrary units (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

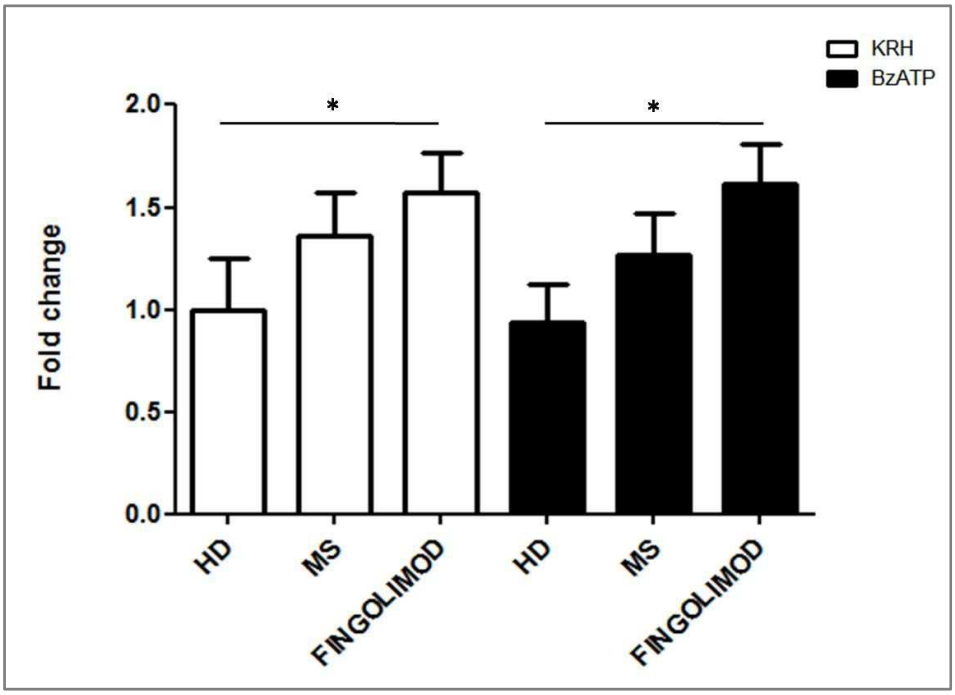


Figure 16. Purinergic P2X7 receptor expression level detected by RT-PCR in unstimulated KRH solution and BzATP-stimulated monocytes in HDs, MS and Fingolimod treated patients. Data are expressed as mean ± SEM of fold change values(*p<0.05; **p<0.01; ***p<0.001).

3. *Fingolimod effect on IL-1βgene expression*

Having assessed that monocytes shed MVs upon BzATP stimulation, we examined mRNA levels for the inflammatory cytokine IL-1β, which production is driven by activation of P2X7R. Comparing untreated MS with HDs, no significant differences were observed for IL-1β mRNA levels, both in unstimulated and BzATP-stimulated conditions. After 12 months of Fingolimod treatment, IL-1β expression is downregulated both in unstimulated and BzATP-stimulated monocytes compared to HD and MS patients, as reported in **figure 17**.

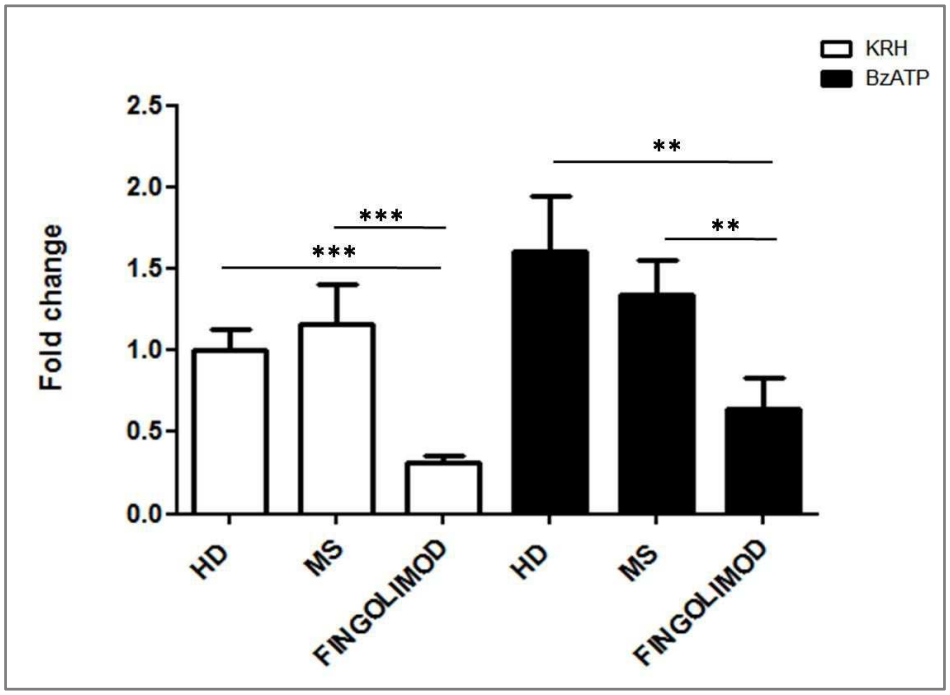


Figure 17. IL-1βexpression level detected by RT-PCR in unstimulated KRH solution and BzATP-stimulated monocytes in HDs, MS and Fingolimod treated patients. Data are expressed as mean ± SEM of fold change values(*p<0.05; **p<0.01; ***p<0.001).

4. *Fingolimod reduced A-SMase enzyme activity but had no effect on A-SMase gene expression*

To investigate the effect of Fingolimod on A-SMase dependent MVs production, we measured enzyme activity and gene expression by Real-Time PCR in monocytes (**Fig.18**). The effect of Fingolimod on A-SMase activity was examined. It was evident a lowering of A-SMase activity in Fingolimod treated-patients in BzATP stimulated monocytes ($p<0.001$) after 12 months of treatment compared to MS patients (**Fig.18A**). The analysis of the real-time performed on A-SMase gene expression showed no differences between HD, MS patients and after Fingolimod treatment (**Fig.18B**).

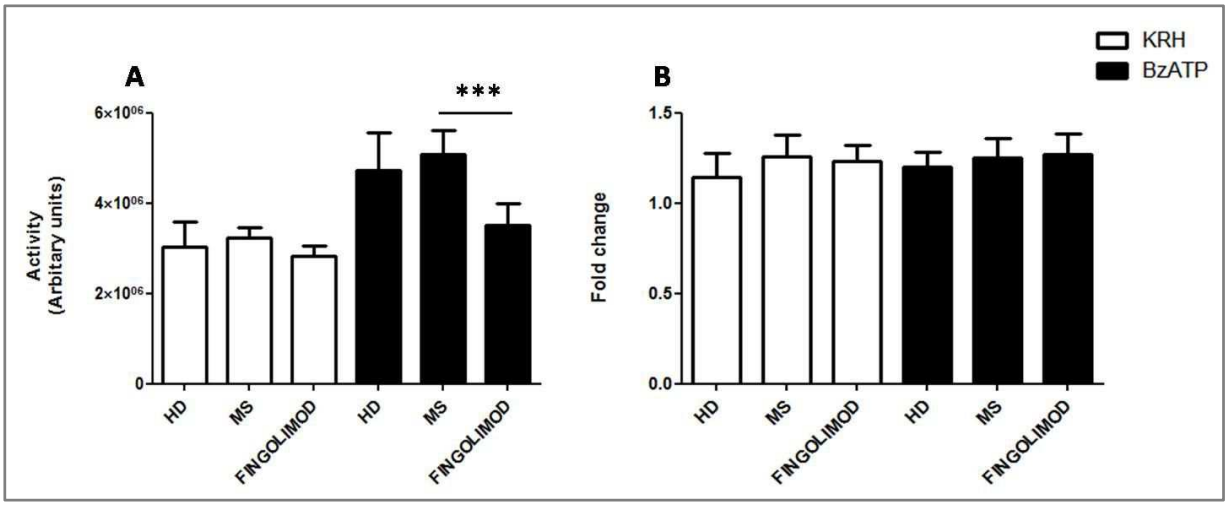


Figure 18. Analysis of A-SMase activity and gene expression. A) A-SMase activity detected by Amplex Red Sphingomyelinase assay kit. Graph showed levels of A-SMase activity in unstimulated KRH and BzATP-stimulated monocytes from HDs, MS and Fingolimod treated patients. Data obtained by spectrophotometric analysis are expressed as mean ± SEM of fluorescence arbitrary units. **B**) A-SMase expression levels by RT-PCR in unstimulated KRH and BzATP-stimulated monocytes in HDs, MS and Fingolimod treated patients. Data are expressed as mean ± SEM of fold change values(*p<0.05; **p<0.01; ***p<0.001).

5. *miRNAs expression levels in EVs from BzATP-stimulated monocytes of HDs and RRMS patients*

Since miRNAs are non-coding sequence expressed in several cells and in EVs, we evaluated the possibility that a set of miRNA were present in EVs. Considering the explorative nature of this study and the small sample size, here we reported some preliminary results about individual (MV/EXO) data without statistical comparison between the subjects' group. In attempt to set a protocol to evaluate the miRNAs expression in EVs from monocytes of HDs and MS patients, one case control and 2 patient cases are considered. The miRNA contents of monocyte-derived EVs were analysed independently, therefore the relative expression was showed as dCT, where RNU1A1 is considered as the endogenous control. Preliminary data showed that miRNAs are not always detectable in MVs, especially in HDs, where MVs are less. In the EXOs fraction, whereas, miRNAs seemed to be more abundant than MVs and are always detectable, both in HDs and MS patients [**Fig.19**].

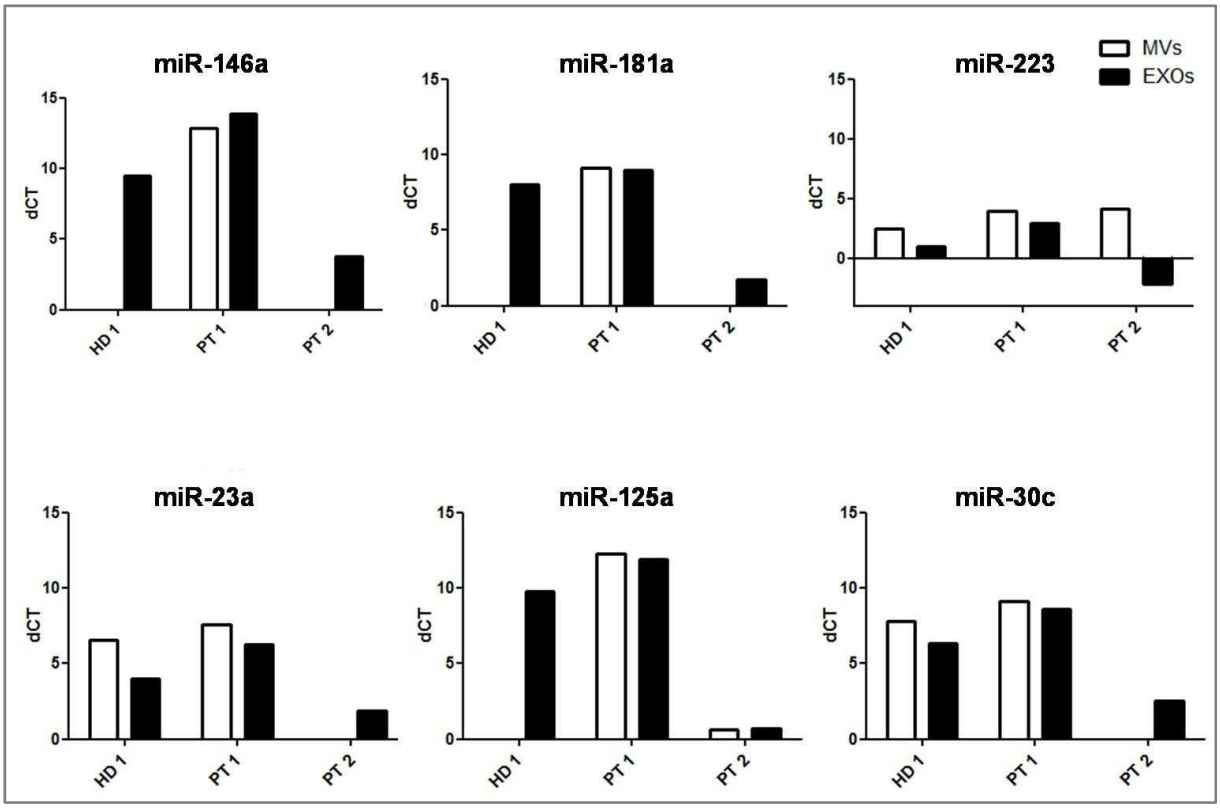


Figure 19. Analysis of miRNAs contents in monocyte-derived EVsby RT-PCR. One case control (HD1) and 2 patient cases (PT 1 and PT 2) are considered. Data are expressed as dCT: CT_{target}- $CT_{reference\ gene}$, where RNA1A1 is considered as the endogenous control.

6. *miRNAs expression levels in pool of EVs from Bz-ATP stimulated monocytes of HDs and RRMS patients*

The same type of analysis was performed in pools of EVs from Bz-ATP stimulated monocytes of HDs and RRMS patients. Pools of EVs in Trizol were collected before RNA extraction. In particular, two pools for HDs were collected, one of 4 HDs, other of 3 HDs. One pool of 5 RRMS EVs patients is collected. Pools collection criteria are based on number of isolated monocytes. In MVs pools from HDs there were no detectable levels of miRNAs, except for miR-223 (**Fig.20**). In EXOs pools from HDs, whereas, a set of miRNAs is detectable, except for miR-181a and miR-125a. In the pool of MVs from RRMS patients miR-146a, miR-181a, miR-223, miR-23a, and miR-30c, were noticed. Analysis performed in EXOs pools revealed expression of all miRNAs tested (**Fig.20**).

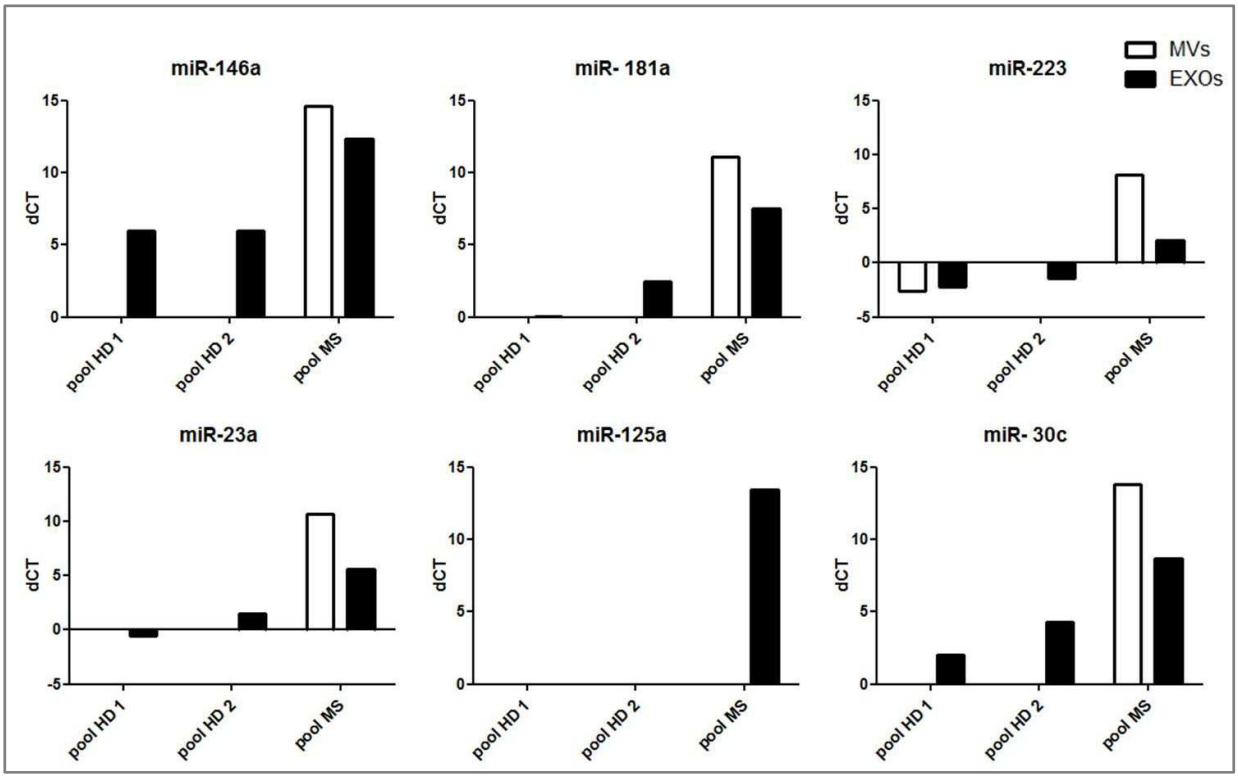
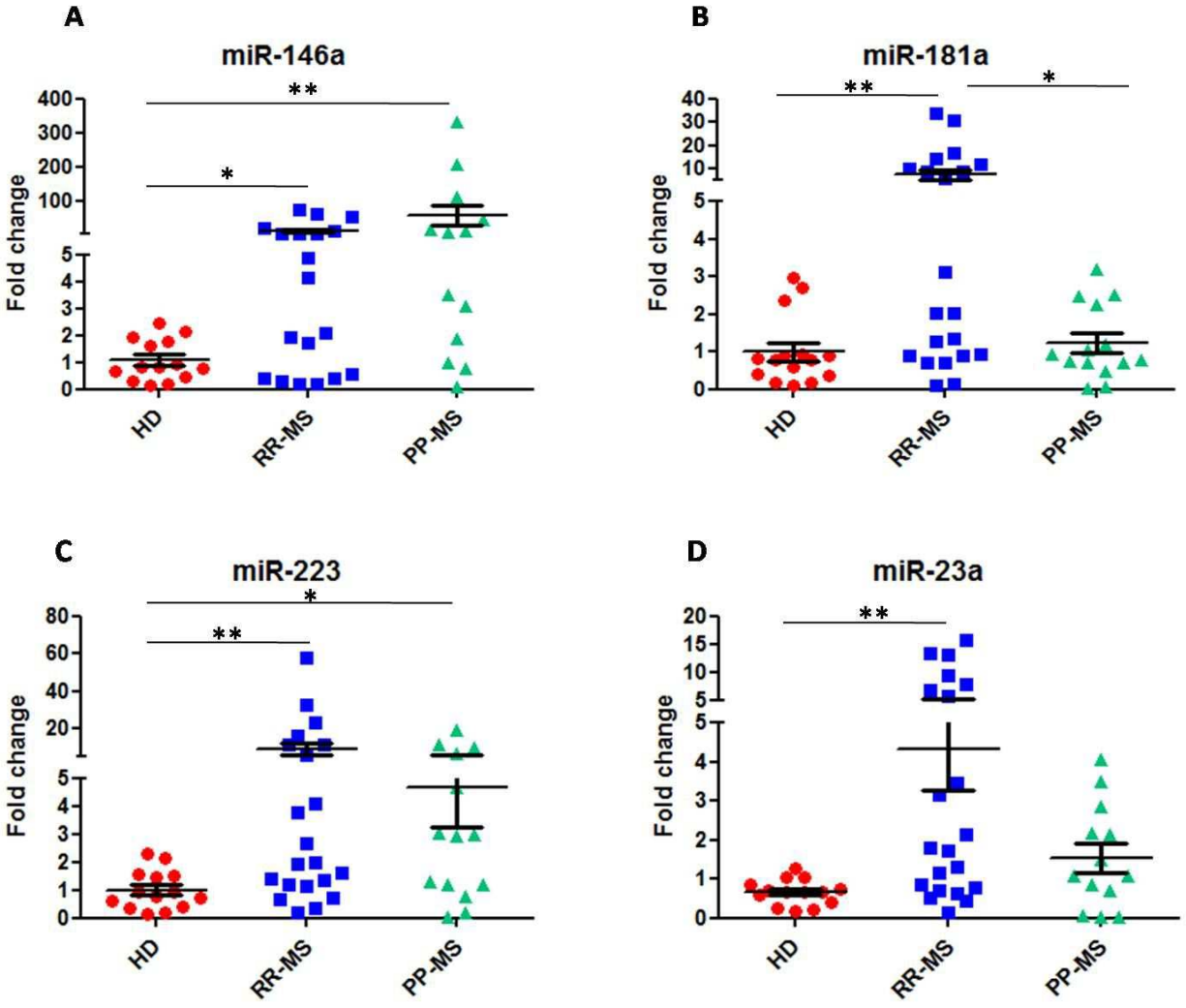


Figure 20. Analysis of miRNAs contents in pool of monocyte-derived EVsby RT-PCR. Pool HD 1 = pool of 4 HDs; Pool HD 2= pool of 3 HDs; and pool MS= pool of 5 RRMS patients. Data are expressed as dCT: CT_{target}-$CT_{reference\ gene}$, where RNA1A1 is considered as the endogenous control.

7. miRNAs expression levels in BzATP-stimulatedmonocytes of HDs and MS patients

Considering that EVs content reflects that of the source cell, we investigated miRNA expression levels in the parental cells. For this type of analysis, we included PPMS patients in order to define a distinct miRNA expression profiling among different form of the disease. miRNAs expression analysis revealed a dysregulation of certain miRNAs in MS patients as compared to controls. In particular, significantly increased expression levels of miR-146a were found in RRMS ($p<0.05$) and PPMS ($p<0.01$) patients as compared to controls (**Fig.21A**). Considering miR-181a, elevated levels were detected in RRMS patients compared to HDs ($p<0.01$) and PPMS patients ($p<0.05$) (**Fig.21B**) . Likewise, miR-223 is overexpressed in RRMS ($p<0.01$) and PPMS patients ($p<0.05$) compared to controls (**Fig.21C**). MiR-23a significantly augmented only in RRMS patients compared to HDs ($p<0.01$), nevertheless a similar trend was observed in PPMS patients compared to HDs (**Fig.21D**). The analysis also revealed a significant upregulation of miR-125a (**Fig.21E**) and miR-30c (**Fig.21F**) in MS patients compared to HDs.

On the contrary, decreased expression of miR-155 was observed in PPMS patients compared to HDs ($p<0.001$) and RRMS patients ($p<0.05$)(**Fig.21G**). Similarly, reduced levels of miR-124 were found in PPMS patients compared to HDs ($p<0.001$) and RRMS patients ($p<0.05$) (**Fig.21H**).



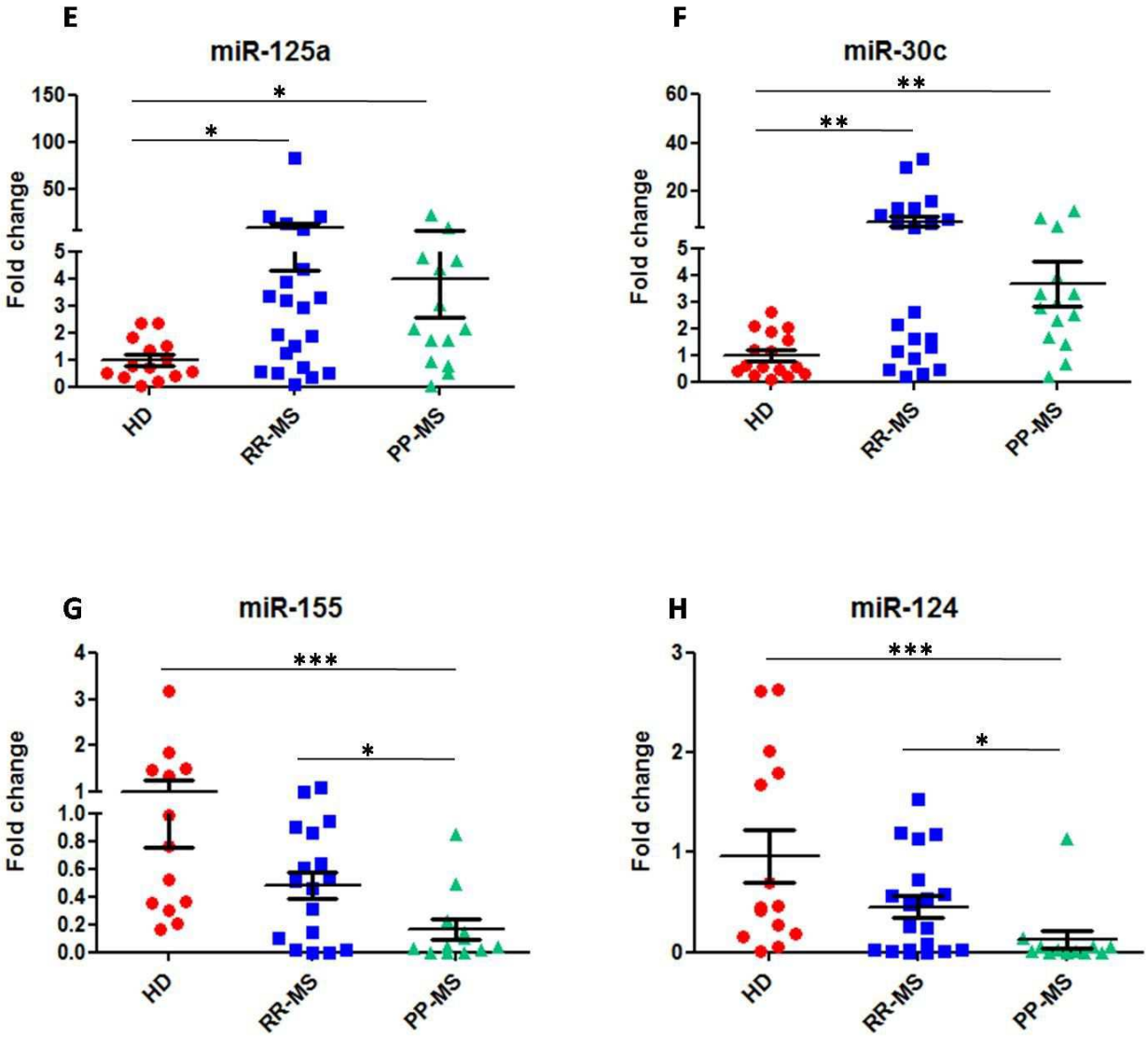


Figure 21. miRNAs expression levels in monocytes of HDs and MS patients by RT-PCR. A) miR-146a; **B)** miR-181a; **C)** miR-223; **D)** miR-23a; **E)** miR-125a; **F)** miR-30c; **G)** miR-155; **H)** miR-124. Data are expressed as mean ± SEM of fold change values (*p<0.05; **p<0.01; ***p<0.001).

DISCUSSION

In the last decade, there was a growing interest for EVs (MVs and EXOs), described as a specialized structures for cellular communication. Several studies have suggested the potential role of EVs derived from different cell types in the development of MS pathology, and in its animal model EAE. Elevated number of EVs have been reported in the serum/plasma and/or in the CSF of MS patients as compared to HDs. Since apparently the level of EVs in biological fluids is associated with the activation of cells involved in MS pathogenesis, it would be very advantageous to identify them as new potential predictive biomarkers, which can also act as potential therapeutic targets. For this reason monocyte-derived EVs production (with particular attention to MVs), their cargo and their pharmacological modulation have been the focus of our investigation. The aim of studying monocytes either in unstimulated or BzATP-stimulated conditions was to report the specific effects of treatment on MVs release in conditions mimicking both inactive (remission) or active (relapse) phase of the disease. Even considering the obvious limitations of this sort of observation *in vitro*, the treatment effect were mostly evident in BzATP-stimulated conditions.

Previously we have shown as MS drugs, particularly IFN-β and TF, interfere with the production of MVs from monocytes of MS patients, as well as with P2X7R and IL-1β expression. Although we have also included a little cohort of Fingolimod treated patients with preliminary results [*Blonda, 2017*], in this study we aimed to better investigate the effects of Fingolimod on the release of MVs considering a larger cohort of patients. Furthermore, due to its specific ability to inhibit A-SMase activity [*Dawson and Qin, 2011*], the key enzyme controlling P2X7R-dependent MVs release [*Bianco, 2009*], Fingolimod results to be a more specific molecule in the modulation of MVs compared to other drugs.

From the analysis of our data, it was recognizable that the mean fluorescence value, which indicates the amount of MVs shed, was higher in MS patients than HDs and Fingolimod. Treatment reduced MVs production compared to MS patients in both conditions and brought

back fluorescence values to physiological conditions similar to those of HDs. Furthermore, we observed that, upon BzATP stimulus, HD and Fingolimod monocytes presented a significant increase in fluorescence, as if the monocytes of MS patients were already *per se* stimulated and further stimulus did not redefine their status. Our results are consistent with data published by Verderio et al. [*Verderio, 2012*] which showed a microglial MVs reduction to baseline levels in the CSF of EAE mice with the administration of Fingolimod.

Subsequently we decided to evaluate the involved MVs release pathway according to the model proposed by Bianco et al. [*Bianco, 2009*] in which the stimulation of P2X7R induced the release of MVs and their cargo.

First of all we evaluated P2X7R expression in MS patients observing a slight non-significant increase. This confirmed in part its role in MVs shedding, nevertheless the possibility that there are other MVs release pathways should not be ruled out, as suggested by Colombo et al. [*Colombo, 2018*]. A significant increment in expression levels was observed in Fingolimod patients compared to HDs. We speculated that the increased expression of P2X7R in Fingolimod patients is an attempt by the cells to make up for the reduced MVs production induced by the drug. However, the mechanism by which Fingolimod may act to modulate P2X7R expression remains to be elucidated.

The downregulation of MVs release correlated with upregulationof P2X7R expression levels suggested that Fingolimod could take part in intracellular pathways inducing a MVs reduction. It's known that Fingolimod inhibits Acid sphingomyelinase (A-SMase) [*Dawson and Qin, 2011*], the enzyme controlling MVs production. These observations suggested that inhibiting MVs release from myeloid cells (microglia or monocyte-macrophages) with Fingolimod administration was possible to reduce in part pathogenic mechanisms induced by MVs. Consistent with this finding, it was worth noting that Fingolimod was able to decrease A-SMase activity in BzATP stimulated monocytes. As suggested by Dawson and Qin [*Dawson and Qin, 2011*], A-SMase activity reduction by Fingolimod inhibition involves the proteolytic degradation

of the enzyme complex. Interestingly, to confirm this mechanism, we observed that A-SMase expression levels by RealTime PCR did not change with Fingolimod treatment, indicating that the target drug mechanism is post-translational. The evaluation of A-SMase activity, but not its related protein levels, is a limitation to the drawing of clear conclusions. However our results confirmed data obtained *in vitro* and in EAE mice, suggesting a novel mechanism of action of Fingolimod.

Since it has been observed that IL-1β was included in MVs [*Bianco, 2009*] and, on the other hand, the activation of P2X7 receptor triggers the inflammasome assembly which may induce the release of IL-1β [*Di Virgilio, 2007*], we decided to evaluate the IL-1β expression levels. IL-1β mRNA levels are reduced in Fingolimod treated patients compared to MS untreated patients and HDs either in unstimulated and BzATP-stimulated monocytes. Our data are in line with results obtained by Noda et colleagues [*Noda, 2013*]. They reported that Fingolimod reduced microglial production of pro-inflammatory cytokines, including IL-1β, after LPS stimulation in a dose-dependent manner.

Secondly, we established to investigate EVs cargo starting from the analysis of a set of miRNAs. Our preliminary results showed that miRNAs are not always detectable in MVs. This is expected especially in HDs, where MVs are fewer. In the EXOs fraction, whereas, miRNAs seemed to be more abundant than MVs and are always detectable, both in HDs and MS patients. The same type of analysis was performed in pools of EVs from monocytes of HDs and MS patients. Also in this case, evaluation in MVs pools of HDs did not reveal miRNAs levels detectable, except for miR-223. In EXOs pools from HDs, whereas, a set of miRNAs was noticed, except for miR-181a and miR-125a. Analysis of EVs pools from RRMS patients showed miRNAs expression levels detectable both in MVs and in EXOs. Although the analysis comprises a very limited number of samples without statistical comparison, miRNAs are more easily detectable in EXOs than in MVs and we could speculate they seem to prefer EXOs as a intercellular communication path. In fact, many researchers focus on EXOs rather than on MVs.

Despite the increasing importance of EVs, the study of the *in vitro* content of EVs derived from a single cellular type has many limitations, mainly due to small sample size and lack of standardized procedures to characterize them. For this reason, many studies gave attention on the analysis of EV cargo from PBMCs or serum/plasma, where the number of EVs is higher. For this purpose, we performed a pilot-study in which MVs and EXOs derived from serum sample of a little cohort of subjects (CIS patients and HDs) were submitted to deep small-RNA sequencing. Data obtained from an in-home bioinformatics platform showed distinct classes of non-coding RNAs in both EVs subsets. Among them, miR-23a and miR-223 are the 20 most abundant miRNAs in the MV and EXO libraries [*Nuzziello, 2017*], according to data obtained from our monocyte-derived EVs. Also a recent study proposed by Prada et colleagues [*Prada, 2018*] revealed the presence of miR-146a, miR-181a and miR-223 in pools of CSF-derived EVs of MS patients. Although it is difficult to detect miRNAs in monocyte-derived EVs, this does not exclude the possibility that they exist and can be spread by EVs to perform their functions on the target cells. Surely a larger starting sample size could overcome these limitations but it is important to consider that the amount of EV-derived miRNAs is small, anyway. To confirm this, Selmaj et al. [*Selmaj, 2017*] found 350 to 400 different miRNAs in serum exosomes per RRMS and HCs sample, which represented on average 0,7% of all exosome RNA sequences.

Given the complexity to perform an analysis on the EV miRNAs and considering that EV content reflects that of the source cell, we investigated miRNA expression levels in the parental cells. For this type of analysis, we included PPMS patients in order to define a distinct miRNA expression profile among different form of the disease.

As regards miR-146a, a miRNA key player in the regulation of innate immunity, we observed a relevant increase of expression level in RRMS and PPMS patients compared to HDs. These results are in line with data obtained from research of Fenoglio and collaborators in PBMCs from MS patients [*Fenoglio, 2011*]. They found an increased level of miR-146a in RRMS and PPMS patients compared to HDs. miR-146a has emerged as anti-inflammatory molecule by inhibiting

NF-kB activity in various cell types. In particular, in human monocytes, miR-146a dowregulates NF-kB activity by inhibiting two signal transducers: TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK 1) [*Wu, 2015*]. In this way, miR-146a can act as a brake on inflammation inducing M2 polarization in monocytes-macrophages [*Essandoh, 2016*]. The anti-inflammatory activity of miR-146a is conceivable with the fact that tested patients are in remission phase, where inflammatory process is reduced compared to relapsing phase of the disease. In PPMS patients, miR-146a had the same expression levels of RRMS, but in this group of patients it may control synaptic functionality and therefore could be responsible of neurodegenerative process. In fact, it has been reported that miR-146a is enriched in microglia and inhibits the expression of neuron-specific targets Nlgn 1, Nova 1, and Syt 1 [*Jovičić, 2013*]. Therefore, if it was conveyed by EVs may alter synaptic functions. Prada and co-workers, recently, demonstrated that rat microglia-derived MVs enriched in miR-146a induce important alterations of synaptic structure and function in neurons. In particular, the transfer of miR-146a from microglia to neurons via EVs downregulates Syt 1 and Nlgn 1 expression. This phenomenon has not yet been demonstrated in humans, but it could be the subject of future plans.

miR-181a is involved in the regulation of the immune system [*Seoudi, 2012*]. Xie and collaborators [*Xie, 2013*] demonstrated that miR-181a was predicted to have several targets site of inflammatory factors. In humans, especially, miR-181a could target the 3'-UTRs of IL1-α, MAPK1, TNF-α, and TLR4. In addition, miR-181a mimics inhibited increment of IL-1β, IL-6, and TNF-α in monocytes and macrophages. Therefore, miR-181a may exert its anti-inflammatory effects by simultaneously targeting [*Xie, 2013*]. Its higher expression levels in RRMS patients compared to HDs and PPMS explains the possibility of this miRNA to exert its anti-inflammatory functions in patients who are in remission phase. miR-181a is very expressed at medium spiny neuron synapses [*Saba, 2012*]. Saba et al. found that miR-181a binds GluA2 subunit of AMPA-Receptors. In addition, miR-181a overexpression reduced GluA2 surface

expression, spine formation, and miniature excitatory postsynaptic current frequency in hippocampal neurons, suggesting that miR-181a could regulate synaptic functions [*Saba, 2012*]. Therefore, its upregulation in RRMS patients, could suggest additional functions compared to anti-inflammatory ones, opening the possibility that miR-181a included in EVs of RRMS patients could alter synaptic functionality in the case of BBB damage. It has also been demonstrated by Liu Y et al. [*Liu, 2013*] that miR-181a binds the 3'UTR of CREB1 regulating its gene expression. They showed that miR-181a suppress the expression of the transcription factor CREB1 and the development of hippocampus neurons. Their findings suggest that miR-181a could be a potential target for preventing neurodegenerative diseases.

As concerning miR-223, its expression increased in RRMS and PPMS patients compared to HDs. Our results are in line with those proposed by Ridolfi et al [*Ridolfi, 2013*], which have observed miR-223 upregulation in PBMCs of RRMS patients compared to HDs. Their results also showed miR-223 lowering trend in PBMCs of PPMS patients compared to RRMS patients, according to data observed by our laboratory. MiR-223 is highly expressed in myeloid cells and is involved in inflammatory response via NF-kB pathway [*Ridolfi, 2013*] and via STAT5 [*Fenoglio, 2016*]. Furthermore, miR-223 is responsible for M2 polarization in monocyte/macrophages [*Essandoh, 2016*] and its delivery by MVs can modulate monocyte/macrophage differentiation [*Ismail, 2013*]. Its high expression, therefore, could be responsible for a modulation of the inflammatory response. It has been observed that this miRNA can also regulate different functions in neurons. In particular, miR-223 may control immature neurons differentiation [*Harraz, 2014*] and negatively regulate Mef2c, which have been observed to stimulate neuronal differentiation [*Jovičić, 2013*]. To sum up, high levels of miR-223 could reduce inflammatory response induced by monocytes, on the other hand, its EV-dependent release, could alter neuronal differentiation.

As previously observed by Fenoglio et al. [*Fenoglio, 2013*], miR-23a increased in RRMS patients. Fenoglio et colleagues also demonstrated miR-23a modulation by Fingolimod treatment

[*Fenoglio, 2016*]. Interestingly, miR-23a is known to target FGF-2 gene, involved in several biological processes, such as nervous system growth, and its levels are reported to be high in CSF of MS patients, particularly those with active disease [*Ismail, 2013*]. Additionally, miR-23a negatively regulates laminB1 [*Lin, 2013*], important factor for myelin maintenance and oligodendrocyte differentiation. These observations suggested that miR23a could be considered as an inflammation marker and possible biomarker investigation in oligodendrocyte differentiation. At the same time, it could serve as treatment response marker.

Also miR-125a augmented in RRMS and PPMS patients compared to HDs. Its upregulation could reduce monocyte migration through BBB cells and inflammatory cytokine secretion [*Reijerkerk, 2013*]. It has been shown that miR-125a promotes M2 polarization inhibiting M1 polarization via KLF4 [*Essandoh, 2016*]. Moreover, this miRNA is involved in dendritic spine maturation targeting PSD-95 [*Stefanovic, 2015*]. Therefore, miR-125a could exert an anti-inflammatory response in monocytes, on the other hand, its EV-dependent transport could impact synapse function, although it has not been demonstrated in humans.

Little is known about the role of miR-30c in the inflammatory response. Recently, Ceolotto et al. investigated the role of miR-30c-5p in atherosclerosis pathways, suggesting an anti-inflammatory capacity of this miRNA. miR-30c reduction seemed to stimulate inflammation, as evidenced by the production of IL-1β [*Ceolotto, 2017*]. Intriguingly, we speculate that high expression levels of miR-30c may influence inflammatory response mostly in RRMS patients that are in remission phase, but it could play an inflammatory role also in PPMS patients, although neurodegenerative process are more evident than inflammatory process. Nevertheless, we did not exclude miR-30c role in neuron functionality. It has been showed that, miR-30c negatively correlated with sema3A in neurons [*Sun, 2016*], therefore high levels of miR30c could affect adult neurogenesis. Likely, we supposed that miR-30c could have a negative role in the repair processes following neuronal damage.

Of the most studied miRNAs implicated in MS, miR-155 is important for its role in both immune and brain cells. MiR-155 expression was elevated in circulating CD14+ monocytes and active lesions [*Moore, 2013*]. More specifically, miR-155 is a M1 promoting polarization marker, relevant in MS disease. It's also known that miR-155 promotes demyelination acting indirectly on astrocytes and oligodendrocytes [*Jagot and Davoust, 2016*]. We hypothesize that probably its low expression in PPMS monocytes compared to HDs and RRMS could denote how inflammatory response is more attenuate than neurodegenerative process.

Finally, it is known anti-inflammatory role of the miR-124 in promoting M2 polarization [*Essandoh, 2016*]. Conversely, studies showed also its high expression in demyelinated hippocampi from patients with MS, suggesting that miR-124 may play a role in the MS pathogenesis [*Dutta, 2013*]. Moreover, miR-124 is a brain-specific miRNA implicated in neuronal differention during CNS development and adult neurogenesis, but it acts also as a key regulator of microglia quiescence [*Ponomarev, 2011*]. The low expression of miR-124 in monocytes of PPMS patients could be a marker of inflammation, suggesting an activated status of cells.

In summary, our results showed that miRNAs, most of which have an anti-inflammatory role promoting M2 polarization, increased in RRMS patients, revealing a monocyte trend to establish an anti-inflammatory response in remission phase of the disease.

To date an increasing role of EVs is recognized in several biological processes, but the information about their cargo and precise molecular effects on target cells are still incomplete. Current literature suggests that study of EVs content may represent a very promising strategy to gain pathogenic information, but limitations due to small sample size make difficult to perform analysis *in vitro*. Nevertheless, to perform the analysis of miRNAs or proteins of interest in the parental cells could be a good starting point. However, until now, the role of monocyte-derived EVs in MS remains to be elucidated.

Our results show for the first time that Fingolimod seems to interfere with MVs release from human monocytes inhibiting A-SMase activity. In addition, our analysis of a set of miRNAs in EVs suggests that, although many restrictions, they are detectable. The study of the same miRNAs in parental cells provides information about their role in inflammatory response and suggests that they could be considered as markers of disease activity. Moreover, their possible involvement in the synaptic function, in MS as well as in OND, may certainly deserve consideration for future investigation. The challenge facing future research will be the optimization and standardization of methods to isolate and characterize EVs content in order to consider them as a possible biomarker.

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