ABSTRACT

Multiple sclerosis (MS) affects about 2.5 million people worldwide. MS is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and a common cause of neurologic disability in young adults.Clinically, the course of MS can be relapsing-remitting (RRMS), primary progressive (PPMS), non-relapsing or relapsing secondary progressive (SPMS). Experimental Autoimmune Encephalomyelitis (EAE) is the prime animal model for MS study and it has been instrumental for the development of four immune modulatory MS therapies thus far. MicroRNAs (miRNAs) are a novel class of small, non-coding RNAs recently discovered to regulate gene expression post-transcriptionally. MicroRNAs are now extensively studied as possible clinical biomarkers. They also represent potential therapeutic targets as they have been implicated in the pathogenesis of several human diseases, including autoimmune diseases as MS. Dysregulation of several miRNAs has been observed in blood cells and CNS tissues of MS compared to control subjects by microarray profiling. Given these premises the aims of my work are: 1) Perform a wide gene expression analysis for miRNAs and targets mRNAs in mononuclear cells from the blood (PBMC) of MS patients and healthy controls; 2) Analyze deeply miRNAs which are known to be highly correlated in the subpopulation of CD4+ cells; 3) Determine whether serum levels of extracellular microRNAs are altered in MS; 4) Evaluate the role of miR-223 in vivo during EAE. First 104 miRNAs transcripts were found to be deregulated between MS cases and controls. Among the best hits, let-7g and miR-150 were confirmed to be deregulated using quantitative PCR in a second sample of MS and controls; genome wide mRNA expression levels of 37.794 transcripts have been obtained on the same two cohorts and correlated with miRNAs expression profile to understand their in vivo effect and to identify potential novel targets of disease. Furthermore we also found that three best characterized miRNAs in CD4+ T cells, miR-21, miR-146a and –b, were statistically significant higher expressed in the PBMCs of RRMS patients as compared with controls. In the analysis of circulating miRNAs the expression profile of 88 best characterized miRNAs was performed in a discovery cohort consisting of serum from 7 MS samples and 3 controls.The results identified three significantly downregulated miRNAs: miR-15b, miR-23a and miR-223. After data validation and replication in two independent populations, only miR-15b and miR-223 were confirmed to be significantly downregulated in MS patients versus controls. Since several lines of evidence suggest that miR-223 is involved in the MS pathogenesis we performed an active EAE experiment in miR-223 knock out (miR-223 KO) mice showing that miR-223 KO display a less severe EAE clinical course compared to littermate control mice. In conclusion, data here presented suggest that miRNAs dysregulation may contribute to the pathogenesis of MS and highlight the possibility to define different disease entities of MS with specific miRNAs profile. This aspect needs to be further investigated considering also that alteration in miRNAs expression in blood or body fluids may lead to the identification of new disease biomarkers of therapeutic targets.

SOMMARIO

La sclersosi multipla (SM) colpisce circa 2.5 milioni di persone nel mondo. La SM è una malattia cronica infiammatoria demielinizzante che colpisce il sistema nervoso centrale ed è la piu' comune causa di disabilità neurologica che esordisce tipicamente nell'età giovane adulta. Il decorso clinico dell'SM può essere suddiviso in tre forme distinte: recidiva – remittente (RRSM), primariamente progressiva (PPSM) e secondariamente progressiva (SPSM). L'encefalomielite autoimmune sperimentale (EAE) è il principale modello animale usato per lo studio della SM, quattro delle terapie immunomodulatorie contro l'SM derivano da esperimenti condotti usando questo modello. I microRNAs (miRNAs) sono una nuova classe di piccoli RNA non codificanti che regolano l'espressione genica a livello posttrascrizionale.I miRNA rappresentano un potenziale target terapeutico e sembrano essere implicati nella patogenesi di diverse malattie comprese le malattie autoimmuni come la SM. Diversi profili di espressione dei miRNAs sono stati analizzati nel sangue e nei tessuti del SNC in pazienti con SM comparati con controlli sani. Con queste premesse gli obbiettivi del mio lavoro sono: 1) Valutare l'espressione genica dei miRNAs e dei target mRNA nelle cellule mononucleate del sangue (PBMC) di pazienti con SM e in controlli sani, 2) Studiare i miRNAs che sono maggiormente espressi nella sottopopolazione di cellule T CD4+ in soggetti con SM, 3) Stabilire se i livelli sierici di mirRNAs extracellulari sono alterati in pazienti SM; 4) Valutare il ruolo di miR-223 in vivo nel modello EAE.Nella prima parte di questo lavoro 104 miRNAssono stati trovati essere deregolati nelle PBMC di pazienti SM comparati a controlli. I due miRNAs trovati più significativi, let-7g e miR-150, sono stati confermati essere deregolati in una seconda popolazione di SM e controlli attraverso analisi di RealTime PCR.Sulla stessa popolazione l'espressione 37,794 mRNA è stata correlata al profilo dei miRNAs per l'identificazionedi nuovi potenziali bersagli di malattia. Inoltre tremiRNAs, miR-21, miR-146a e-b, largamente espressi delle cellule T CD4+, sono stati trovati statisticamente elevati nei PBMC di pazienti RRMS rispetto ai controlli sani.Nella seconda parte del lavoro, per lo studio dei miRNA circolanti,è stato analizzato il profilo di espressione di 88 miRNA nel siero di 7 campioni MS e 3 controlli.Tre miRNAs sono stati trovati deregolati nei pazienti SM: miR-15b, miR-23a e miR-223. Dopo aver convalidato e replicato il dato in due popolazioni indipendenti, solo i miR-15b e miR-223 si sono confermatisignificativamente deregolati nei pazienti SM rispetto ai controlli. Poichè diverse evidenze suggeriscono che il miR-223 è coinvolto nella patogenesi della SM è statoeffettuato un esperimento di EAE attiva in topi mutati nel miR-223 (topi miR-223 KO). I topi miR-223 KO sono risultati più resistenti alla malattia rispetto ai topi controllo. In conclusione, i dati qui presentati suggeriscono che una deregolazione dei miRNAs possa contribuire alla patogenesi della SM. Tale aspetto deve essere ulteriormente indagato considerando anche che l'alterata espressione dei miRNA nel sangue o nei fluidi corporei dei pazienti potrebbe portare in futuro all'identificazione di nuovi biomarcatori e bersagli terapeutici.

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LIST OF ABBREVIATIONS

3'UTRs	3' untranslated regions
BCL-2	B-cell chronic lymphocytic leukemia 2
CLSPN	Claspin Xenopuslaevis human homolog
CNS	Central nervous system
CSF	Cerebrospinal fluid
DGCR8	Di George syndrome critical region gene 8
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Status Scale
EPHB2	EphrinB2 gene
Exp5	Exportin-5
FBS	Fetal bovin serum
FDA	U S Food and Drug Administration
GA	Glatiramer acetate
GWAS	Genome-wide association study
GWS	genome wide significance
H&E	Hematoxylin and eosin
hAGO2	human argonaute protein 2
HLA	Human leukocyte antigen
HTATIP2	HIV-1 Tat interactive protein 2
IFN	Interferon
IL	Interleukin
KIF-1B	Kinesin family member 1B
KO	Knockout
LFB	Luxol Fast Blue
Ly6C	Lymphocyte antigen 6 complex, locus C

Ly6G	Lymphocyte antigen 6 complex, locus G
MAF	Minor allele frequency
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
Mef-2c	Myocyte enhancer factor 2C
MHC	Major histocompatibility complex
miRNAs	MicroRNAs
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic Resonance Imaging
MS	Multiple sclerosis
NF-Kb	Nuclear factor kappa B
PAZ	Piwi Argonaute Zwille
PBMCs	Peripheral blood mononuclear cells
PPMS	Primary progressive multiple sclerosis
QPCR	Quantitative PCR
RISC	RNA-induced silencing complex
RRMS	Relapsing-remitting multiple sclerosis
SNP	Single nucleotide polymorphism
SOCS1	Suppressor of cytokine signaling-1 gene
SPI1	Transcription factor PU.1
SPMS	Secondary progressive multiple sclerosis
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factors
TRBP	Trans activating Response RNA Binding Protein
Treg	Regulatory T cells
VLA-4	4 subunit of very late antigen-4 receptor

1. INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) affects about 2.5 million people worldwide. MS is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and a common cause of neurologic disability in young adults [1]. It's more common in women than man ratio 3:2. It is an immune mediated disease characterized by presumed autoimmune disruption of myelin and varying degrees of axon injury within the CNS.

The depiction of "a remarkable lesion of the spinal cord accompanied with atrophy" by Robert Carswell in 1838 anticipated a more or less complete description of the pathological anatomy and clinical features of multiple sclerosis (named thus in 1955) by the last decades of the 19th century.

MS expresses itself in four clinical forms: relapsing remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS (PRMS). Approximately 87% of patients present with RRMS, characterized by acute attacks (relapses) followed by partial or full recovery (remission). Patients can manifest with a heterogeneous group of symptoms including changes in vision (unilateral visual loss, diplopia), weakness, dyscoordination, sensory loss or distortions, or changes in bowel and bladder function. Progression of disease may eventually lead to severe disability.

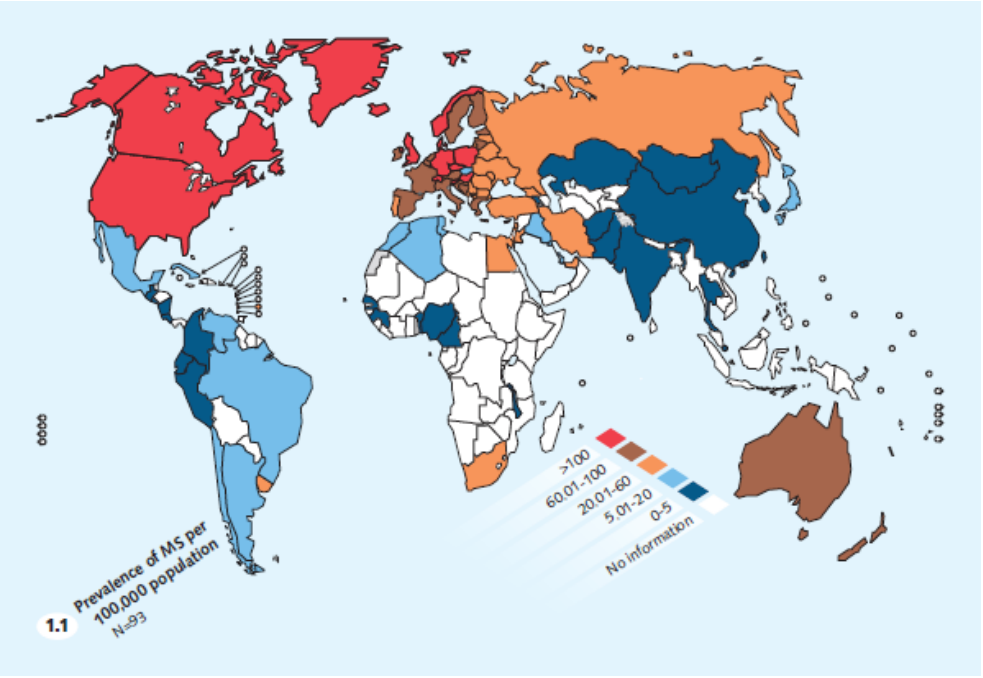
Inflammation of central nervous system is the primary cause of damage in MS. The specific elements that start this inflammation are still unknown. Studies have suggested that genetic, environmental and infectious agents may be among the factors influencing the development of MS. Many immunological studies have been done on the animal model for human MS known as the experimental autoimmune encephalomyelitis (EAE). Based on this model and observations of MS in humans, roles of several immunological pathways involved in MS are being explored. Oligodendrocytes, the myelin producing cells in the CNS, seem to be the main target of the immune response to the self. In MS, T cells infiltrating the CNS are activated to one or more antigens in CNS myelin [myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG)]. They migrate from the peripheral circulation within the CNS at the level of post-capillary venules, diffusely infiltrate parenchyma and cause multifocal demyelination and axonal degeneration [2-5].

1.1.1 Epidemiology and role of environment in MS

The cause of multiple sclerosis involves environmental exposure and genetic susceptibility. Arguing the merits of one faction versus the other is unproductive. Each is clearly implicated, together with the cultural condition of age at which the interplay between genes and the environment occurs.

Environmental Factors

The global distribution of multiple sclerosis can be generalized as increasing with distance north or south of the equator, but that summary conceals many places with disproportionately high or low frequencies (scheme 1) [6]. MS is common in regions populated by northern Europeans but this distribution is modified by where individuals who are at risk of disease live early in life. Several studies have also reported increasing incidence of multiple sclerosis over time, although these data can be confounded by heightened awareness of the disease and new diagnostic techniques. Orton and colleagues [7] report a significant increase in incidence of multiple sclerosis in Canadian women, but not men, over the past 30 years, causing a change in the female to male ratio to more than 3:1. What environmental factor this finding shows, however, is not so clear. Migrations involving large numbers of people affect t the distribution of multiple sclerosis. Studies from South Africa, Israel, Hawaii [8-10], and of immigrants to the UK [11] correlate the risk of multiple sclerosis with place of residence in childhood (scheme 1). Migration from high-risk to low-risk regions in childhood is associated with a reduced risk, and from low to high prevalence parts of the world with an increased risk of developing multiple sclerosis by comparison with the population of origin. However, analysis based on a homogeneous Australian population shows no effect of age-at-migration, with 15 years as the point of stratification, suggesting that the risk of exposure spans a wider age range than was originally suggested [12]. Patients with multiple sclerosis report being infected with measles, mumps, rubella, and Epstein-Barr virus at later ages than do HLA-DR2 matched controls [13]. These data lend support to the so-called hygiene hypothesis where by individuals not exposed to infections early in life, because of a clean environment, make aberrant responses to infections when encountering these challenges as young adults. Lang and colleagues [14] describe a basis for molecular mimicry between Epstein-Barr virus and a self-protein, so that an immune response to the virus in advertently cross-reacts with myelin and induces demyelination; four DRB1 restricted T-cell receptor peptide contacts are identical for myelin basic protein and Epstein-Barr virus. Studies investigating pathological changes suggest that a high proportion of B cells, accumulating in lesions of chronic multiple sclerosis, are infected by Epstein-Barr virus [15]. Frustrated by the low dividend from systematic searches for candidate infectious agents with sophisticated methods for virus detection, some commentators have suggested other environmental triggers such as low sunlight, vitamin D deficiency, diet, geomagnetism, air pollutants, radioactive rocks, cigarettes, and toxins [16].



Scheme 1.MS Epidemiology [MS Atlas, WHO & MSIF, 2008]

The genetics

Multiple sclerosis has a familial recurrence rate of about 20%. Overall, the reduction in risk changes from 3% in first-degree relatives (siblings, 5%; parents, 2%; and children, 2%), to 1% in second-degree and third-degree relatives. These confer relative risks of 9・2, 3・4,and 2・9, respectively, compared with a back ground age adjusted risk in white northern Europeans of 0・3% [17, 18].Population-based series of multiple sclerosis in twins from Canada and the UK show higher clinical concordance rates in monozygotic than in dizygotic pairs (25% *vs* 5%) [19, 20]. Conversely, studies from Italy provide equivalent rates irrespective of zygosity [21]. Individuals with multiple sclerosis, who were adopted soon after birth and those having affected members of their adoptive family, have the same risk as does the general population and, therefore, a substantially lower frequency than that observed in the biological relatives of index cases [22]. The same is true for step-siblings of index cases [23]. The age-adjusted risk for half-siblings is lower than that for full siblings and with no difference in risk for half-siblings reared together or apart. The recurrence risk is higher for the children of conjugal than single-affected parents [24]. Together, these studies implicate genetic factors in determining familial clustering and individual susceptibility to multiple sclerosis.

The only solid finding for a long time was the MHC region, which is indisputably the strongest genetic risk factor for MS. However, as a result of recent GWASs there are currently close to 50 non-MHC loci, which are associated with MS with

genome-wide significance (GWS) (P-value $5x10^{-08}$). Most of these are located near genes with immunological functions.

Major Histocompatibility Complex (MHC)

First associations of HLA protein variants, which are encoded by HLA-genes within the MHC region, with MS were described nearly four decades ago [25, 26]. The since identified DNA risk haplotypeHLA-DRB1*1501-DQA1*0102-DQB1*0602 has been widely replicated [27] and is estimated to explain 17%-62% of MS heritability [28]. However, it still remains unclear which of the genes is the main determinant of increased risk for MS, and whether there are several susceptibility genes within the MHCII region. The HLA-genes within MHCII encode molecules expressed on the surface of APCs and present antigens to CD4+ cells, but the mechanisms by which the MS associated variants result in increased susceptibility are currently unknown. In addition to MHCII, there is also evidence for independent risk factors in the MHCI region [29-31].

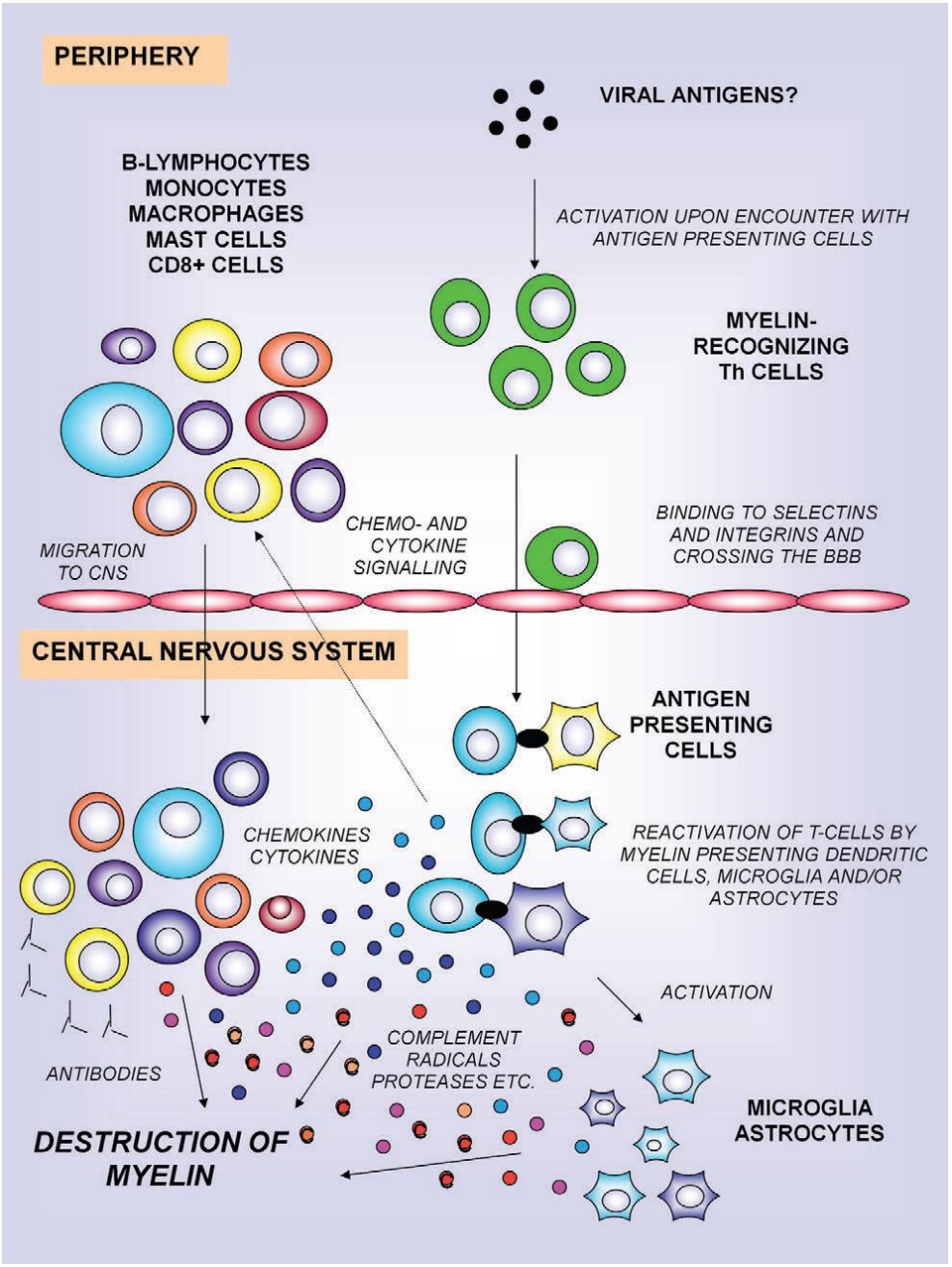
Non-MHC loci

As now seventeen non-MHC loci had been reported to be associated with MS with GWS. However, the reported ORs for all confirmed risk variants are low (1.1-1.4) and even together with the MHC region, much if not most of the genetic variants contributing to MS heritability still remain to be identified. Further fine mapping and functional studies are also required in order to point the functionally relevant variants and genes in the currently known loci, many of which extend over several hundred kb and contain several genes. Many candidate genes in these loci have immunological functions (*CD58*, *CD6*, *CD86, CLEC16A*, *CLECL1, GPR65, EOMES, IL12A*, *IL12B,IL2RA, IL22RA2, IL7R, IRF8*, *MALT1, MERTK, SP140, STAT3*, *TAGAP,TNFRSF1A*, *TNFSF14*, *TYK2*), although interestingly some genes relevant for neurological functions have also emerged (*AHI1*, *GALC*, *KIF21B*). Strikingly many of the established loci overlap with those identified in other autoimmune diseases [32].

1.2 Pathogenesis

We believe that the disease process starts with increased migration of autoreactive lymphocytes across the blood‑brain barrier. The transition from physiological surveillance to a pathological cascade arises from regulatory defects that allow these cells to set up an immune response within the brain. Regulatory lymphocytes from people with multiple sclerosis fail to suppress effector cells [33]. These autoreactive cells do not effectively apoptose on stimulation, because of overexpression of β-arrestin 1, which is a key promoter of naive and activated CD4+ T-cell survival [34]. Presumably, failure of local regulatory mechanisms within the brain accounts for the particular sites of inflammation, dominated by perivascular CD8+ cell infiltrates, causing so-called plaques that cluster around the lateral ventricles and corpus callosum, in the cortex and subcortical white matter, the optic nerves and brainstem, and throughout the spinal cord. Investigators have recently discovered that the key role assigned historically to T-helper 1 (Th1) (interferon-g secreting) cells in EAE was misplaced. Rather, inflammation is driven

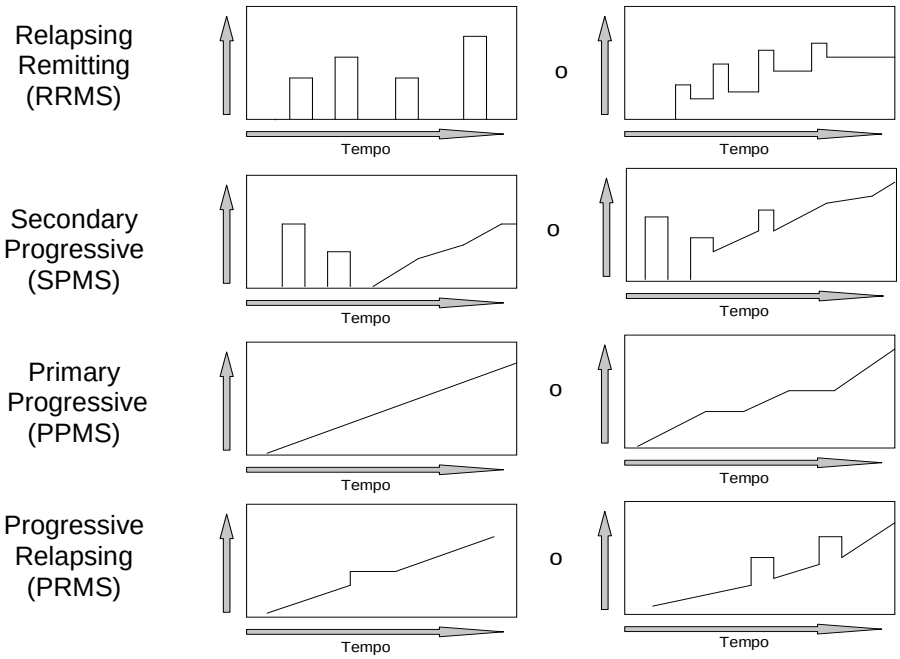
by a newly-designated T-lymphocyte subtype that secretes interleukin-17 under interleukin-23 control [35]. Interleukins 17 and 22 disrupt the human blood-brain barrier, allowing efficient penetration of Th17 cells into the brain where they can kill human neurons [36]. The antigen specificity of these immune responses is unresolved, not least because many auto reactive lymphocytes can be detected in healthy individuals. Originally, myelin proteins were favored as candidates for initiation of the disease process in multiple sclerosis, but other specificities are now also implicated. As T and B lymphocytes, plasma cells, and macrophages accumulate, pro-inflammatory cytokines amplify the immune response through recruitment of naive microglia. Contact is established between activated microglia and components of the oligodendrocyte myelin unit that isopsonised with ligands for microglial Fc and complement receptors. A lethal signal is then delivered through cell surface bound tumor necrosis factor α (TNFα) [37]. Acute demyelinating lesions also show extensive axonal injury with transaction [38] that correlates with T-cell and microglial infiltration. With onset of the secondary progressive stage, areas of demyelination coexist with diffuse axonal and neuronal degeneration, associated with the accumulation of hyper phosphorylated and insoluble tau [39]. Lesions seem to grow slowly by radial expansion as focal brain inflammation fades into diffuse parenchymal microglial activation resulting in extensive abnormalities of the normal appearing white matter, in which a delicate balance exists between anti-inflammatory genes up-regulated in oligodendrocytes and pro-inflammatory pathways activated in microglia [40]. B-lymphoid follicles accumulate in the meninges sustaining a compartmentalized humoral immune response that can drive intrathecal antibody production and damage nearby cortex; these are the cells that harbour Epstein-Barr virus [15]. Pathological changes of primary progressive multiple sclerosis are characterized by reduced plaque load, less evidence for inflammation, and the absence of lymphoid follicles. Remyelination (scheme 2) accounts for the appearance of shadow plaques. It is most active during the acute inflammatory process coinciding with phagocytic removal of myelin debris, but also occurs in the progressive phase. The mature nervous system maintains a pool of oligodendrocyte precursors that can migrate in response to semaphorin 3A and 3F [41]. Undifferentiated oligodendrocyte precursors surround the lesions of multiple sclerosis and presumably act as the source of cells having the potential to remyelinate naked axons [42]. In 20% of people with multiple sclerosis, plaques are eventually remyelinated. Clearly, remyelination is less successful in other instances, with cycles of demyelination and remyelination apparently exhausting the capacity for tissue repair.



Scheme 2.Hypothetic scenario of events leading to demyelination in MS. Based on text and figures in a review by Sospedra and Martin (2005).

1.3 Clinical course

Clinically, MS can be relapsing-remitting (RRMS), primary progressive (PPMS), non-relapsing or relapsing secondary progressive (SPMS) (scheme 3). 80% of patients present with an acute episode affecting one (or occasionally several) sites, which is known as the clinically isolated syndrome. If accompanied by white matter abnormalities detected by Magnetic Resonance Imaging (MRI) at clinically unaffected sites, the chance of a second attack of demyelination subsequently occurring, and so fulfilling the diagnostic criteria for relapsing-remitting multiple sclerosis, increases from 50% at 2 years to 82% at 20 years [43]. New episodes occur erratically but the rate seldom exceeds 1·5 per year. With time, recovery from each episode is incomplete and persistent symptoms accumulate. Eventually, around 65% of patients enter the secondary progressive phase; in 20%, the illness is progressive from onset. In both these situations, progression starts at around 40 years of age [44]. Primary and secondary progressive multiple sclerosis often manifest as spinal disease, but syndromes that are attributable to dysfunction of optic nerves, cerebrum, or brain stem can also occur. Children with multiple sclerosis are usually girls and presentation is commonly with encephalopathy. They take longer from onset to reach the secondary progressive stage than adults, but nevertheless do so at a younger age [45]. In all cases, the clinical course usually evolves over several decades. Death is attributable to multiple sclerosis in two-thirds of cases and to the increased risk and complications of infections particularly of skin, chest, and bladder in people with advanced neurological disability. The median time to death is around 30 years from disease onset, representing a reduction in life expectancy of 5–10 years.



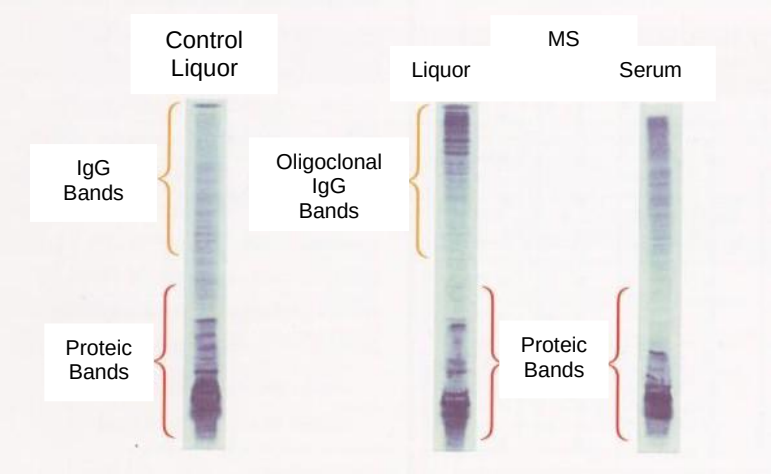
Scheme 3.MS subtypes by Lublin e Reingold [46]

1.4 Diagnosis

Magnetic Resonance Imaging (MRI)
Using MRIs have made diagnosing MS both simpler and more complex. Since MS is an inflammatory disease of the CNS, MRIs are able to visualize changes in a patient. However, the changes seen in MRIs are changes in proton density (water content) and therefore are nonspecific. MRI changes should therefore never be solely used to diagnose MS, irrespective of the claims of the radiology report. However, certain changes in MRI are very indicative of MS. MRI changes seen associated with the disease correlate with the gross and histologic pathology of the disease [47].

CSF Analysis
Up to 90% of people with clinically definite MS have spinal fluid that exhibits changes of low-grade inflammation. These changes are both measured directly in CSF, such as the immunoglobulin G (IgG) concentration and oligoclonal bandings, are calculated from measuring CSF and serum IgG, and albumin (scheme 4). Using certain formulae, calculated values include the IgG index and IgG synthesis rate. The presence of these changes is influenced by both the progression of the disease and the anatomic location of the inflammatory lesions. Those near ventricular surfaces will result in spinal fluid problems. However, with greater involvement of the blood-brain barrier, the specificity of the calculated changes seen in MS CSF (like IgG synthesis rate) decreases [48]. Early during the

progression of the disease, the spinal fluid is often normal [49] but can deteriorate over time [50]. Spinal fluid changes at this stage are imperative in diagnosing MS. In up to 20% of patients with clinically definite MS, the spinal fluid is normal probably due to differences in the disease pathogenesis in these individuals [51]. However, the presence of a low-grade inflammation in CSF without the occurrence of other diseases could cause similar changes which would support an MS diagnosis.



Scheme 4. Positive IgG Oligoclonal bands in the serum and CSF of MS patients

1.5 Therapy

There are currently 8 FDA approved agents for relapsing forms of MS. No agents are FDA approved for PPMS. FDA approved agents include four preparations of interferon-beta (*Avonex, Rebif, Betaseron and Extavia*),glatiramer acetate (*Copaxone*), mitoxantrone (*Novantrone*), and natalizumab (*Tysabri*) and the recently approved first oral medication fingolimod (*Gilenya*) [52]. Many other immunologically active agents are used off label and others are nearing study completion and FDA application. The differing types and durations of immunologic effects of these agents will increase the complexity and likely risks of future MS care.

Interferon beta: Interferon beta (IFN-beta) was first approved by the FDA for MS treatment on 1993. The clinical efficacy of IFN-beta is greater in RRMS than in SPMS. Interferon beta-1b products (*Betaseron*and the identical *Extavia*) are recombinantly produced by Escherichia coli bacteria. Interferon beta-1a (*Avonex*and *Rebif*) are recombinant peptides produced in Chinese hamster ovary cells and are identical to natural human interferon-beta.

Glatiramer Acetate: Glatiramer acetate (GA), formerly known as copolymer 1, is a random polymer of glutamic acid, lysine, alanine and tyrosine, the most common amino acids in myelin basic protein. There is one formulation of GA marketed as *Copaxone*, and it is currently approved for the treatment of RRMS.

Monoclonal Antibodies

Natalizumab (*Tysabri*): it is a humanized monoclonal antibody with an IgG4 framework. It was specifically designed for the treatment of MS and was FDA approved on 2004. Its target molecule is CD49, the 4 subunit of very late antigen-4 (VLA-4) receptor. VLA-4 interacts with vascular cell adhesion molecule-1 so that immune cells can migrate through the blood brain barrier. By binding to CD49, natalizumab prevents the adhesion between the endothelial cell and the immune cell, thus migration of leukocytes into the central nervous system is blocked.

Rituximab (*Rituxan*): it is a chimeric murine/human IgG1 monoclonal antibody. Its target is CD20, an antigen produced only on mature B cells and not on the Ab-producing plasma cells. This monoclonal antibody is FDA approved for the treatment of rheumatoid arthritis and B cell lymphoma and it remains an investigational agent for treatment of MS.

Cytotoxic and other agents

Mitoxantrone (*Novantrone*) is a synthetic anthracenedione that intercalates into DNA. It causes cross-linking and strand breaks and inhibits topoisomerase II, thus interfering with DNA repair. Besides causing generalized immunosuppression, mitoxantrone inhibits monocyte and lymphocyte migration, induces apoptosis of dendritic cells and decreases the secretion of proinflammatory cytokines such as tumor necrosis factor, interleukin-2 and interferon-g [53]. It also inhibits B cell function, increases T cell suppressor function and inhibits macrophage mediated myelin degradation. Mitoxantrone was approved by the FDA on 2000 for SPMS and worsening RRMS [53].

Methotrexate: Methotrexate inhibits the synthesis of DNA, RNA and protein by inhibiting dihydrofolic acid reductase, which is necessary for the synthesis of

thymidylate. Through this reaction, lymphocyte production is inhibited, thus reducing inflammation. Currently methotrexate is used only off-label for RMMS.
Intravenous Immunoglobulin: Treatment with intravenous immunoglobulin (IVIG) has been reported as beneficial for treatment of patients with RRMS.

Emerging Treatments
Fingolimod (FTY720): it is a new oral medication with a recently approved FDA application and is awaiting wider use. It is a structural analogue of sphingosine. It targets receptors of sphingosine-1-phosphate (S1P). S1P is an important signaling lipid involved in the migration of lymphocytes from secondary lymphoid organs to the periphery. Recently a study showed that patients treated with fingolimod had a lower annualized relapse rate (0.20) compared to patients treated with interferon (0.33) [54]. Moreover, MRI findings supported the clinical results.

1.6 Animal Model

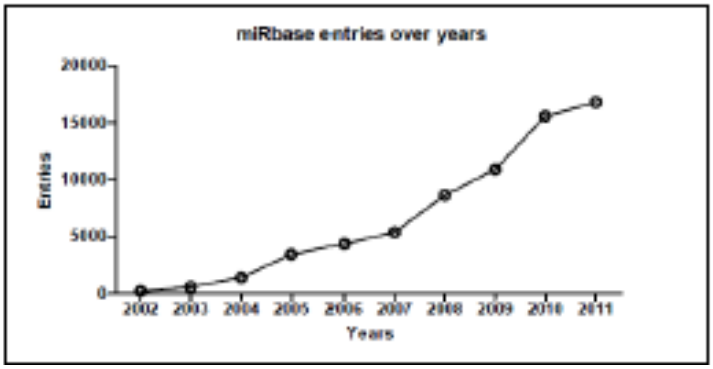
Autoimmune inflammatory demyelination and variable degrees of axon damage also occur in the best characterized animal model of MS, EAE. EAE shares several similarities to MS including its CNS histopathology, relation to certain MHC II, and different clinical courses (relapsing-remitting or chronic depending upon species, strain and myelin antigen used for induction). Although EAE is not a perfect model for MS, it has been instrumental in better understanding the human disease and it was crucial in the development of three FDA-approved MS medications (glatiramer acetate, natalizumab, mitoxantrone), as well as several other MS drugs in the pipeline [55]. In EAE and possibly MS, there are two main phases of immunopathological events: an initial priming/induction phase in which auto-aggressive lymphocytes are activated and a subsequent recruitment and effector phase in which such cells invade the CNS and cause tissue damage [56, 57]. Many different immune mechanisms can lead to demyelination and axonal injury in MS and EAE. Besides specific mechanisms provided by adaptive immune responses ($CD4^+$, $CD8^+$ T cells or specific Abs), tissue injury can also be induced by activation of effector cells via stimuli mediated by innate immunity.

1.7 MicroRNAs

1.7.1 MicroRNAs gene structure

Since their first discovery, in 1993, by Victor Ambros research group, microRNAs partially revealed what for years remained in shadow: the role of “junk” DNA in eukaryotes genome. In fact what for years has been called “junk”, now it is not anymore [58]. MicroRNAs represent a class of small non-coding RNAs with peculiar functions in control of gene expression at post-transcriptional level [59]. The micro-RNAs mature form is a single stranded RNA, 19-22 nucleotides long, whose maturation steps take place in part in the nucleus and in part in the cytoplasm. MicroRNAs are transcribed from their own genes scattered in all chromosomes in humans, except for Y chromosome [60].To date, the microRNAs database (miRBase) (www.miRbase.org), in which annotated sequence of miRNAs genes are reported and constantly updated, shows that 1424 miRNAs genes are

scattered throughout the human genome; of note, this number is continuously growing up both in man and in other species. Since the initial miRBase publication, the number of annotated sequences has shown an astonishing growth, from 218 entries in 2002 to over 16000 entries in 2011 (Scheme 5).
Most miRNA genes are located in intergenic regions (intergenic miRNAs) almost >1kb away from annotated/predicted genes, although some miRNAs were found in intronic regions, within protein coding-genes or in non-coding genes (intronic miRNAs) [61]. Intergenic miRNAs are transcribed as autonomous units with their own promoter/regulatory region, from both RNA Polymerase II or III; about a 50% of intergenic miRNA are found in close proximity to other miRNAs, forming extended clusters which are transcribed as single polycistronic unit [62]. Intronic miRNAs, residing within protein coding genes or non-coding genes, seem to be transcriptionally related to the expression of their host gene and processed in consequence of the spliceosome formation [63]. Transcription of intergenic miRNAs, controlled by their own promoter, generates several Kb long pri-miRNAs with CAP structures and poly(A) tails, which allow its subsequent processing reactions. On the other hand, the transcription of intronic miRNAs underlies the control of their host mRNAs using the same promoter, and involves protein complexes of mRNAs splicing machinery [64].



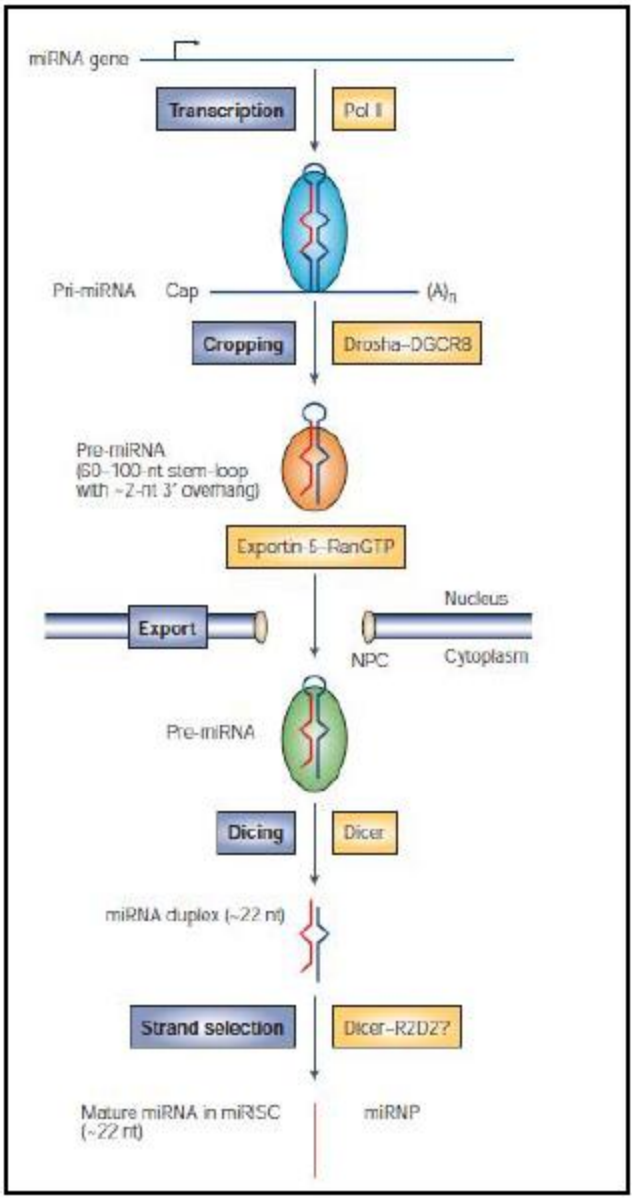
Scheme 5.Number of miRNAs annotated sequences in miRNA database miRBASE, from 2002 to 2011.

1.7.2 MicroRNAs biogenesis and maturation

Transcription of miRNA gene is mediated by RNA polymerase II or III, which produce a primary transcript, called primiRNA. This transcript contains the 7-methylguanosine cap and a poly (A) tail. Pol II-dependent transcription enables temporal and positional control over miRNAs expression so that a specific set of miRNAs can be expressed during several different cellular stages and under specific conditions and in specific cell types. PrimiRNAs are long primary transcripts that contain a local stem-loop structure, which is essential for following processing reactions. This stem-loop structure is cleaved in the nucleus by the RNase III Drosha to release the pre-cursor of miRNA (pre-miRNA). Drosha is a large protein of 160 kDa, which is conserved in animals that requires a cofactor,

the Di George syndrome critical region gene 8 (DGCR8) proteins in humans [65]. Because the next processing enzyme is confined to the cytoplasm, the Drosha product pre-miRNA, needs to be exported to the cytoplasm. Export of pre-miRNA is mediated by one of the Ran-dependent nuclear transport receptors, exportin-5 (Exp5) [66]. Once in the cytoplasm, pre-miRNAs are processed into 22-nt miRNA duplexes by the cytoplasmic RNase III Dicer, a highly conserved protein of 200 kDa. The cleavage products are thought to be quickly unwound by helicase (Argonaute protein), and a single mature strand can be asymmetrically incorporated into the RNA-induced silencing complex (RISC) where they can then act by translational repression (by a cleavage-incompetent RISC) or mRNA degradation (by a cleavage-competent, Slicer-containing RISC). The counterpart of the mature miRNA from the duplex that is generally regarded as a passenger strand called miRNA* (miRNA "star"), whose regulatory capacity has not been systematically examined in vertebrates, is usually degraded.

In particular, mature miRNAs are incorporated into the effector complex, which is known as, miRISC (miRNA containing RNA-induced silencing complex) by associating with Argonaute proteins. The RISC complex composition is still not completely clear, but what is known is that it is composed by Dicer and a dsRNA-binding protein named TRBP (Trans activating Response RNA Binding Protein) that recruits argonaute proteins like hAGO2 (human argonaute protein 2). The Argonaute protein family was first identified in plants, and members are defined by the presence of PAZ (Piwi-Argonaute-Zwille) and PIWI domains [67]. It can be divided into the Ago subfamily and the Piwi subfamily. Ago proteins are ubiquitously expressed in many organisms. In mammals, for example there are eight Argonaute genes: human Ago1, Ago3 and Ago4 genes are clustered on chromosome 1, whereas the Ago2 gene is located on chromosome 8. The human PIWI subfamily comprises HIW1, HIW2, HIW3 and HILI that are encoded by genes on chromosomes 12, 11, 22 and 8, respectively. Studies on isolated PAZ domains from different organisms revealed that this domain contains a specific binding pocket that anchors the characteristic two nucleotide 3"-overhang that results from digestion of RNAs by RNase III [68]; instead PIWI domains show extensive homology to RNase H. Indeed, biochemical in vitro studies have shown that some are endonucleases, and these are often referred to as 'slicers'. In humans, only Ago2 has slicer activity [67]. After processing operated by Dicer, only a strand of the dsRNA produced is selected as leading strand for post-transcriptional gene silencing. Ago2 plays a fundamental role in leading strand selection: thermodynamic stability of the dsRNAs ends establishes which strand is selected. The strand with relatively unstable pairing at 5" end is generally chosen and thus remains as component of the silencing complex (Scheme 6) [69].

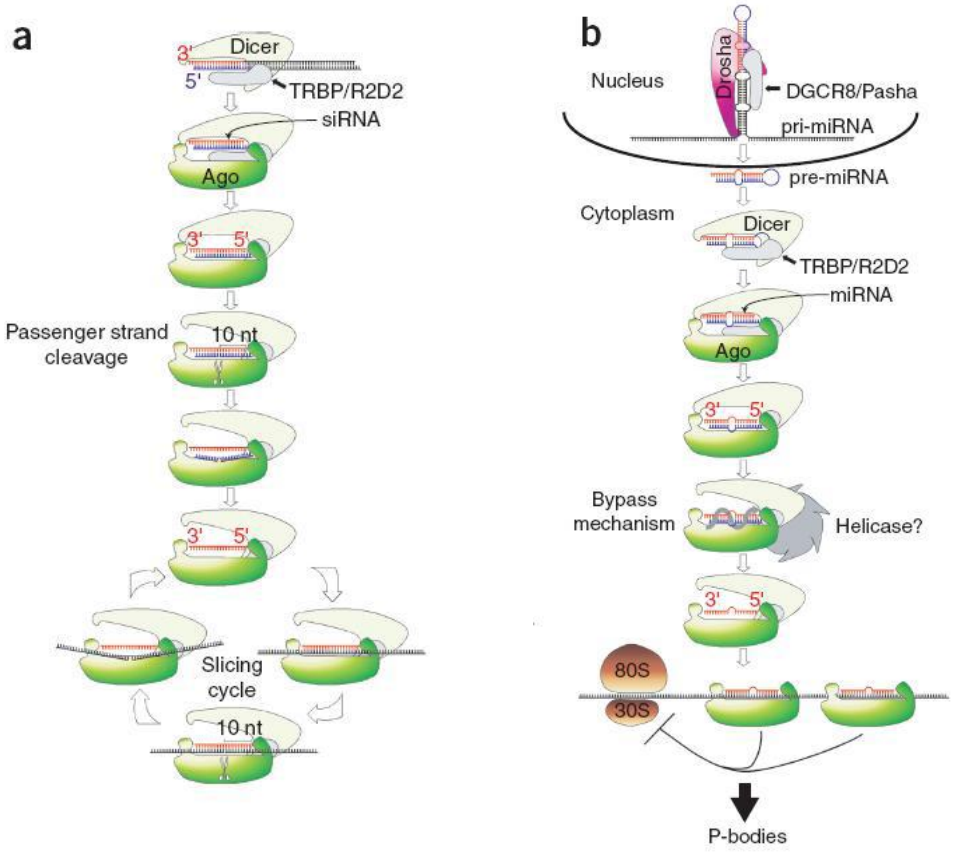


Scheme 6. Mechanism of miRNAs maturation: from transcription to miRNA strand selection

1.7.3 Post-transcriptional regulation mechanism

MiRNAs negatively regulate gene expression at post-transcriptional level by mediating translational repression or degradation of the mRNA targets. The main actor of these two different mechanisms is the protein complex RISC. Translational repression is thought to occur when the leading strand, through an imperfect

pairing to the target mRNA, directs RISC to bind the 3' untranslated regions (3'UTRs) of the targets. This imprecise matching causes a bulge in the duplex formed between the miRNA and its mRNA and results in blocking the target mRNA (usually the 3'UTR) from being translated and in protecting the target mRNA from destruction by endonucleases (Scheme 7b). This complexes are then transported into particular cytoplasmic loci called p-bodies (processing bodies) which are deprived of the translational machinery but retain proteins involved in mRNA degradation. P-bodies are sites in the cytoplasm in which, in addition to the mRNA component, essential components of the mRNA degradation pathway (Argonaute proteins and miRNAs) are localized, together with additional proteins, including the mRNA decapping enzymes (Dcp1/Dcp2), the 5'-3' exonuclease, Xrn1, Dhh1p, Pat1p and, in mammalian cells, GW18 that are responsible of the transcript de-capping through which the mRNAs capture and degradation occur [70].This mechanism is used by miRNAs for the endogenous regulation of different gene products. In contrast, mRNA degradation or "slicing" is thought to occur by siRNAs which form perfect/near perfect interactions with their target mRNAs, which results in the cleavage of the mRNA known as RNA interference (Scheme 7a). The perfect complementation presumably triggers the action of Slicer, which in mammals is putatively thought to be the Argonaute2 (Ago2) endonuclease acting either alone or together with other Argonaute or unknown proteins within the RISC complex. Slicer mode of action is by cleaving the target mRNA between the 10th and the 11th nucleotide from the 5' end of the miRNA. Seven nucleotides at 2-7 positions (relative to the 5' end of miRNA), known as seed sequences, are crucial in binding to the target mRNA. These series of events lead to the silencing of target genes [71].



Scheme 7. A. Mechanism of mRNAs degradation upon perfect pair matching between miRNA and mRNA target. B. Translational inhibition by imper-fectly matching between miRNA and mRNA.

1.7.4 MiRNAs and disease

In the human genome, miRNAs are involved in the regulation of approximately 30% of all of the protein-coding genes, evidencing their pivotal role in the regulation of cell functions. Most of the evidences of the involvement of microRNAs in diseases come from cancer research. It has been demonstrated that about 50% of human miRNA genes are located in cancer associated regions or at fragile sites of chromosomes. Many genomic profiling studies indicated a deregulation of miRNAs expression in various tumors, and some miRNAs are reported as partially responsible for altered cell replication in cancer (e.g. miR-17-92 or miR-155 in B-cell Lymphomas). Recent studies on the tumor-suppressing or tumor-promoting activities by miRNAs, support the hypothesis that miRNAs could exploit roles as oncogenes or as onco-suppressor genes; this is the case of miR-15 and miR-16

which have a role as suppressor of BCL-2 pro-apoptotic gene that is frequently overexpressed in B-cell chronic lymphocytic leukemia (B-CLL) [72]. Although miRNAs have been found as important modulators of tumor cell growth, they have been also involved in many other diseases. Indeed, they have been characterized as implicated in the regulation of several functions of the immune system and some of their alterations were directly linked to immunological disorders. Autoimmune diseases represent a group of immunological disorders in which altered miRNA expression has been found to play important roles. For example, miR-146a, miR-155 and miR-16 have been found altered in T-cells isolated from rheumatoid arthritis patients and miR-326 has been found to be downregulated in peripheral blood mononuclear cells (PBMCs) of patients with autoimmune multiple sclerosis [73, 74].

1.7.5 MiRNAs and MS

Overall is becoming clear that miRNA deregulation contributes to human autoimmunity diseases, including MS [75]. Several studies have recently explored the involvement of miRNAs in MS by studying their expression in peripheral blood cells (whole blood cells, peripheral blood mononuclear cells or in the T and B cell subset) mainly by microarray profiling [76, 77]. Results have been heterogeneous possibly due to the differences in methods of quantification, types of patients included and tissues used. MiRNA deregulation was also found by microarray profiling of MS lesions in the CNS. Alterations in miRNAs in tissue lesions might be related to immune cell infiltration and/or changes in miRNA expression on CNS-resident cells. MiR-155 and miR-326 were found up-regulated in active compared to inactive MS lesion [78]. These miRNAs target the 3' un-translated region of CD47, a protein that is ubiquitously expressed and inhibit phagocytosis of the cell by macrophages. This suggest that one mechanism could be that altered miR-155 and miR-326 levels in MS lesions reduce CD47 on CNS- resident cells, leading to a release of macrophages from inhibitory controls. Interestingly, miR-326 and miR-155 have also been reported to play important roles in modulating T cell responses in the EAE model. In vivo silencing of miR-326 resulted in fewer Th-17 cells and a less severe EAE, while in contrast over-expression resulted in more Th-17 cells and worse EAE. Moreover, miR-326 expression correlates with disease severity in patients with MS and mice with EAE [74]. Another study showed that mice with miR-155 deletion were highly resistant to EAE. The function of miR-155 is to promote inflammatory properties of T-cells including Th-17 and Th-1 cells subsets [79].
There is evidence suggesting that miR-223 could be also involved in MS pathogenesis. Several reports showed altered expression of miR-223 in MS. MiR-223 was demonstrated to be up-regulated in whole blood cells [80] and T regulatory cells [81] in MS subjects compared to healthy controls. MiR-223 was also over expressed in CNS active MS lesions compared to normal CNS specimens [78]. MiR-223 is mainly expressed in myeloid cells and its function was originally described in the regulation of granulopoesis. It modulates the NF-Kb pathway, thus altered expression of this miR-223 might affect macrophage activation [82]. MiR-223 deficient mice display an expansion of the granulocytes compartment and granulocytes are hyper mature, hypersensitive to activating

stimuli [83]. Mir-223 was found to be up-regulated on neutrophils infiltrating the CNS in mice after spinal cord injury [84]. The role of miR-223 in the EAE has not been reported yet.

2. AIMS

Aim 1: Perform a wide gene expression analysis in PBMC of MS patients by:

- 1a: Testing the expression profile of 1145 miRNAs in PBMC of different subtypes of MS patients compared to healthy controls in a discovery cohort.

- 1b: Validating the results obtained in the aim 1a in a second larger MS population.

- 1c: Studying the potential impact of deregulated miRNAs by performing a whole genome mRNA profiling on the discovery cohort of the aim 1a by looking at predicted targets according to online database.

Aim 2: Analyze deeply miRNAs which are known to be highly correlated in immune processes, especially in the subpopulation of CD4+ cells by:

- 2a: Testing the expression profile of candidate's miRNAs: miR-21, miR-146a and b, in the PBMC of different subtypes of MS.

- 2b: Carrying out an association analysis for miR-146 rs2910164 single nucleotide polymorphism (SNP) in a larger population of MS patients and controls.

Aim 3: Determine whether serum levels of extracellular microRNAs are altered in MS by:

- 3a: Performing miRNAs profiling in a discovery population of serum collected from MS samples and controls.

- 3b: Validating the best dysregulated miRNAs in a further independent validation sample.

- 3c: Trying to replicate the best miRNAs in a larger independent replication sample.

Aim 4: Determine the effects of mir-223 deficiency in vivo during EAE by:

- Comparing EAE in miR-223 deficient versus control mice using active immunization.

3. METHODS

3.1. Populations

The overall population available in our Institute consists of samples from 300 patients with MS and 300 age-matched controls. DNA from the overall population was available whereas serum, plasma and PBMC were available from a cohort followed over time consisting of 100 MS samples.

Aim 1: Population: the discovery cohort consisted of 19 MS patients (7 RRMS, 6 SPMS and 6 PPMS) and of 15 healthy controls. Three RRMS and 1 SPMS patients were in an acute phase at time of sampling. Regarding the treatment, among RRMS patients, 2 were treated with interferon-g-1a, 1 with azathioprine, and 4 untreated; among SPMS patients, 3 were treated with interferon-g-1b, 1 with azathioprine, 1 with interferon-g plus cyclophosphamide, and 1 untreated; among PPMS patients, 1 was in treatment with azathioprine, 1 with methotrexate, and 4 had no treatment. Data have been validated in a replication sample of 10 MS patients, of whom 5 with RRMS (2 treated with Interferon-g-1a and 3 untreated), 2 with SPMS (1 treated with azathioprine and 1 untreated) and 3 with PPMS untreated, and 10 age- and sex-matched controls.

Aim 2: Population: the miRNAs expression analysis population consisted of a cohort of 29 out of 346 patients: 16 RRMS patients in relapse phase, 6 SPMS, 7 PPMS and a cohort of 19 out of 339 age- and gender-matched controls. All these subjects underwent blood withdrawal for RNA isolation from peripheral mononuclear cells. Patients were not under any treatment at the time of the blood collection.

Aim 3: Population: we enrolled 3 RRMS, 4 PPMS and 3 controls as discovery population. The validation sample consisted of 8 RRMS, 5 PPMS and 11 healthy controls. The replication sample consisted of 28 MS patients, 16 RRMS, 13 PPMS and 30 controls. Patients were not under any treatment at the time of the blood collection.

All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory test, brain Magnetic Resonance Imaging. All patients fulfilled the McDonald‘s criteria [85]. Age of controls did not significantly differ from that of patients, as well as gender distribution. Informed consent to participate in this study was given by all subjects.

3.2. mRNA and miRNAs extraction from PBMCs

For each subject mRNA and miRNAs were extracted from 14 ml of blood using gradient centrifugation and Trizol reagent according to manufactured protocol. RNA quantification and integrity were assessed using Nanodrop-2000 spectrophotometer and gel electrophoresis.

3.3. MiRNAs whole genome micro-array screening and quantitative PCR (QPCR) analysis for miRNA

The expression profile of miRNAs was determined using the Illumina® BeadArray technology. We used the Universal-12BeadChip® to analyze 1145 miRNAs for each sample. The protocol was performed following the manufacturer's instructions. Bead-Chips are imaged using the Illumina BeadArray Reader®, measuring the fluorescence intensity at each addressed bead location. We used the Illumina GenomeStudio® software (version 2010.1) to assess fluorescent signal, whose intensity corresponds to the quantity of the respective miRNA in the original sample. Each sample has been tested in technical replicate.

For the QPCR the reverse transcription (RT) reaction was performed using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol. The quantitative Real Time PCR was performed on the ABI7500 FAST system (Applied Biosystems). MiRNAs were detected with single TaqMan® miRNA assays (Applied Biosystems). Reaction and amplification protocols were performed according to the instruction of the manufacturer. As endogenous control gene was used the small nuclear RNA RNU48 and the relative quantification was calculated with the ΔΔCt method.

3.4. mRNA whole genome micro array screening

Whole genome expression analysis was performed using the Illumina® BeadArray technology. The mRNA expression profile was obtained using the Illumina Total Prep RNA Amplification Kit (Ambion) and the HumanHT-12 Expression BeadChip® following the manufacturer's instructions. Each individual array on the chip targets more than 25,000 annotated genes with more than 48,000 probes derived from the RefSeq (Build 36.2, Rel 22) and UniGene (Build 99). BeadChips were imaged using the Illumina Bead Array Reader and the Illumina GenomeStudio® software was used to assess fluorescent hybridization signals and to perform the quality control filters. Each sample has been tested in technical replicate.

3.5. DNA extraction and SNP analysis

High molecular weight DNA was isolated from whole blood using Flexigene Kit, as described by manufacturer. The amount of DNA was determined by measuring the optical density at 260 nm wavelength on a spectrophotometer. rs2910164 was analyzed using TaqMan methodology on the ABI7500 FAST system.

3.6. MiRNAs isolation and qPCR analysis from human serum samples

Whole blood samples were allowed to sit at room temperature for a minimum of 30 min and a max of 2 hrs, after collection. Separation of the clot was done by centrifugation at 1,000–1,300 x g at room temperature for 15–20 min. The serum was removed and dispensed in aliquots of 400µl into cryo tubes. Specimens were stored at -80°C. 400µl of human serum was thawed on ice and lysed with equal

volume of 2X Denaturing Solution (Ambion). To allow sample-to-sample normalization, synthetic *C. Elegans* miRNAs *cel-miR-39*, *cel-miR-54* and *cel-miR-238* (synthetic RNA oligonucleotides synthetized by Qiagen) were added (as a mixture of 25 fmol of each oligonucleotide in 5 µl total volume) to each denatured sample. RNA was isolated using *mir*VANA PARIS kit following the manufacturer's protocol for liquid samples (Ambion).

3.7. Screening of miRNAs in human serum by Human RT^{2}miRNA PCR Array (MAH-001, SABiosciences)

RNA was retrotranscribed with RT^{2}miRNA First Strand kit (SABiosciences) according to the instruction of the manufacturer. For Real Time PCR experiments the Human RT^{2}miRNA PCR Array (MAH-001) was used and runs were performed in an Applied BioSystems 7500 FAST system. The array profiles the expression of 88 most abundantly expressed and best characterized miRNA sequences in the human miRNA genome. Human RT^{2}miRNA PCR Arrays included built-in positive control elements for the proper normalization of the data (SNORD48, SNORD47, SNORD44 and RNU6-2), for the quality of RNA samples and for general PCR performance of the array.

3.8. Measurement of miR-23a, miR-15b and miR-223 levels in human serum by Taqman qRT-PCR assays

1.67 µl of RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems, ABI) in a small-scale RT reaction. The RT product was diluted and the protocol from Mitchell et al was used [86]. Data were analyzed with SDS Relative Quantification Software version 2.2.2 (ABI), with the automatic Ct setting for assigning baseline and threshold for Ct determination.

3.9. Statistical analysis

The SABiosciences PCR Array data analysis was based on ΔΔCt method with normalization of the raw data to housekeeping genes (using the software available at http://www.sabiosciences.com/pcarraydataanalysis.php). *P* values were calculated based on a Student's t-test of the replicate 2^(- ΔCt) values for each gene in the control group and MS group. The data obtained were calculated as Fold-Change [2^(- ΔΔCt)], which was the normalized gene expression [2^(- ΔCt)] in the MS group divided by the normalized gene expression [2^(- ΔCt)] in the control group. Comparisons between miRNAs levels in the different groups were performed using a One-Way Anova. Spearman test was used for correlations with clinical data.

3.10. Mice

Protocols were approved by the Animal Studies Committee of Washington University. MiR-223knock out (miR-223 KO) and control mice were transferred from the laboratory of Dr Fehinger and bred at our animal facility. These two mouse

lines were initially derived from littermates, and were subsequently bred in parallel. These mice were first engineered from Johnnidis and colleagues and further backcrossed for twelve generations to C57BL/6 mice [83].

3.11. EAE induction

Mice were immunized subcutaneous with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ (MOG aa 35–55: MEV GWY RSP FSR VVH LYR NGK; Sigma Genosys, Woodlands, TX, USA), emulsified in IFA 50 ug/mouse Mycobacterium tuberculosis, strain H37RA. In all experiment, mice also received 300ng/mouse pertussis toxin (PTX; List Biological Laboratories, Campbell, CA, USA) intra venous at the time of immunization and 2 days post immunization. The development of EAE was followed and graded on a scale of 0–5 as described previously [87].

3.12. CNS histology

Mice were perfused with buffered 4% paraformaldehyde, CNS tissue-dissected, and embedded in paraffin. Thick sections (4 um) on slides were stained with H&E to assess inflammation and Luxol Fast Blue (LFB) to assess demyelination. Scoring for inflammation and demyelination was done in a blinded manner by a single observer on a scale of 0–5, as previously published [16]. For inflammation, the scoring system was: 0 - no inflammation; 1 - a few inflammatory cells in the leptomeninges; 2 -organization of inflammatory cells around blood vessels; 3 - extensive perivascular cuffing with extension into the underlying parenchyma; 4 - large regions of white matter inflammation extending into the parenchyma covering between one-fourth and one-half cord white matter; 5 -extensive inflammation covering more than one-half cord white matter. For demyelination, the following scale was used: 0- none; 1 - a few subpial fibers affected; 2 - partial rim of subpial involvement, not into the parenchyma; 3 - extension beyond the subpial region into the parenchyma; 4 - large regions of white matter involvement, less than one-half cord cross-section; 5 - one-half or more than one-half cord white matter cross-section involved.

3.12. Proliferation assays and cytometric bead array (CBA)

Spleen cells were isolated from immunized mice and cultured at 2.5 x 106 cells/ml in complete RPMI-1640 medium, 5% FBS, with or without 10 ug/ml antigen ($MOG_{35–55}$). Incorporation of 3Hthymidine (0.5 uCi/well) during the final 18 h was counted (Betaplate 1205, Wallac, Gaithersburg, MD, USA), and the stimulation index was calculated as the ratio between count per minute (cpm) with antigen and cpm in medium alone. For CBA analysis spleens from the mice were removed at Day 30 post immunization. Single-cell suspensions were prepared, and cells were cultured with medium alone or with $MOG_{35–55}$ (5 or 10 ug/ml). Aliquots of cell culture supernatant were removed at 48, 72, and 96 h of culture and frozen at –80° for subsequent analysis. IL-17, TNF, IFN-g, and IL-6, present in cell culture supernatants, were analyzed by CBA (BD Biosciences, Franklin Lakes, NJ, USA).

3.13. FACS analysis of spleen cells

FACS analyses were performed on spleenocytes that were stained for 15 min with FITC-conjugated anti-mouse CD11b antibody (clone M1/70, 561688 BD Pharmingen), phycoerythrin (PE)-conjugated anti-mouse Ly-6C antibody (clone AL-21, 562728 BD Horizon), PerCP-Cy 5.5-conjugated anti-mouse Ly-6G (clone 1A8, 551461, BD Pharmingen), (APC)-conjugated anti-mouse MHC class II (clone M5/114.15.2, 47-5321 eBioscence) phycoerythrin (PE)-conjugated anti-mouse SiglecF (clone E50-2440 562068, BD Pharmingen), FITC-conjugated anti-mouse CD4 (clone H129.19, 553651 BD Pharmingen), PerCP-Cy 5.5-conjugated anti-mouse CD19 (clone ID3, 340865 BD Pharmingen) and (APC)-conjugated anti-mouse CD8 (clone 53-6.7, 561093 BD Pharmingen). Cells were subsequently washed with excess medium and re-suspended in 2% FCS. Flow cytometry was conducted on a FACSCalibur (BDBiosciences).

4. RESULTS

4.1 MicroRNA and mRNA expression profile screening in multiple sclerosis patients to identify new potential biomarkers

4.1.1 MiRNAs whole-genome micro-array screening and validation

MiRNAs represent key regulators of several biological phenomena, including cell proliferation and differentiation, apoptosis, signal transduction and organ development. Several articles have been published in the last years exploring the role of miRNAs in multiple sclerosis, suggesting an involvement of miRNAs in the pathogenesis and course of MS. Here is reported our study on 1145 miRNAs in the PBMCs of MS patients along with healthy controls, with the aim to identify novel potential biomarkers and link miRNAs to specific genes that are dysregulated in MS.

Expression profile of 1145 miRNAs was determined using the Illumina® technology in PBMCs from 19 patients with MS and 14 controls (discovery sample). A total of 805 miRNAs (70.0% of the total) passed the quality control filter, characterized by the presence of a detection p-value <0.01 in cases or in controls. Among them, a total of 104 miRNA transcripts were significantly deregulated ($P< 0.05$), of which 45 upregulated and 59 downregulatedin MS patients versus controls. After excluding the non-annotated miRNAs (HS 176 and HS 204.1) and miR-653:9.1 which was not present in the miRBase Targets Release Version 5, we list the best 10 hits in Table 1. Among the 10 miRNAs which ranked highest, 6 (let- 7g, miR-150, miR-374a*, miR-363, miR31* and miR192) have been tested in the replication sample, two of which being significantly deregulated with same direction in such sample (let-7g ($p = 0.001$) and miR-150 ($p = 0.004$)) (Figure. 1).

4.1.2 Target genes of identified deregulated miRNAs

Identified deregulated miRNAs were conceived to be instrumental for the discovery of novel putative genetic targets and biomarkers potentially implicated in the disease using two alternative approaches. In the first approach, we searched putative targets of let-7g and miR-150 using bioinformatic prediction algorithm as implemented in miRBase Targets database using a stringent cut-off of $p < 0.001$. A total of 301 and 120 target genes have been predicted for let-7g and miR-150, respectively. Since the two miRNAs were down-regulated in MS cases compared to controls (Figure 1), we further restrict the list to those targets which were found to be significantly upregulated in MS versus controls ($p < 0.05$) according to genome-wide expression (GWE) data available on the same cohort of cases and controls (Table 2). In the second approach, we used a clustering algorithm as implemented by the GenomeStudio® software to directly correlate miRNA and mRNA expression levels. According to cluster analysis, let-7g was negatively correlated in both patients and controls with leucine-rich repeat kinase 1(LRRK1) mRNA levels (r: -0.70) and, in MS patients, with a putative gene (C9orf109; r: -0.85), whose function is still unknown. In patients, but not in controls, miR-150

expression was negatively correlated with claspin *Xenopuslaevis* human homolog (CLSPN) mRNA levels (r: -0.73). However, none of these genes was predicted to be targets of let-7g or miR-150 according to miRBase Targets database, and they were not represented in the list of the differentially expressed mRNA transcripts ($p < 0.05$) obtained in our GWE study. Stratified analyses did not reveal any major effect exerted by the gender and disease course, even if analyses were inflated by the small sample size in the categories. As regards the type of treatment, let-7g was significantly different in MS cases and controls even restricting analyses on 9 MS patients with no treatment ($p = 0.003$), while miR-150 was not confirmed as significant ($p = 0.31$).

Let-7g, it was suggested that it targets the expression of the Toll-like receptor 4 (TLR4) which plays a key role in host immunity. Let-7g was negatively correlated with LRRK1 transcript, which, according to available bio informatic prediction algorithms, it is not a predicted target of let-7g. Therefore, there are likely other factors or pathways not yet discovered which potentially contribute to the observed association. In the list of genes targeted by let-7g according to miRBase Targets database ($p < 0.001$) and upregulated in GWE data (Table 2), the most interesting candidate is the HIV-1 Tat interactive protein 2 (HTATIP2 or TIP30) which was found to be over-expressed in MS chronic lesions and, according to in vitro experiments, to prevent the maturation of oligodendrocyte precursor cells into oligodendrocytes possibly explaining the remyelination failure of MS lesions [88]. Moreover, this protein inhibits the transcription of osteopontin, a pro-inflammatory cytokine implicated in the generation of Th17cells [89].

MiR-150 is known to be involved in the maturation of B cells [90]. Among predicted targets of miR-150 upregulated in GWE data (Table 2), it is of interest the presence of the suppressor of cytokine signaling-1 gene (SOCS1) which affects immune processes within the central nervous system (CNS) [91]. Furthermore, the SNP rs243324, located in the 5 regulatory region of the SOCS1 gene was reported to be associated with MS [92]. Moreover, transgenic mice overexpressing SOCS1 in oligodendrocytes develop a more severe experimental autoimmune encephalomyelitis (EAE). Other interesting targets include the SPI1 gene, a transcription factor implicated in the emathopoiesis and in the maturation of the immune system, and the EphrinB2 gene (EPHB2), a receptor of ephrins which mediate numerous developmental processes, particularly in the nervous system. No correlation between in silico findings and GWE based approach was found, since CLSPN transcript was not among predicted targets.

4.2 Expression and genetic analysis of miRNAs involved in CD4+ cell activation in patients with multiple sclerosis

4.2.1 MiRNAs expression analysis from whole PBMC of MS patients and healthy controls

It's known by literature that miRNAs have unique expression profiles in cells of the innate and adaptative immune system, and play pivotal roles in the regulation of both cell development and function, In particular, miRNAs targeting key molecules involved in processes in the immune system are critical for MS susceptibility. Some

miRNAs seem to be implicated in the activation and proliferation of lymphocytes such as the miR-21 and the miR-146 family. MiR-21 is implicated in the activation of CD4+ lymphocytes [93]. The miR-146 family is composed of two members, miR-146a and -b, both involved in the innate immune response, for which miR-146a seems to act as negative regulator .By these evidences, we sought for differences in the expression profile of miR-21, miR-146a and -b, which are known to be highly correlated in immune processes, in different subtypes of MS.

MiRNAs expression analysis revealed a deregulation of certain miRNAs in patients as compared with controls. In particular, significantly increased expression levels of miR-21, miR-146a and -b were found in RRMS patients as compared with controls (1.44 ± 0.13 vs 0.79 ± 0.06, $P = 0.036$; 1.50 ± 0.12 vs 0.84 ± 0.08, $P = 0.039$; 1.54 ± 0.15 vs 0.72 ± 0.08, $P = 0.001$, Fig. 2).No differences were found considering miRNAs expression levels in SP and PPMS.
According to our results, a dysregulation of miR-21, miR-146aand -b seems to occur in RRMS patients as compared with controls, suggesting that these molecules might contribute to the pathogenesis of the disease. Intriguingly, all RRMS patients considered were in the relapse phase, thus the observed dysregulation appears to be specific of the acute phase of disease, where the inflammatory events are predominant. This is in accordance with previous observations showing that miR-21, miR-146a and -b are involved in the differentiation and regulation of CD4+ cells. In particular, it was previously shown that the expression level of miR-21 is elevated in activated T cells in vitro, which represents the main pathological event leading to the disease. Considering miR-146a, increased levels were already found in other autoimmune diseases such as rheumatoid arthritis and psoriasis. Recently miR-146 was found over expressed by regulatory T cells (Treg) [94], a highly specialized T cell subset responsible for controlling self-reactive T cells, a process which appears to be impaired in MS. Intriguingly, according to our results, miR-21, miR-146a and –b did not seem to be dysregulated in the progressive phase of the disease (SP and PPMS) since their expression level is comparable to the one found in controls. These differences could be related to a different pathogenesis underneath different subtype of disease. In particular in RRMS, inflammatory events are predominant and occur in response of an aberrant activation of the immunesystem towards myelin proteins supposed to act as autoantigens. Nevertheless, in progressive forms of MS, neurodegenerationis prominent, as demonstrated by the development of motor disability, cognitive deficits and brain atrophy [95]. This makes conceivable that the dysregulation of miRNAs specific of the activation and differentiation of the immune system seems to be predominant in the acute phase of the disease.

4.2.2 Association analysis for miR-146 rs2910164 SNP

In addition, with the premise that gene polymorphisms may play a role in the biogenesis and action of the mature miRNA, we carried out an association analysis for miR-146 rs2910164 single nucleotide polymorphism (SNP) in a large population of MS patients and controls.
Genes encoding for the above mentioned miRNAs were subsequently screened for SNPs through a bioinformatics approach (www.patrocles.org). The analysis

revealed that only miR-146a gene presents a common variant, rs2910164, which constitutes in a *G* to *C* substitution possibly influencing the processing of the miRNA or altering the target binding affinity and specificity [96]. Rs2910164 was subsequently tested for association with MS. Data analysis failed to detect any differences either considering allelic and genotypic frequencies in patients as compared with controls (MAF: 28% vs 25%, Table 2, P> 0.05). Stratifying according to gender or disease subtype, no differences were found as well (P > 0.05). Moreover, no influence seems to be exerted by rs2910164 in miR-146a and -b levels since stratifying according to rs2910164 status, no significant differences have been found in miR-146 levels between carriers and non-carriers of rs2910164 C allele (P > 0.05) (Table 3).

4.3 Decreased circulating miRNA levels in patients with multiple sclerosis

4.3.1 Screening of 88 miRNAs in the serum of MS patients and healthy controls

The course of MS is largely unpredictable, and, as opposed to other diseases, clear quantitative criteria for management of both treatment and response to it are lacking. The mechanism of action of immunomodulatory agents is not clearly understood, and there are no reliable clinical or biological markers that accurately predict response to therapy. In this setting of variable responsiveness and clinical heterogeneity the identification of biomarkers for immunomodulatory treatment efficacy would allow to predicting the course of the disease, the efficacy of treatments in modulating inflammatory events ongoing and the clinical response to different drugs. A promising class of prognostic biomarkers for MS could be microRNAs (miRNAs). Recently miRNAs have been found also in the extracellular environment, such as serum, plasma and other body fluids [97, 98]. These miRNAs are stable and show distinct expression profiles among different fluid types. The origin and the function of these circulating extracellular miRNAs remains poorly understood. They are likely used as mediators of cell-cell communication, although many more studies are needed to clarify this process [97].
For these reasons we decide to analyzed the expression profile of the 88 most abundantly expressed and best characterized miRNAs (Figure 3A) in the serum of a discovery cohort consisting of 7 MS and 3 control subjects. An overall down regulation of miRNAs in MS compared to control subjects was observed (Figure 3 B). The trend was even stronger when PPMS patient miRNA levels were compared to controls (Figure 3C).
Results from array analysis led to the identification of the three most significantly down regulated miRNAs: miR-15b (0.31 fold changes over controls $P<0.029$), miR-23a (0.41 fold change over controls, $P<0.047$) and miR-223 (fold change 0.19 over controls, $P<0.004$). Stratifying according to disease subtype, when PPMS patients were considered, the downregulation still remained significant for miR-223 (fold change 0.29 over control, $P<0.024$), whereas for miR-15b and miR-23a the significance threshold value was borderline (miR-15b) or not reached (fold change 0.23, $P<0.05$ and fold change 0.30, $P<0.084$, respectively). Considering only

RRMS patients, a significant downregulation was observed only for miR-223 levels (fold change 0.29 over controls, *P*<0.024).
Upon identification of the candidate dysregulated miRNAs, we proceeded with the identification of putative target genes through predictive algorithms that led to the identification of genes potentially implicated in MS pathogenesis. Target prediction based upon TargetScan 6.1, www.microRNA.org and www.pictar.org websites led to the identification of several target genes of possible relevance to MS pathology. Both miR-15b and miR-23a target *FGF-2* gene, a member of the fibroblast growth factor family. FGF-2protein has been implicated in several biological processes, such as limb and nervous system development, wound healing, and tumor growth. FGF-2 levels are reported elevated in CSF of MS patients, particular those with active disease and the gene was found to be differentially expressed in active and chronic MS lesions in postmortem tissues suggesting FGF-2 as marker of inflammation in MS lesions.
Another interesting target gene of miR-15b is *KIF-1B* (Kinesin family member 1B) which encodes a motor protein that transports mitochondria and synaptic vesicle precursors. *KIF*-1B gene was extensively investigated in the context of MS as a possible gene influencing MS susceptibility, though results remain controversial.
Among the genes targeted by miR-223 is the transcription factor mef-2c (myocyte enhancer factor 2C). Mutations and deletions at this locus have been associated with severe mental retardation and epilepsy [99].
MiR-223 plays a role in the regulation of granulopoiesis by targeting mef-2c and modulates the NF-Kb pathway, thus its downregulation could modulate immune inflammatory responses [82].

4.3.2 Validation analysis

Results were subsequently validated in an independent population consisting of 13 MS and 8 control subjects. Significant downregulation of miR-15b, miR-23a, and miR-223 levels in MS patients when compared to controls was confirmed (miR-15b: 0.47±0.16 vs 2.35 ± 0.82 mean fold change over controls ± SEM, miR-23a: 0.45 ± 0.10 vs 1.59 ± 0.26 mean fold change over controls ± SEM and miR-223: 0.29 ± 0.07 vs 1.01 ± 0.14 mean fold change over controls ± SEM, *P*<0.001, Figure 4A, B and C). Moreover, stratifying according to disease subtype, the downregulation still remained significant in both RR- and PPMS patients versus controls (*P*<0.01, Figure 4A, B and C).

4.3.3 Replication analysis

Once validated, results were further considered for replication in an independent larger cohort consisting of 28 MS and 30 healthy subjects. MiR-15b was confirmed to be significantly downregulated in MS samples compared with controls (mean fold change ± SEM over controls: 1.62 ± 0.25 vs 2.49 ± 0.34, *P*<0.02, Fig 5A). Conversely, stratifying according to disease subtype a significant downregulation of miR-15b levels was confirmed in PPMS (mean fold change over controls ± SEM: 1.21 ± 0.21 versus 2.49 ± 0.34, *P*<0.01) but not in RRMS patients (mean fold change over controls ± SEM: 1.98 ± 0.41 vs 2.49 ± 0.34, *P*>0.05, Figure 5A).

Similarly, results were replicated for miR-223. In particular, a significant downregulation was observed in MS samples compared with controls (mean fold change over controls ± SEM: 3.25 ± 0.41 versus 5.16 ± 0.70, *P*< 0.03, Figure 5C). Interestingly, miR-223 levels results were downregulated the most in PPMS patients (mean fold change over controls ± SEM: 2.09 ± 0.36 versus 5.16 ± 0.70, *P*=0.002 Figure 5C).
In contrast, the replication study failed for miR-23a. No significant differences in miR-23a levels were found in MS patients when compared to controls, even when the RR and PPMS groups were considered separately (*P*>0.05, Figure 5B). The reason for this failure could be attributed to the different environmental exposures in the populations considered, or a spurious finding in the initial smaller cohorts. Despite that both MS validation and replication populations were Caucasian and age-gender matched, they reside in different geographical areas with exposures to different environmental stimuli, such as air pollution, chemicals, heavy metals and bisphenolA that have been shown to influence miRNA transcription.
Stratifying miRNA levels according to gender, significantly downregulated levels of miR-223 were found both in PPMS male patients and PPMS female patients as compared with the respective gender-matched controls (2.14 ± 0.54 versus 5.42 ± 1.20 mean fold change over controls ± SEM, *P*=0.03 and 2.05 ± 0.50 versus 4.98 ± 0.88 mean fold change over controls ± SEM, *P*=0.04).

4.3.4 Correlation with clinical data

A correlation analysis with clinical data and miRNA levels was performed considering the validation and replication MS cohorts together. No significant correlations were found between miRNA levels and age, or disease duration at blood drawn.
Conversely, significant correlations were found with the Expanded Disability Status Scale (EDSS), as a measure of clinical disability, at time of blood drawn.
MiR-15b, miR-23a and miR-223 robustly directly correlated with the EDSS score in the overall PPMS sample (r=0.61, *P*<0.007; r=0.59, *P*<0.009; r=0.86, *P*<10^{-4}) (Figure 6). When the RRMS group was considered, a statistically significant direct correlation was observed for miR-23a only (r=0.67, *P*<0.001, data not show). On the contrary, when the overall MS population was considered for the analysis, the only significant direct correlation was found between EDSS score and miR-23a levels.
Strikingly data arising from the present study indicated the down regulation of miR-15b and miR-223, in MS patients, which was driven by PPMS subjects of both genders. The correlation between the levels and disability, as measured by the EDSS score, was direct. This was unexpected because the actions of miRNA to down regulate gene expression and the genes being targeted might suggest that lower miRNA would enhance inflammation. Thus, the direct correlation of higher circulating miRNA levels with worse EDSS was not expected. The correlation was driven by the PPMS subset, which is known to involve less inflammation than RRMS. In PPMS, higher miRNAs levels correlated with worse disability status. With the expectation that increased miR-15b and miR-223 would diminish inflammation, the direct association with disability score in PPMS is reminiscent of a concept that inflammation may provide a beneficial effect and might relate to

differences between RRMS (where remissions are seen) and PPMS (where remissions are not seen) in certain situations in MS [100].

4.4 Role of miR-223 in the EAE model

4.4.1MiR-223 knock-out mice develop attenuated EAE in comparison to littermates control mice

In our work we have found that miR-223 is down regulated in the serum of MS patients compared to healthy controls. MiR-223 was also found to be up-regulated by microarray analysis in whole blood [80] and in active brain lesions in MS patients compared to control subjects [78]. MiR-223 is highly expressed in myeloid cells [101] and it modulates the NF-Kb pathway [82], with subsequent effects on inflammatory and immune responses. All these evidences suggest a possible role of miR-223 in disease mechanisms and prompted us to test disease susceptibility in miR-223 knock out (miR-223 KO) mice in the animal model for MS, the EAE model. EAE model is a critical step for a better understanding the relevance of miR-223 in all the pathogenic process.

MiR-223 knock-out (KO) mice were obtained from Dr. Fenhiger Todd. The first EAE experiment was performed using 8 male miR-223 KO mice and 4 littermates control mice (n=4). These mice are available on a C57BL/6 background, thus EAE was induced with MOG_{35-55} using protocols of immunization which are routine in our laboratory (see methods). Overall miR-223 KO mice developed delayed and significantly less severe disease compared to littermate control mice ($P<0.0001$ by two-way ANOVA) (Figure 7). Table 2 summarizes EAE clinical characteristics. MiR-223 KO mice displayed a delayed disease onset and a reduced mean maximum and cumulative clinical score. The EAE experiment was repeated twice with the same results.

4.4.2 Deletion of miR-223 decreases CNS inflammation, demyelination, and axonal damage during EAE

The degrees of CNS inflammation and demyelination were evaluated at day 30 post immunization in the upper and lower spinal cords of miR-223 KO mice (n=8) and the littermate controls (n=3) (Figure 8A). Inflammation and demyelination were graded in a blinded manner. Inflammation was not reduced significantly in miR-223 KO mice compared with control mice. Demyelination was reduced but not significantly in miR-223 mice compared with controls (Figure 8B). Extensive areas of demyelination were detectable in control mice, and little demyelination was observed in miR-223 KO mice.

4.4.3 Deletion of miR-223 does not inhibit T cell priming in the EAE-induction phase

At the end of the experiment on day 30 post-immunization we tested lymphocyte activation and cytokine production against MOG_{35-55} in vitro. For this purpose,

splenocytes were isolated from miR-223 KO and control mice and re-stimulated in vitro with different concentration of $MOG_{35-55}$for 72h. No differences in lymphocyte proliferation were noted between the two groups. IL-2, TNF-α, IFN-γ, IL-17, IL-10, and IL-6 production was assayed in cell culture supernatants. No significant differences in proliferation and cytokine production were detected between spleen cells from miR-223 KO versus control mice in two experiments (data not shown).

4.4.4 MiR-223 KO mice showed a higher number of monocytes and granulocytes/neutrophils compared to controls mice

In parallel, splenocytes harvested for flow cytometer analysis, the staining was performed using a 4 colors FACSCalibur with three different set of antibodies anti-mouse: first CD11b, MHC classII, Ly6C/Ly6G, Ly6C, second CD11b, SiglecF, Ly6C/Ly6G, Ly6C and CD4, CD8, CD19. The staining showed a significantly higher number of granulocytes/neutrophils (Ly6C/Ly6G positive cells),eosinophils (SiglecF positive cells) and monocytes (Ly6C single positive cells) in the miR-223 KO compare to WT mice, this results are in agreements with the previous report that showed an unusual neutrophilia in miR-223 mice [83] (Figure 9A). While the number of CD8 positive cells in the miR-223 KO mice is significantly lower compare to the WT mice. No differences in the number of CD4 and CD19 positive cells between the two groups were seen (Figure 9B).

5. FIGURES

	miRNA ID	Media ± SEM in MS	Media ± SEM in controls	Fold change	p-value
1	miR-363	4756.6 ± 289.5	5894.0 ± 149.1	−1.33	$1.44x10^{-6}$
2	miR-31	780.2 ± 163.0	1141.0 ± 141.6	−1.54	$8.69x10^{-5}$
3	miR-524-3p	174.7±12.5	151.7 ± 4.8	1.25	$2.35x10^{-4}$
4	miR-876-3p	233.5 ± 37.5	385.1 ±47.7	−1.54	$7.97x10^{-4}$
5	let7g	20106.8 ± 212	20745.7±141.8	−1.05	$1.10x10^{-3}$
6	miR-223	3875.7 ± 130.4	3341.8±136.1	1.16	1.38x10
7	miR-550	1290.4 ± 375.8	825.2 ±77.6	2.02	$1.65x10^{-3}$
8	miR-181	1934.1 ± 426.1	2887.7 ±545.8	−1.56	$1.73x10^{-3}$
9	miR-374	1014.1 ± 148.4	1914.1 ±157.0	−1.49	$1.97x10^{-3}$
10	miR-150	24507.8± 197	25893.8 ±162.7	−1.03	$2.06x10^{-3}$

Table 1.Ranked list of 10 most differentially expressed miRNAs in MS cases versus controls.

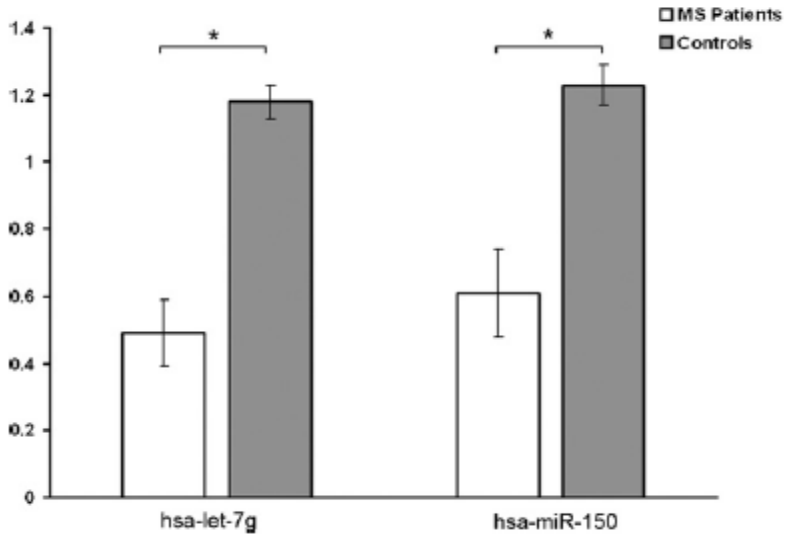


Figure 1. Expression levels of replicated miRNAs in PBMC from MS patients and controls. Values are expressed as mean relative miRNA levels ±SEM. y-Axis represents the relative expression level taking an arbitrary reference sample as 1.0.

miRNA	N. of target genes predicted using mirbase (*p*<0.001)	Target genes upregulated in MS vs controls (*p*<0.05) in GWE data	Fold Change	Differential *p*-value	*r*
Let-7g	301	KCTD14	2.07	0.0328	-0.65
		GNG5	1.44	0.0006	-0.52
		ATOX1	1.60	0.0004	-0.51
		ZNF341	1.25	0.0228	-0.48
		HTATIP2	1.16	0.0130	-0.41
		JOSD2	1.47	0.0056	-0.41
		HBEGF	2.27	0.0006	-0.36
		ESPL1	1.10	0.0125	-0.35
		C19orf56	1.27	0.0014	-0.27
		CYB561D2	1.17	0.0080	-0.2
miR-150	120	ASGR1	1.46	0.0373	-0.52
		SP5	1.17	0.0029	-0.48
		DCPS	1.46	0.0185	-0.43
		SPI1	1.55	0.0032	-0.40
		EPHB2	1.30	0.0006	-0.38
		COG7	1.23	0.0037	-0.34
		TRPM4	1.32	0.0006	-0.33
		SLC6A12	1.62	0.0318	-0.29
		SOCS1	159	0.0185	-0.21
		IER5	1.39	0.0011	-0.21

Table 2. Expression levels of genetic targets of two deregulated miRNAs selected using in silico approach and found up regulated in MS vs controls (p<0.05) in GWE data.

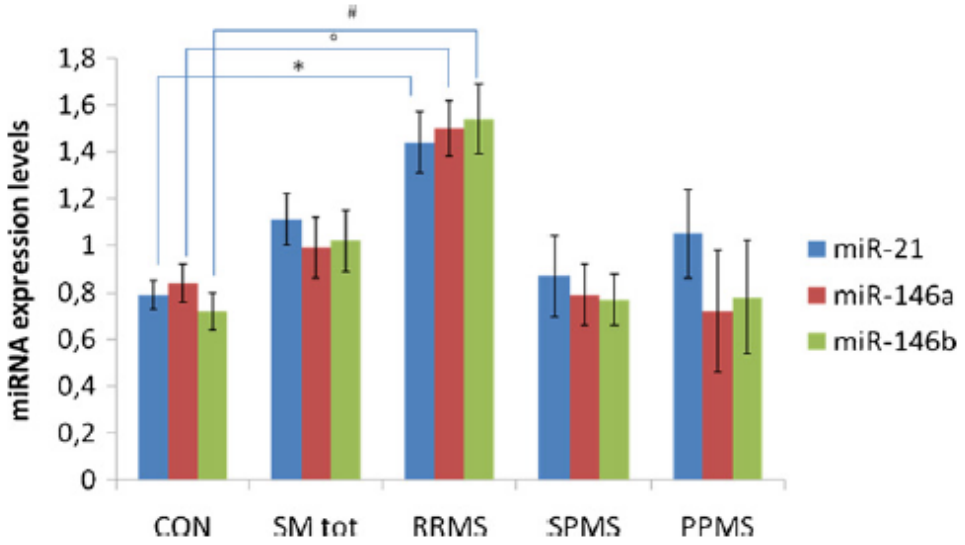


*Figure 2.miR-21, miR-146a and -b expression levels in PBMC from MS and controls, expressed as fold increase ± SEM. *P = 0.036, ◦P = 0.039, #P = 0.001 RRMS patients vs controls.*

miR-146 rs2910164 frequencies	Controls (n = 339)	MS patients (n = 346)
Allele		
G	505 (75.0)	497 (72.0)
C	173 (25.0)	195 (28.0)
Genotype		
GG	195 (57.5)	181 (52.3)
GC	115 (34.0)	135 (39.0)
CC	29(8.5)	30(8.7)

Table 3. Allele and genotype frequencies expressed as n (%) of miR-146 rs2910164 SNP in patients and controls.

Array Layout

miR-142-5p	miR-16	miR-142-3p	miR-21	miR-15a	miR-29b	let-7a	miR-126	miR-143	let-7b	miR-27a	let-7f
miR-9	miR-26a	miR-24	miR-30e	miR-181a	miR-29a	miR-124	miR-144	miR-30d	miR-19b	miR-22	miR-122
miR-150	miR-32	miR-155	miR-140-5p	miR-125b	miR-141	miR-92a	miR-424	miR-191	miR-17	miR-130a	miR-20a
miR-27b	miR-28b	miR-148a	miR-200c	miR-99a	miR-19a	miR-23a	miR-30a	let-7i	miR-93	let-7c	miR-106b
miR-101	let-7g	miR-425	miR-15b	miR-28-5p	miR-18a	miR-25	miR-23b	miR-302a	miR-186	miR-29c	miR-7
let-7d	miR-30c	miR-181b	miR-223	miR-320a	miR-374a	let-7e	miR-151-5p	miR-374b	miR-196b	miR-140-3p	miR-100
miR-103	miR-96	miR-302b	miR-194	miR-125a-5p	miR-423-5p	miR-376c	miR-195	miR-222	miR-28-3p	miR-128	miR-302c
miR-423-3p	miR-185	miR-30b	miR-210	SNORD48	SNORD47	SNORD44	RNU6-2	miRTC	miRTC	PPC	PPC

Figure 3A.. List of the plat position of tested miRNAs.

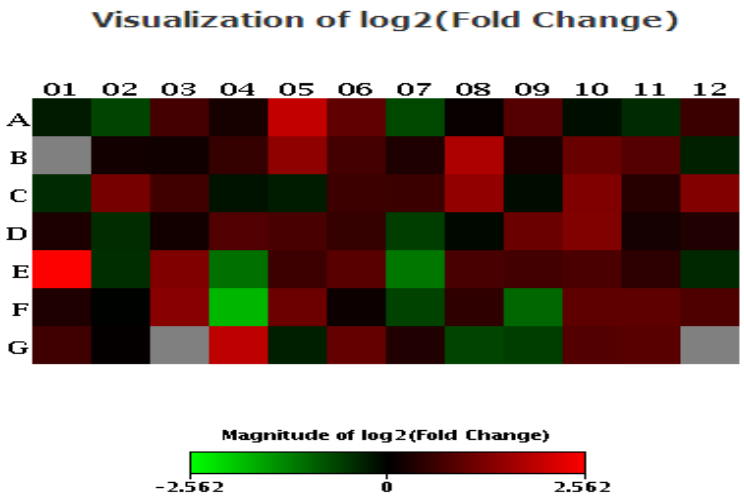


Figure 3B.Cluster diagram of fold change values for the 88 investigated miRNAs from the array profiling in the discovery sample. Fold change values in RRMS patients versus controls.

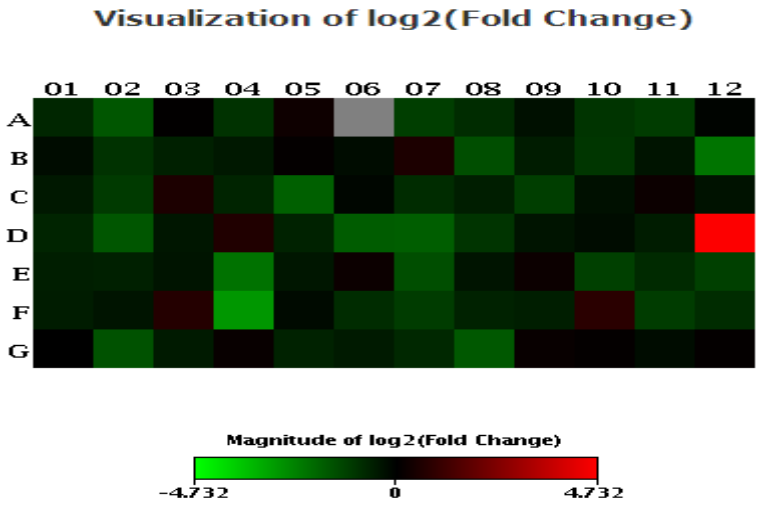


Figure 3C. Cluster diagram of fold change values for the 88 investigated miRNAs from the array profiling in the discovery sample. Fold change values in PPMS patients (3C) versus controls. Each square represents a single miRNA. Green squares represent lower than median level of miRNA expression; black squares represent median level of miRNA expression and red squares represent higher than median level of miRNA expression.

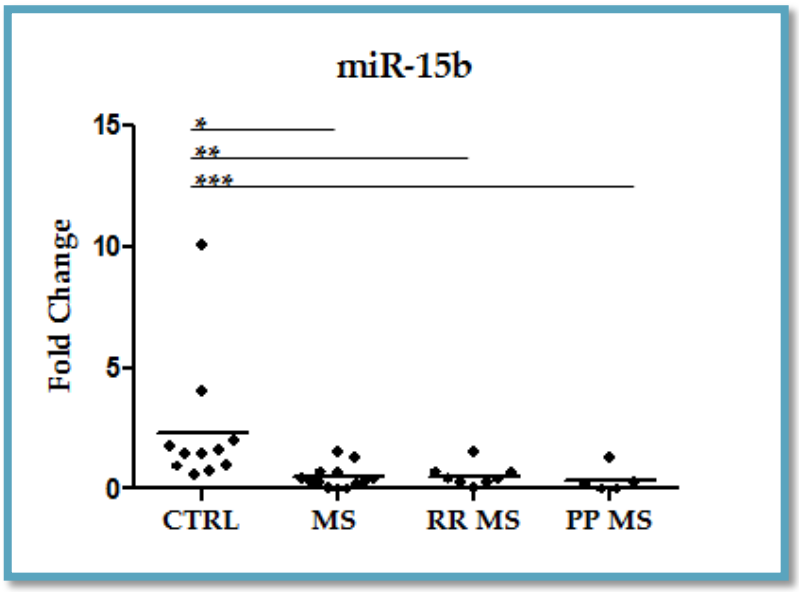


Figure 4A.

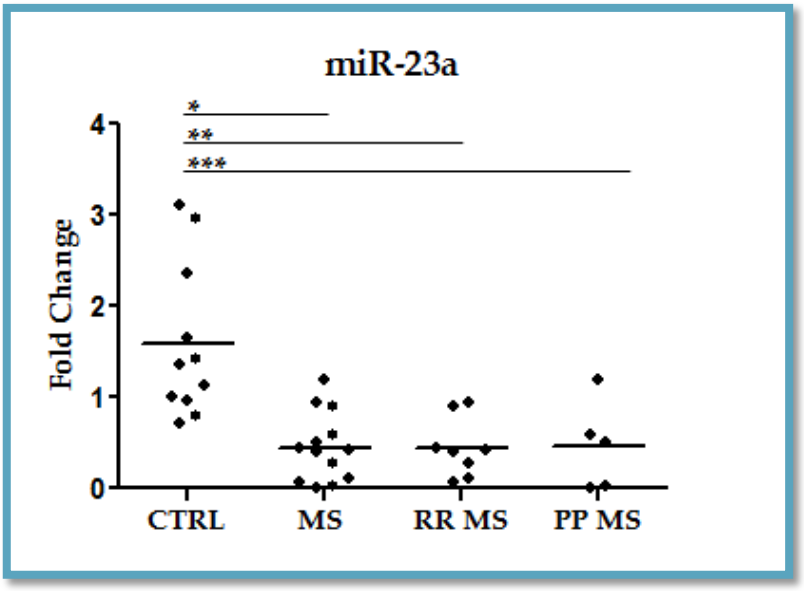


Figure 4B.

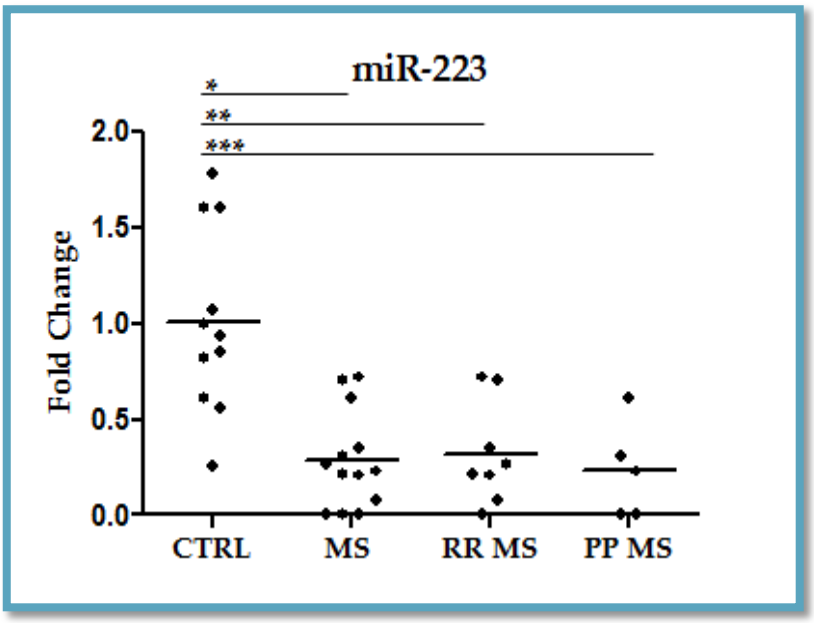


*Figure 4C. Scatter plot of the distribution of miR-15b (3A; *P<0.001, **P=0.003 and ***P=0.009), miR-23a (3B; *P<0.001, **P=0.001 and ***P=0.016) and miR-223 (3C; *P<0.001, **P=0.002 and ***P=0.005) serum levels in MS patients and healthy controls in the validation sample.*

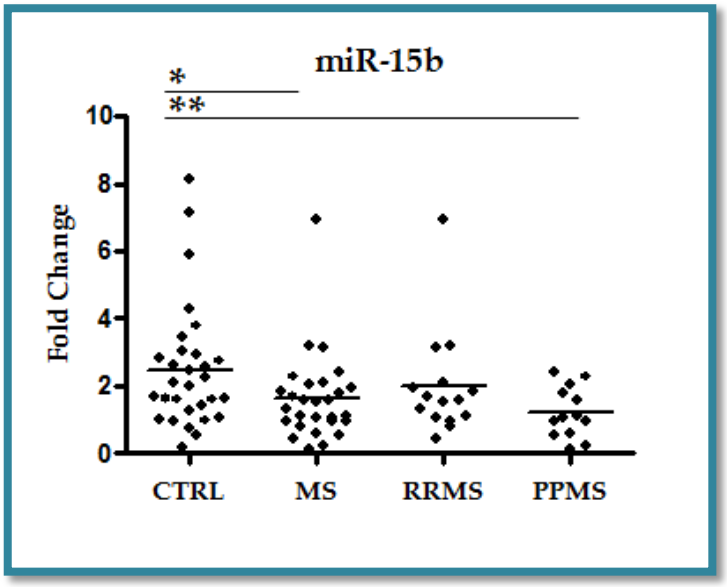


Figure 5A.

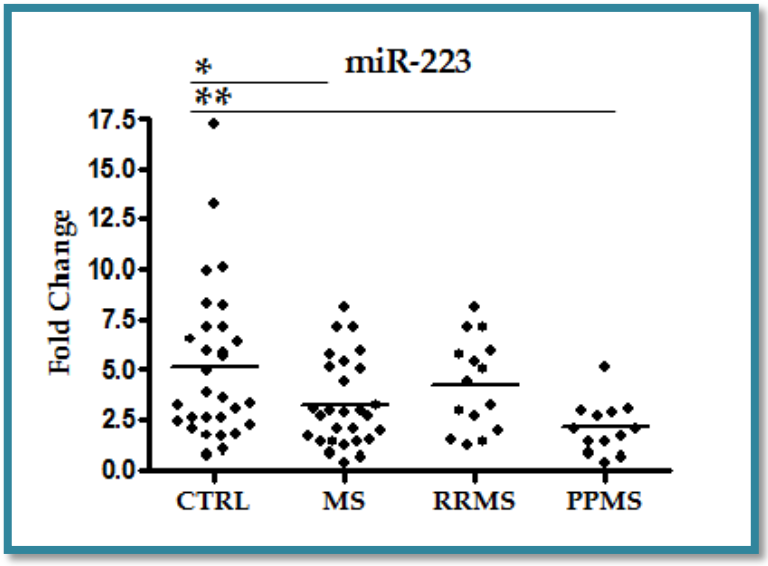


Figure 5B.

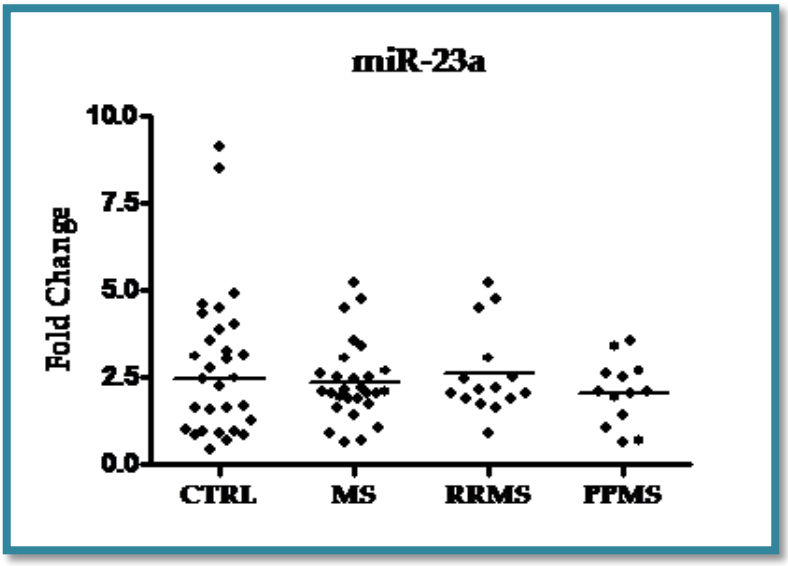


*Figure 5C. Scatter plot of the distribution of miR-15b (4A; *P<0.02 , **P<0.01), miR-23a and miR-223 (*P<0.03, **P=0.002) serum levels in MS patients and healthy controls in the replication sample.*

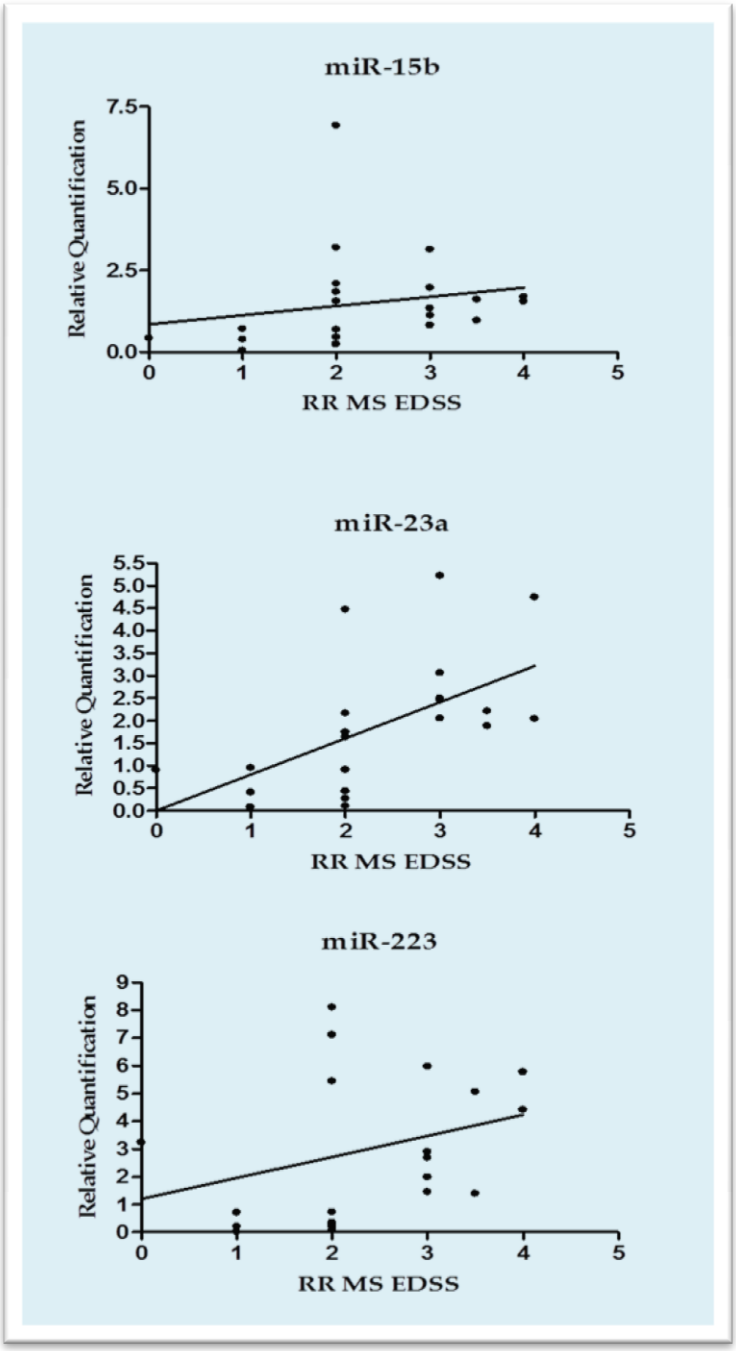


Figure 6.Correlation between relative quantification of miR-15b, miR-23a and miR-223 versus EDSS score in the total PPMS population

	Incidence	Mortality	Day of onset	Maximum clinical score	Cumulative clinical score
MiR-223 KO	8/8	0/8	15.4±2.9	2,75 (2-4)	26.6±5.9
WT mice	4/4	¼	12.8±2.1	4 (3-5)	36.8±5.9
P value				0,02	

Table 2.EAE clinical parameters. Cumulative clinical score was calculated over the first 30 days

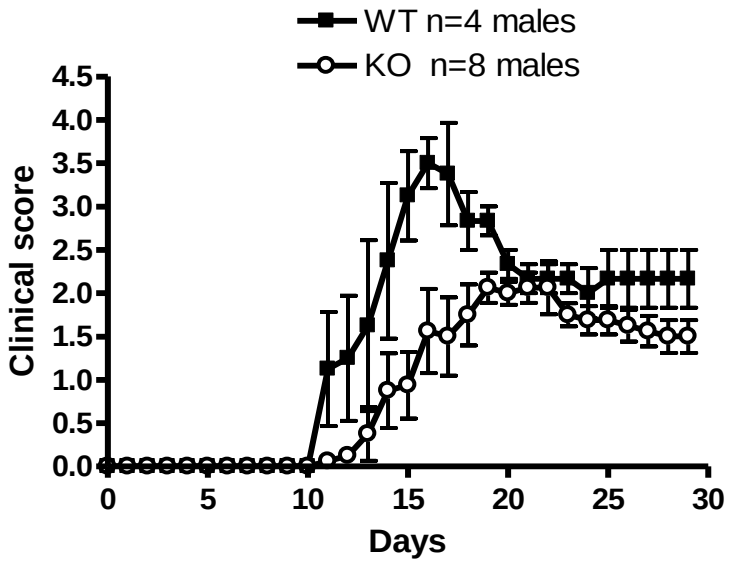


Figure 7.MiR-223 knock-out mice immunized with MOG35-55 presented a less severe disease course compared to littermate control mice. The difference was significant with P<0.0001 by two-way ANOVA. Results are expressed as mean disease scores ± SEMs.

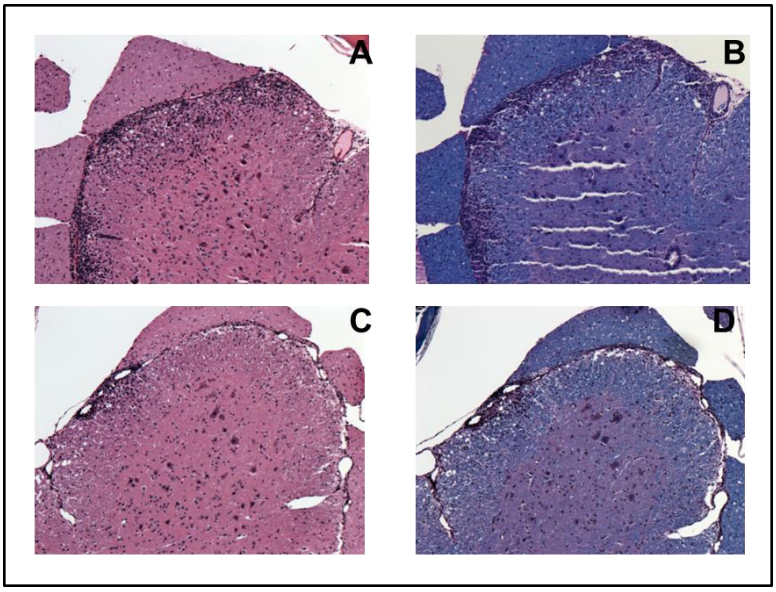


Figure 8A.Spinal cord inflammation and demyelination in miR-223 KO and controls mice. Spinal cords were obtained on Day 30 p.i. Representative mice for control (A and B) and miR-223KO (C and D) groups are shown. LFB (B and D) and H&E-stained sections (A and C).Original Magnification 10X.

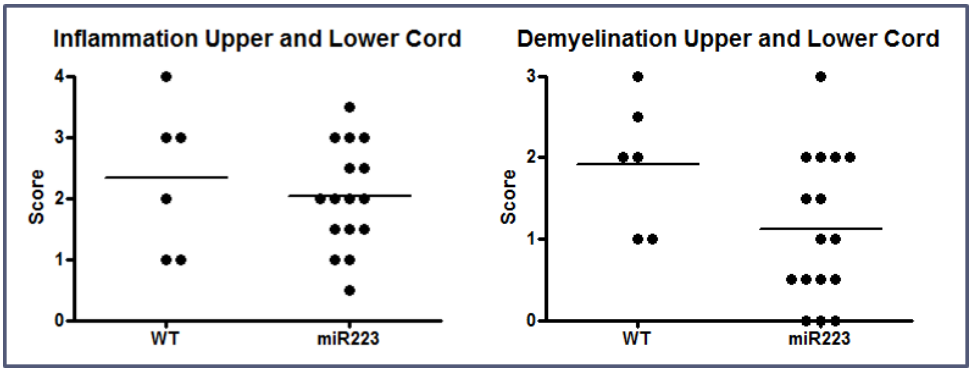


Figure 8B.Inflammation and demyelination quantified in a blinded manner. Horizontal lines indicate median values.

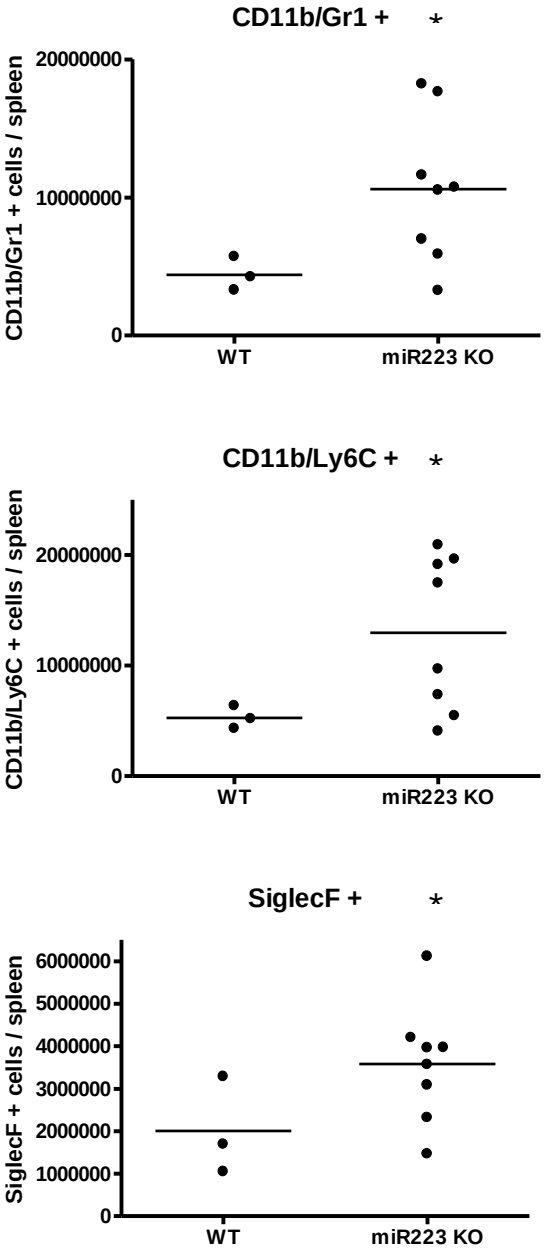


*Figure 9A.Scatter Plot of the flow cytometer analysis from splenocytes of miR-223 KO and WT mice. The results are showed as the absolute number of each cells subset / spleen. (*P<0.04)*

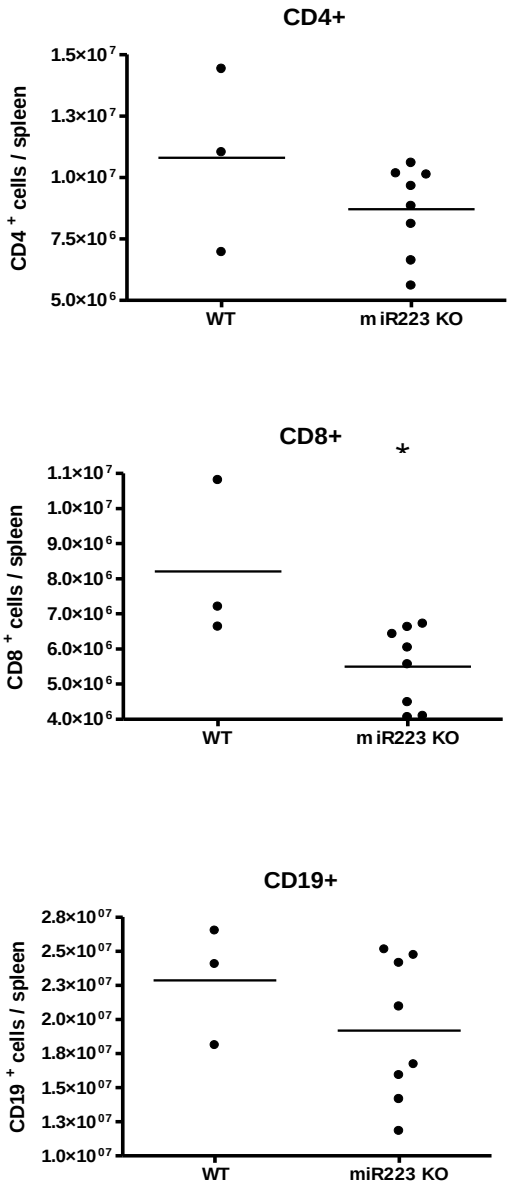


*Figure 9B.Scatter Plot of the flow cytometer analysis from splenocytes of miR-223 KO and WT mice. The results are showed as the absolute number of each cells subset / spleen. (*P<0.04)*

6. CONCLUSIONS

It is increasingly recognized that miRNAs are involved in several different human diseases, including MS. In the first part of this project we characterized miRNA signature in the PBMCs of patients with MS versus healthy controls, identifying let-7g and miR-150 to be down regulated in MS versus controls. Promising targets and biomarkers of disease were identified by correlating data from genome wide mRNA expression analysis and miRNAs expression on the same cohorts of subjects. In addiction miR-21, miR-146a and –b, three of the best characterized miRNAs involved in the activation of $CD4^+$ T cells, were found dysregulated in the PBMCs of MS patients versus controls. Functional studies of cellular pathways targeted by miRNAs, by in vitro approaches, are needed to provide more insights to their impact in the cellular regulatory networks, which may also help to understand the MS pathogenesis.

Recently, the identification of miRNAs differently expressed in blood and lesions of MS patients versus controls made miRNAs to be considered as new potential biomarkers for MS. This idea was more reliable with the recent discovery of stable miRNAs in biological fluids, including plasma, serum, urine, and saliva (5). Secreted miRNAs have many requisite features of good biomarkers: stability in biological fluids, and easy detection by quantitative PCR. For this purpose, the second part of my thesis was focused on analysis of circulating miRNA in sera of MS and healthy control subjects. The results obtained indicated altered levels of cell-free miR-15b and miR-223 in MS patients compared to controls, in two separate populations. MiRNA profiling in MS patients could potentially identify biomarkers for disease prognosis and clinical subtype, to aid therapeutic decisions or monitor therapeutic effects. In addition, miRNA analysis of peripheral blood is a non invasive, reproducible and easy to carry out. Future studies should also include examination of serum miRNA levels in relation to response to therapy in MS.

Several lines of evidence suggest that miR-223 showed an up regulation of this miRNA in the context of MS disease. The miR-223 was found up regulated in the blood of MS patients compared to healthy controls as well as in the $CD4^+CD25^+$Treg cells from MS subjects and also in active MS lesions of the brain compared to normal brain specimens. For this purpose our findings on miR-223 were translated into the in vivo MS animal model. The EAE model is the most commonly used MS animal model; it has been instrumental for the development of four approved MS therapies thus far and it led to greater insights into the utility of miRNAs in the pathogenesis and the clinical settings of MS. In this analysis active EAE was induced in miR-223 KO mice and littermates controls by immunization with the MOG peptide. We found that mice deficient in miR-223 exhibit a delayed course and reduced severity of the disease and less inflammation in the CNS. These are only preliminary results that have to be further characterized with other analysis, using both active and adoptive transfer EAE model. In conclusion, given the ameliorating effect of miR-223 deficiency in EAE experiments, silencing or inhibiting of miR-223 may be an effective therapeutic approach in the treatment of MS.

The reliability of my thesis is sustained by the statistical approach, the validation and replication in different independent cohorts of sample and by the congruent results in the animal model analysis.

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