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RELIABLE BIOMARKERS FOR CLINICAL FORMS OF MULTIPLE SCLEROSIS: DEVELOPMENT OF PERSONALIZED STRATEGIES BASED ON IFN TYPE I IMMUNE SIGNATURE.

BIOMARCADORES DE LAS FORMAS CLÍNICAS DE ESCLEROSIS MÚLTIPLE: DESARROLLO DE ESTRATEGIAS PERSONALIZADAS BASADAS EN PERFILES INMUNOLÓGICOS DE LA VÍA DEL INTERFERÓN DE TIPO I.

TESIS DOCTORAL DE:

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MATTHEW L. ALBERT

Madrid, 2013

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A mis padres

La fixité du milieu intérieur est la condition d'une vie libre et indépendante

Claude Bernard

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ABBREVIATIONS

ACTB	Actin beta
APC	Antigen presenting cell
BBB	Blood-brain barrier
CD	Cluster of differentiation
CDMS	Clinically definite MS.
CIS	Clinically isolated syndrome
CCL	C-C motif chemokine ligand
CNS	Central nervous system
CSF	Cerebrospinal fluid
CLU	Clusterin
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DCs	Dendritic cells
DMT	Disease-modifying therapy
DPP	Dipeptidyl peptidase
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein Barr virus
EDSS	Expanded disability status scale
EGF	Epidermal growth factor
EIF2AK2	Eukaryotic translation initiation factor 2- alpha kinase 2
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
FGFb/2	Fibroblast growth factor basique or 2
FKBP8	FK506-Binding protein 8
GA	Glatiramer acetate
GADPH	Glyceraldehyde 3 phosphate dehydrogenase
Gd	Gadolinium
GUS	Beta-glucuronidase
HLA	Human leukocyte antigen
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HHV-6	Human herpes virus 6
HPRT	Hypoxantine guanine phosphoribosyltransferase
IFN	Interferon
IFNAR	Interferon alpha, beta and omega receptor
Ig	Immunoglobulin
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IL-1RN	Gene that encodes IL-1RA
IRF	Interferon regulatory factor
ISGs	Interferon stimulated genes
ISREs	Interferon stimulated response elements
IP10	Interferon gamma-induced protein 10
JAK	Janus kinase
LDLR	Low density lipoprotein receptor
MBP	Myelin basic protein
MCP-1	Monocyte chemotactic protein 1
MDA5	Melanoma differentiation associated gene 5
mDCs	Myeloid dendritic cells
MHC	Major Histocompatibility Complex
MIF	Macrophage migration inhibitory factor
Mig	Monokine Induced by Gamma Interferon
MIP	Macrophage Inflammatory Proteins
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
MS	Multiple sclerosis
MOG	Myelin oligodendrocytes glycoprotein
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic Acid
MX1	Myxovirus Resistance 1
NAWM	Normal-appearing white matter
NDEL1	Nude like protein 1
NK	Natural killer cell
NO	Nitric oxide
RR-MS	Relapsing-remitting MS
OB	Oligoclonal bands
OPCs	Olygodendrocyte precursor cells
OR	Odds ratio
PBMCs	Peripheral blood mononuclears cells
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PHA	Phytohemagglutinin

Abbreviation	Meaning
PMA	Phorbol Myristate Acetate
PP-MS	Primary progressive MS
RA	Rheumatoid arthritis
Rantes	Regulated upon activation, normally T expressed and presumably secreted
RIGI	Retinoic Acid Inducible Gene I
RSAD2	Radical S-Adenosyl methionine domain containing protein 2
RR-MS	Recurrent remitting MS
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphim
SP-MS	Secondary progressive MS
STAT	Signal transducer and activator of transcription
TBP	TATA box binding protein
TCR	T-cell receptor
TGFβ	Transforming growth factor beta
Th	CD4+ T helper cells
TIMP1	Tissue inhibitor of metalloproteinase 1
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFSF10	Tumor necrosis factor ligand superfamily, member 10
TRIM25	Tripartite motif-containing protein 25
Treg	Regulatory T lymphocyte
USP18	Ubiquitin-specific protease 18
VCAM-1	Vascular cell adhesión molecule-1
WHO	World Health Organization

SUMMARY

Multiple sclerosis (MS) is the most common cause of neurological disability in young population after trauma and represents a significant personal, social and economic public health burden. The clinical course and response to therapy of MS is highly heterogeneous, but most patients go from a relapsing-remitting disease course, where patients may respond to immunomodulatory drugs, to a steady progression and neurodegeneration that is unresponsive to any currently available treatment. Around 20% of MS patients present with the primary-progressive form, for which there is no therapy.

To date, reliable biomarkers that enable to distinguish among the different clinical forms of MS are lacking. Such biomarkers are highly desirable given the need to define which patients could evolve to progressive forms and the disappointing failure to stop primary and secondary progressive MS.

Recent evidence [1] suggests that treatment failure with type I interferon (IFN) in hepatitis C may be linked to high dipeptydil peptidase (DPP) cleavage of one of its substrates, the chemokine IP10/CXCL10. Since MS is an organ-specific autoimmune disease in which type I IFN is the gold standard treatment to modify clinical course, we explored the role of DPP4 in MS pathophysiology.

Our main objective was to identify blood biomarkers in MS through the following approaches: the identification of biomarkers that could discriminate between MS patients and healthy controls; biomarkers that could stratify MS patients and reflect pathophysiological differences according to their clinical form; and finally, the identification of biomarkers related with type I IFN signaling pathways that might help in the personalization of future therapeutic strategies for MS and provide mechanistic insight of MS pathophysiology. Secondly, we focused on the use of a combination of biomarkers using logistic regression for their predictive classification power achieving superior sensitivity and specificity.

We studied 182 subjects (129 MS patients and 53 healthy controls) that were consecutively recruited during two years as two independent cohorts. The current investigation with an extensive multiplex set consisting of cytokines, chemokines, soluble receptors, growth and angiogenic factors, was evaluated using bead-based immunoassays (Luminex) and ELISA. The gene expression profiles of a vast array of genes related with type I IFN signature and MS

pathophysiology were studied in peripheral blood mononuclear cells (PBMCs) by BioMark technology.

The data presented here show a significantly decreased expression of DPP4 and DPP activity in the plasma of MS patients with respect to healthy controls, which might interfere in the chemokine immunoregulation in the central nervous system. In addition, DPP activity correlated inversely with clinical disability score in MS. On the other hand, our results demonstrate that the divergent clinical and histology-based MS forms are associated with distinct profiles of circulating biomarkers, mostly chemokines and growth/angiogenic factors (HGF, Eotaxin, MCP-1, Rantes, EGF, MIP-1β, VEGF and FGFb); and with different gene expression levels in their PBMCs (CLU, IRF2 and LDLR). Responder patients to type I IFN displayed high levels of plasma IP10 and MCP-1, and a specific expression pattern of IFN stimulated genes.

In summary, this study has contributed to the identification of novel biomarkers and their combinations that might serve as useful tools for the classification of the different clinical forms of MS and to explore the pathophysiological differences between the progressive and the recurrent-remitting phase of the disease.

La esclerosis múltiple (EM) es la causa no traumática más común de discapacidad neurológica en adultos jóvenes y representa un significativo problema personal, social y económico para los que la padecen. La evolución clínica y la respuesta a la terapia es altamente heterogénea, pero la gran mayoría de pacientes pasan de una fase recurrente-remitente, donde los pacientes pueden responder a tratamientos inmunomoduladores, a una progresión y neurodegeneración continua que es intratable con los medicamentos de los que se dispone actualmente. Aproximadamente un 20% de los pacientes con EM debutan con la forma primaria progresiva, para la que hasta ahora no existe terapia.

Por el momento, no existen biomarcadores que permitan distinguir con fiabilidad entre las distintas formas clínicas con EM. Tales biomarcadores serían altamente necesarios para definir aquellos pacientes que podrían evolucionar a formas progresivas de la enfermedad y el consiguiente fallo para detener la progresión en la EM primaria y secundaria.

Evidencia reciente [1] sugiere que el fallo en el tratamiento con interferón de tipo I en la hepatitis C puede estar ligada a una mayor actividad enzimática dipeptidil peptidasa (DPP) en uno de sus sustratos IP10/CXCL10. Dado que la EM es una enfermedad autoinmune órgano específica, en la que el IFN de tipo I sigue siendo el tratamiento más utilizado para modificar el curso de la enfermedad, en este trabajo hemos querido investigar el papel de DPP4 en la fisiopatología de la EM.

El principal objetivo de este trabajo fue el de indentificar biomarcadores de EM en sangre periférica mediante las siguientes aproximaciones: identificación de biomarcadores que pudieran discriminar entre pacientes con EM y controles sanos; biomarcadores que pudieran estratificar los pacientes con EM y reflejar diferencias fisiopatológicas según la forma clínica; y finalmente, la identificación de biomarcadores relacionados con la vía del IFN de tipo I que podrían ayudar a personalizar futuras estrategias terapéuticas para la EM y proporcionar información sobre los mecanismos de la fisiopatología de la EM. En segundo lugar, nos centramos en la combinación de biomarcadores mediante regresión logística para aumentar el poder de clasificación predictivo alcanzando mayor sensibilidad y especificidad.

Hemos estudiado 182 sujetos (129 pacientes con EM y 53 controles sanos) que fueron reclutados consecutivamente durante dos años como dos cohortes independientes. La presente investigación con un amplio set de citoquinas, quimiocinas, receptores solubles y factores de crecimiento solubles y angiogénicos, fue evaluada mediante inmunoensayos con microesferas (Luminex) y ELISA. Los perfiles de expresión génica de un amplio conjunto de genes relacionados con la señalización del IFN de tipo I y la fisiopatología de la EM, se estudiaron en células mononucleares de sangre periférica (PBMCs) por la tecnología BioMark.

Los datos aquí presentados muestran una significativa expresión disminuida de DPP4 y de la actividad DPP en el plasma de los pacientes con EM con respecto a los controles sanos, lo que podría interferir en la inmunoregulación de quimiocinas en el sistema nervioso central. Además, la actividad DPP se correlacionaba inversamente con la escala de discapacidad clínica en EM. Por otro lado, nuestros resultados demuestran que las distintas formas clínicas de EM se asocian con distintos perfiles de biomarcadores clirculantes, en su mayor parte quimiocinas y factores de crecimiento/angiogénicos (HGF, Eotaxin, MCP-1, Rantes, EGF, MIP-1β, VEGF y FGFb); y con diferentes niveles de expresión génica en sus PBMCs (CLU, IRF2 y LDLR). Los pacientes respondedores a IFN de tipo I presentaban niveles elevados de IP10 y MCP-1 en plasma, así como una expresión específica de los genes estimulados por IFN.

En resumen, este estudio ha contribuido a la identificación de biomarcadores y de sus combinaciones que podrían servir como herramientas útiles en la clasificación de las distintas formas clínicas de EM y que permiten explorar las diferencias fisiopatológicas entre las fase recurrente-remitente y la fase progresiva de la enfermedad.

1. INTRODUCTION

1.1. HISTORICAL PERSPECTIVE OF MULTIPLE SCLEROSIS

The first description of multiple sclerosis (MS) dates from the XIV century [2], but it was in the XIX century when the first anatomopathological descriptions were made. Robert Carswell (1838) associated the presence of demyelinating lesions with clinical features and Jean Cruveilhier (1835) was the first to report clinical findings of a patient who would later develop demyelination [3]. But it was some decades later, in 1868, when the French pathologist Jean-Martin Charcot formally describes the disease entity as “sclérose en plaques” and performs the first detailed correlation between clinical and post-mortem findings [4]. Considered the founder of modern Neurology, Charcot‘s pictures illustrated the expansion of lesions from the ventricles into the cerebral hemispheres and he noted the accumulation of inflammatory cells perivascullary in brain and spinal cord white matter of patients [5, 6]. The English physicians added the adjective “disseminated” due to the extension of lesions in the central nervous system (CNS) and the American neurologists the adjective “multiple” due to the multiple lesions and episodes of neurologic dysfunction [7]. The French still conserve the original name given by Charcot, but the most used denomination of the disease is multiple sclerosis (MS). In 1933, Thomas Rivers was the first to induce experimental autoimmune encephalomyelitis (EAE), the animal model of MS, by injecting repeatedly brain emulsions and extracts from rabbits in primates provoking CNS demyelinating lesions. This immunization of mammals with CNS myelin suggests an autoimmune nature of the chronic inflammation present in the disease to self-antigens. In 1948, Elvin Kabat describes increments in oligoclonal immunoglobulins in the cerebrospinal fluid (CSF) of patients with MS, sustaining an inflammatory nature of the disease [8, 9]. Nowadays, the etiology of MS remains elusive, but since the 1990s, several treatments available can modify the disease course of the relapsing form of MS, delaying neurological deterioration of MS, even if the mechanisms of actions of these drugs are not well understood. However, for the progressive forms of MS there is still no effective therapy.

1.2. EPIDEMIOLOGY OF MULTIPLE SCLEROSIS

MS can be defined as a chronic inflammatory demyelinating disease of the CNS in which repeated episodes of inflammatory demyelination result in the formation of persistently demyelinated plaques of gliotic scar tissue associated with varying degrees of axonal loss [10]. Anatomopathologically, it is characterized by the loss and disruption of the myelin sheath that surrounds the axons in brain and spinal cord, producing multifocal lesions in the CNS white matter [11] that can lead to axonal degeneration and to progressive neurological dysfunction. Common symptoms include visual disturbances, loss of balance and coordination, spasticity, sensory disturbances, bladder and bowel incontinence, pain, weakness, fatigue and paralysis.

MS severely compromises the quality of life of the patient and their families, and has a huge adverse socioeconomic impact for MS patients, their families and society as a whole.

1.2.1. Incidence and Prevalence

MS is the most common neurological disease that causes disability in young adults [7]. The disease has an increasing prevalence worldwide that may be attributable to environmental factors or to a higher awareness and more accurate diagnosis.

According to the Atlas of MS performed in 2008 by the World Health Organization (WHO), the median estimated incidence of MS in the world is 2.5 per 100,000 (with a range of 1.1–4.0) [12]. The estimated mean MS prevalence worldwide is 30 per 100,000 inhabitants (with a range of 5-200) (Figure 1). MS is more common in Europe, North America, Australia and New Zealand, with prevalences greater than 60 per 100,000. It is less common in Eastern Mediterranean (14.9), Central and South America (between 5 and 20 per 100,000), the Western Pacific (5), South-East Asia (2.8) and Africa (0.3) [12]. Data from continents like Africa must be taken with precaution as few countries provided data for this report. In Europe, the prevalence is the highest in the world: 80 per 100,000. Scotland has the highest prevalence rate with 200 [13] and the Scottish Orkney islands with 402 [14] per 100,000 inhabitants. Other countries with very high prevalences are: Hungary (176 per 100,000), Slovenia (150) and

Germany (149). Data from this atlas generally confirm the observation stated by the neurologist John Kurtzke, in 1975, of that MS prevalence increases the further countries are from the equator [15]. A north-to-south gradient of declining prevalence of MS seems to be still present in Europe, but there are several exceptions to this rule. For instance, very close geographical regions like Sicily (>60 per 100,000) and Malta (4 per 100,000) have totally different prevalences [16]. Southern European countries like Spain and Italy have also high MS prevalences, between 60 and 100 per 100,000 that have increased in the last years [16]. In the United States of America and Canada, the prevalence is over 130 per 100 000 [12] (Figure 1).

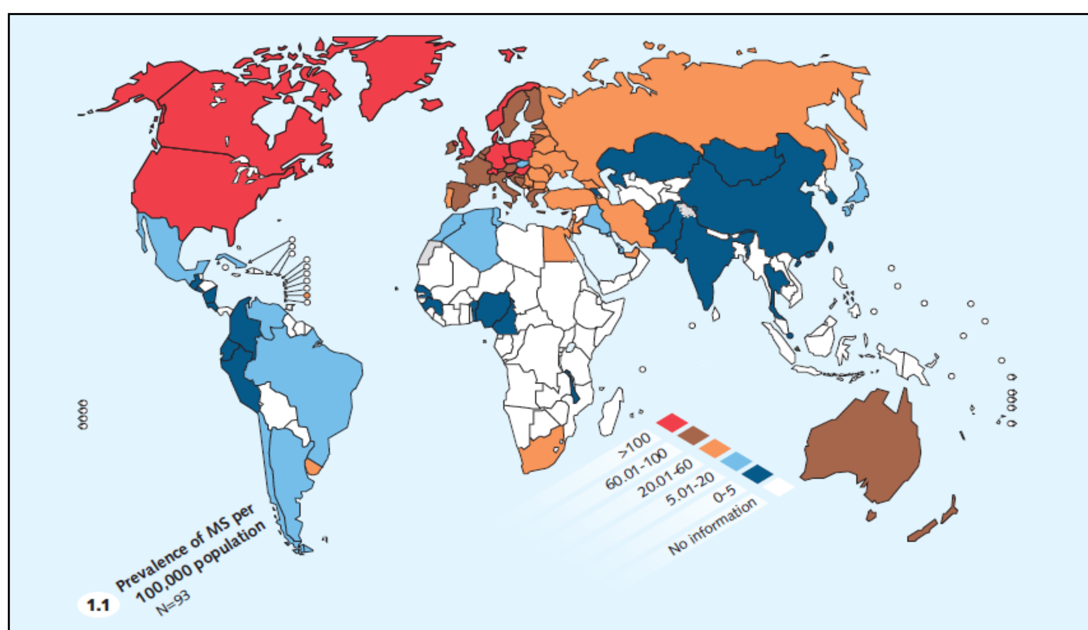

Figure 1 Multiple Sclerosis prevalence in the world. Figure taken from the Atlas of MS 2008 (World Health Organization).

1.2.2. Age of Onset and Sex Dimorphism in Multiple Sclerosis

Although the disease has a broad range of age at onset (85% of cases occur between the ages of 14 and 55), the disease symptoms typically start at childbearing years, around 30 years of age. Globally, the average age of onset is 29.2 years, with an interquartile range of 25.3 and 31.8 years [12]. Children can also suffer from MS, before the age of 16 years old, girls are more frequently affected than in MS adult-onset (female/ male ratio F/M=2.8 vs 1.8). Children

with MS take longer to arrive to a disabling progressive state of the disease but do so at a younger age [17].

As observed in other autoimmune diseases [18-20], MS affects more frequently young women than men and the disease course is modified by pregnancy and decreases after menopause [21]. During pregnancy, the frequency of MS relapses clearly decreases with a subsequent surge at postpartum [20, 22-25]. The current F/M ratio is 2/1 worldwide [12] but some populations (Canadians, Japanese, Northern Finnish, Iranian, Sardinian) have reported a higher incidence in women over the last decades increasing the female/male ratio to more than 3/1 [26-30], while others populations have not (Swedish) [31]. This rapid change in female incidence of MS in some populations is thought to be due to environmental factors rather than to genetic ones. Potential factors underlying the sex-bias in MS are the effects of sex hormones on immune responses [32-34] and the differential distribution of sex hormones receptors in immune cell subsets [35, 36].

1.2.3. Environmental Factors

The cause or causes of MS remain unknown. MS is now considered a complex disorder that is triggered in genetically susceptible individuals by different environmental and stochastic factors [37].

1.2.3.1. Migrations

Several studies from migrations have demonstrated that the risk of developing MS depends on the place of residence in childhood. Children who migrate early in life from low-risk regions to high risk regions have an increased risk of developing MS [38]. In the first generation, Asian immigrants' children from countries with low incidence show a similar incidence of MS to the host country children [39], what reflects the importance of environmental factors rather than of genetic ones.

1.2.3.2. The Hygiene Hypothesis

This hypothesis states that hygiene based reductions of cross infections during infancy explain the rise of autoimmunity or allergies as an adult [40].

In 1966 Leibowitz *et al.* observed that MS patients had lived in cleaner conditions during childhood than their contemporary controls [41]. They proposed that a certain sanitary level early in life might represent a 'threshold' for the risk of developing MS later. They agreed with the hypothesis stated previously by Poskanzer *et al.* [42] that MS might be a manifestation of a subclinical infection acquired later in life by the individual when sanitary conditions were high during childhood. Given the similarity of the epidemiological patterns of poliomyelitis and MS, Poskanzer *et al.* suggested an enteric infection as cause of the disease. This is why the hygiene hypothesis was originally named the poliomyelitis hypothesis. Epidemiological studies have later observed an increase in the incidence of MS associated with a reduction in intestinal parasitic infections [43, 44].

1.2.3.3. The Human Herpes Virus Family

Many epidemiological studies have searched for a causative infectious agent in MS and viruses have been widely associated with MS etiology. Human herpes viruses (HHV), especially herpesvirus 6 (HHV-6) and Epstein-Barr virus (EBV) have been thoroughly studied in the last decades and suggested as possible triggers of MS disease. HHV are double stranded DNA (dsDNA) viruses ubiquitous and neurotropic that induce lytic, persistent and latent infections [45].

HHV-6 is a T lymphotropic herpes virus expressed in neurons and glia from MS and normal brains [46, 47] but only in the oligodendrocytes from MS plaques and not in control brains, what suggests an active replication of the virus [46]. HHV-6 DNA and increased concentrations of IgG and IgM antibodies in blood and CSF have been found only in a minority of patients and seem to correlate with exacerbations in the relapsing-remitting phase of the disease [48-50]. Furthermore, those MS patients without active replication of HHV-6 respond better to IFNβ treatment than those with active replication of the virus [51]. Two polymorphisms in the gene MHC2TA (rs4774C and rs3087456G), gene that codes for the transcription factor CIITA involved in the regulation of HLA class II expression [52], is associated with active replication of HHV-6 in MS [51, 53].

EBV or HHV-4 virus is a B lymphotropic herpes virus transmitted primarily via saliva. EBV provokes a latent infection in B lymphocytes with permanent immunological stimulation through life [45]. EBV infection is very common and

subclinical in children, but in adults it causes an acute febrile syndrome, known as infectious mononucleosis (IM). More than 99% of MS patients have an EBV infection compared to 86-95% in healthy controls [50], and the relative risk of MS for EBV-negative individuals is very low (OR, 0.06; 95% CI, 0.03–0.13) [54]. Pediatric MS and EBV infection have been also associated. In a European cohort of children with MS, 99% were EBV-seropositive compared to 72% age-matched controls [55], while in a North American pediatric cohort with MS, only 86% were EBV-seropositive compared to a 64% in healthy controls [56]. Therefore, children with MS seem to be infected at higher rates of EBV than their age-matched controls. In adults, MS patients who acquired EBV in adulthood, manifested as IM, had a twofold to threefold higher risk of MS [57, 58]. Additionally, seroepidemiological studies have revealed that high levels of EBV-antibodies were associated with higher risk of MS and the elevation of antibody titers to EBV nuclear antigen-1 (EBVNA-1) has been observed years before the first symptoms appear [59]. In a large prospective study with 50 million serum samples from US military [60], only 5% were EBV-seronegative and 10 cases of MS occurred among these EBV-seronegative baseline subjects who developed first symptoms of MS several months after serological positivity for MS. In order to establish whether EBV infection is a putative causative agent of MS, those children EBV-seronegative with MS should be followed up to see whether they evolve to the classical MS adult disease. In addition, Lang *et al.* demonstrated the molecular mimicry between a myelin basic protein (MBP) and an EBV peptide, four DRB1* restricted T cell receptor peptide contacts are identical to both proteins, so the immune response to the virus would cross-react with self-myelin and induce demyelination [61]. Overall, EBV infection increases the risk of developing pediatric MS and in adult-onset MS, late EBV infection, presented as IM, is associated with MS. Molecular mimicry must be considered as a possible pathophysiological mechanism.

In summary, up to date no virus has been isolated or directly linked with MS. However, different pieces of evidence suggest that the disease may be triggered by an infectious agent and evolve to an immune-mediated chronic disease.

1.2.3.4. Vitamin D

The geographical distribution of MS correlates with the duration and intensity to sun exposure [62-64] . UVB radiation is the major source of vitamin D, although this vitamin can also be ingested with the diet (i.e. fatty fish) or from dietary supplements. There are some large longitudinal studies that support the inverse association between vitamin D and risk of MS. A prospective follow-up study for over 20 years of 182,000 female nurses documented 173 MS patients and found a MS risk 33% lower in those women who took the highest vitamin D intake (from food and supplements) than in those in the lowest quintile 0.67 (relative risk [RR]=0.67, 95% CI = 0.40-1.12; p = 0.03). Specifically, women who took daily at least 400 IU of vitamin D from supplements, had a MS risk 41% lower than those with no vitamin D supplement intake ([RR]=0.59, 95% CI 0.38-0.91; p=0.006) [65]. Another nested case-control study in personnel of the US military [66] found that high levels of serum 25(OH) vitamin D levels (>99 nmol/L) in young non-Hispanic white adults had a 62% lower MS risk, with independence of vitamin D status in childhood. Lucas *et al.* studied cumulative lifetime sun exposure by measuring the degree of actinic damage in the dorsum of the hand and its association with a first demyelinating event (FDE) among 216 MS patients and 395 matched controls from the same latitudes in Australia. Higher actinic skin damage was associated with decreased FDE.

CYP27B1 is the enzyme that hydroxilates 25(OH) vitamin D to its active form 1,25-dihydroxycholecalciferol (calcitriol). Homozygous carriers of nonfunctional mutations suffer from rickets type 1, a very rare disease, but in three patients studied in Norway with this disease during childhood, all of them developed MS as adults [67]. Moreover, heterozygous carriers of mutations in CYP27B1 have lower levels of calcitriol and an increased risk of developing MS [68].

1.2.4. Genetics

MS familiar studies have proven the relevance of genetic susceptibility to MS. Monozygotic twins of the index case have a ≈25% risk of developing MS, dizygotic twins a ≈5% and non-twin-siblings have a 20 to 40 fold increased risk [69-71]. Non-biological relatives living with affected patients and MS subjects who were adopted soon after birth have no higher risk than general population,

indicating that sharing the environment with MS is not a risk factor for acquiring the disease and that familial aggregation of MS is genetically determined [71].

The major histocompatibility complex (MHC) exerts the most important contribution to MS susceptibility. The genetic association with MS and the MHC, a cluster of genes on the short arm of chromosome 6, was identified more than 30 years ago [72, 73]. As MHC molecules select the (autoreactive) T-cell repertoire and determine the ability of T-cells to respond to a specific antigen, this association highlights the central role of self-reactive T cells in MS pathogenesis [74]. The strongest and more replicated genetic association with MS is the DR15 haplotype with the corresponding genotype DRB1*1501-DQA1*0102-DRQB1*0602. This allele is very common in countries with high MS prevalence (14-30%) [75], and is present in all populations except for Sardinian and other Mediterranean groups in whom MS associates with DR3 and DR4 haplotypes (DRB1*0301-DQA1*0501-DRQB1*0201 and DRB1*0405-DQA1*0301-DRQB1*0302) [76, 77]. Being heterozygous for the DRB1*1501 allele increases the risk of MS by three fold and homozygosity by over six fold [78]. Interestingly, the haplotype DRB1*03 (ancestral haplotype 18.2) has been recently associated with MS and is restricted to patients with IgM oligoclonal bands (OB), a bad prognostic marker, conferring a risk similar to that caused by DRB1*1501 [79]. Interactions between different MHC-DRB1 alleles, called epistatic interactions, can either increase the risk or confer protection in MS [78].

Genome-wide studies involving large number of patients have identified protective effects endowed by HLA-C5 [80] and HLA-DRB1*11 [81, 82] and increased susceptibility in immunologically important risk alleles: IL-2RA [83], CD58 [84], STAT3 [85], IL-7RA [86]. A recent large-scale meta-analysis of most of the genome-wide single nucleotide polymorphism (SNP) known for MS has validated the described SNPs and discovered three new loci of MS susceptibility and remarked the common presence of some SNPs in other autoimmune diseases and inflammatory conditions [87]. The association of Non-MHC II risk alleles is not as strong as with MHC II alleles. In the Spanish population, an association between MS susceptibility and a polymorphism located in the promoter of the tumor necrosis factor-alpha (TNF) gene, rs1800750 (TNF -376) has been described [88, 89]. This polymorphism was later

on found in a subgroup of Spanish MS patients characterized by the presence of oligoclonal IgM bands against myelin lipids (OCMB) restricted to the CSF [90].

As it is summarized below, MS is a complex autoimmune disease with multiple internal and external factors that may trigger autoreactivity to self antigens in the CNS. The CNS interacts with the immune system and the rest of intrinsic and stochastic factors targeting the disease process [37].

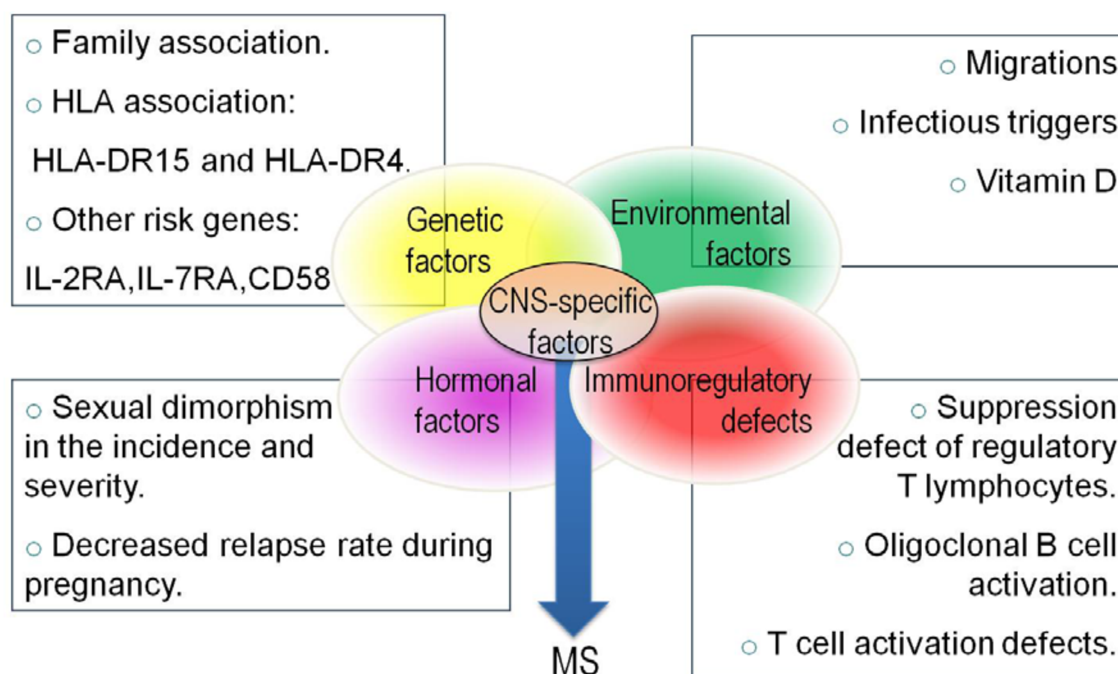

Figure 2 Multiple Sclerosis, a multifactorial disease. Ideas from [91].

1.3. NATURAL HISTORY OF MULTIPLE SCLEROSIS

1.3.1. Clinical Course

MS is a clinically heterogeneous disease that varies according to the location of plaques or lesions in the CNS. Most of the patients with MS (80%) present with an acute attack, called clinically isolated syndrome (CIS), which can affect one or several neurological sites of the organism [92] (Table 1). Neurological sites more usually affected in CIS include the optic nerve, spinal cord, brainstem or cerebellum and rarely a cerebral hemisphere [93]. The most frequent symptoms include optic neuritis in one eye with visual disturbances; paresthesias in the extremities suggesting a spinothalamic abnormality and weakness in feet or

hands reflecting motor dysfunction of the spinal cord. If there are white matter lesions detected by magnetic resonance imaging (MRI), the risk of suffering a second relapse increases [94]. New attacks occur with different frequencies, but rarely exceeding 1.5 per year [95]. This clinical form is known as relapsing-remitting MS (RR-MS), characterized by recurrent relapses with total or partial recovery and a disease inflammatory course that can be modified with therapy. After a median of 15 years from disease onset [92], progression evolves in around 65% of patients and they enter in the secondary-progressive MS form (SP-MS). Around a 20% of patients have a progressive onset without relapses, known as primary-progressive MS (PP-MS) [95] (Figure 3). For the SP-MS and PP-MS evolutive forms, available treatments are unable to stop the progression of the disease. Older age at relapsing-remitting (RR) phase, high relapse frequency in the first two years of disease and short first-inter-relapse interval (< 6 years) are associated with higher risk of more rapid progression to SP-MS [92, 96].

Some patients with RR-MS present with the mildest phenotype of MS called “benign” MS. These patients are not disabled after ten or more years of the disease course and have normal employment and domestic activities. However, this “benign” adjective does not imply that they have no disease symptoms but the disabling is slower and does not happen in all patients [97].

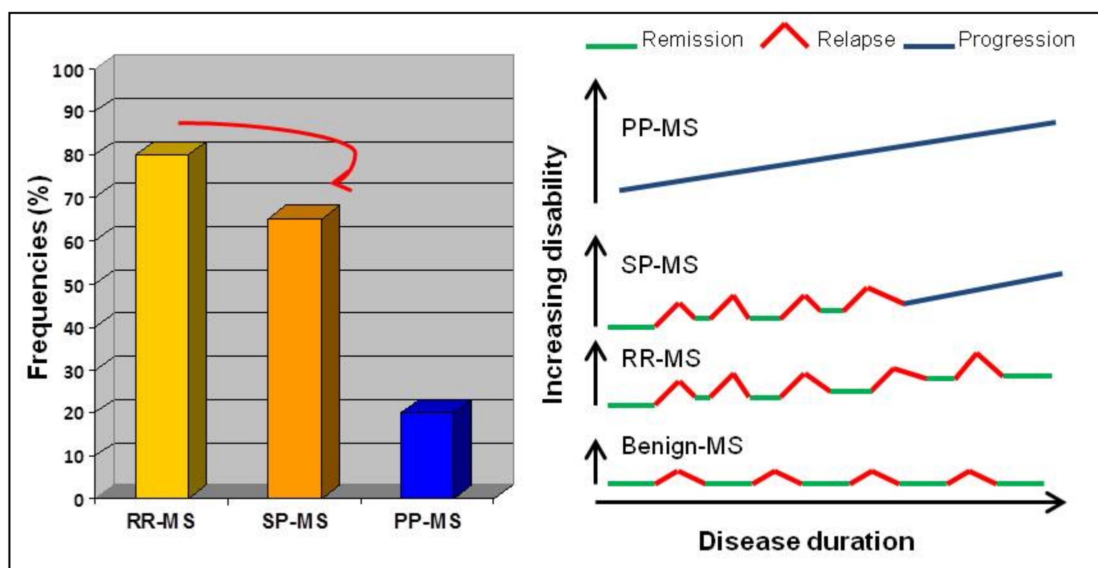

Figure 3 Phenotypes and frequencies of the clinical forms of multiple sclerosis. Frequencies adapted from [95] and figure adapted from [98].

The Expanded Disability Status Scale proposed by Kurtzke in 1983 (EDSS) evaluates the degree of neurologic impairment in MS patients and classifies them in a scale from 0 to 10 as a numerical measure of the disease evolution throughout time. For instance, an EDSS=3 implies a moderate disability, whereas EDSS=6 aid for walking, EDSS=8 wheelchair, and EDSS=10 death from MS. The subdivisions (1.0, 1.5, 2.0 . . . 9.5, 10) correspond to the definition by Functional System grades which are: Pyramidal, Cerebellar, Brain Stem, Sensory, Bowel & Bladder, Visual, Cerebral, and the Sensory and Bowel & Bladder Systems [99]. This scale is largely used as a standard measure to evaluate MS patients, but has some important disadvantages: it gives too much importance to the ability to walk and does not take into account the cognitive dysfunction in MS.

1.4. DIAGNOSIS OF MULTIPLE SCLEROSIS

The cornerstone of the MS diagnosis is based on clinical evidence from a detailed neurological history and physical examination. There are symptoms and signs of the motor, sensory, visual and autonomic systems, but many others can occur (Table 1). The lesions must be disseminated in place (at least two independent lesions in the CNS) and time (two or more episodes of neurologic dysfunction separated at least for 30 days) [95]. There are two characteristic clinical symptoms of MS: Lhermitte's symptom (electrical sensation that runs down the spine or limbs on neck flexion) and Uhthoff phenomenon (transient worsening of symptomatology when the body temperature increases, after for instance exercise or a hot bath) [95]. When clinical manifestations are not sufficient for an accurate diagnosis, paraclinical features can help for the establishment of the diagnosis.

	Symptoms	Signs
Cerebrum	Cognitive impairment, hemisensory and motor, affective (depression).	Deficits in attention, reasoning, executive function; dementia (late). Upper motor neuron signs.
Optic nerve	Unilateral painful loss of vision.	Scotoma, reduced visual acuity and colour vision.
Cerebellum and cerebellar pathways	Tremor, clumsiness and poor balance.	Postural and action tremor, dysarthria. Limb incoordination and gait ataxia.
Brainstem	Diplopia, oscillopia Vertigo Impaired swallowing Impaired speech and emotional lability Paroxysomal symptoms	Nystagmus, internuclear ophthalmoplegias. Dysarthria Pseudobulbar palsy
Spinal cord	Weakness Stiffnes and painful spasms Bladder dysfunction Erectile impotence Constipation	Upper motor neuron signs Spasticity
Other	Pain, fatigue, temperature sensitivity and exercise intolerance	

Table 1 Main symptoms and signs of multiple sclerosis. Adapted from the table "Symptoms and signs of multiple sclerosis by site" by [95].

1.4.1. Paraclinical Tests

Paraclinical tests provide clinicians with more information that help establishing a correct and early diagnosis of MS.

Magnetic Resonance Imaging

Abnormalities of white matter are demonstrated in more than 95% of MS patients. MRI shows the anatomical dissemination of lesions and used serially

over time it can show new plaques in the absence of clinical episodes [95]. MRI positivity by itself, without clinical manifestations, does not make a correct diagnosis because lesions are not exclusively characteristic of MS disease, but also non-specific white matter cerebral lesions appear in people without clinical signs of disease and in people older than 50 years. In contrast, lesions detected in the spinal cord are abnormal at any age [95]. Gadolinium (Gd) is a contrast material and Gd-enhancement in T1 indicates active lesions, inflammation and evidence of breakdown of the blood-brain barrier [100].

Cerebrospinal Fluid

A lumbar puncture is performed so as to better elucidate the aetiology of the clinical episode. The detection of *oligoclonal bands (OB)* of IgG in the CSF and not in the serum of MS patients is the most important CSF test in MS, as OB are seen in most of the patients (>90%) [95]. OB reflect intrathecal synthesis of immunoglobulins. However, the detection of OB is not exclusive to MS and it has diagnostic value once other CNS inflammation causes have been excluded [101]. There are complementary tests, as the *Albumin quotient* (QAlb=CSF albumin/serum albumin, QAlb>$7*10^{-3}$ in 12% of patients), the *IgG index* (increased QIgG=CSF/ serum in 80% of patients) and *cell count* (50% with >4 white cells/µl but only 1% with cell counts >35/ µl) [101] that help in the differential diagnosis of MS.

Evoked Potentials

Evoked potentials measure the CNS response to different stimuli (visual somatosensorial and auditory). The speed of response gives idea of the degree of demyelination.

In summary, MRI and OB are the main paraclinical tools used in clinical practice [95, 102] but imaging techniques and OB have shown weak clinical correlations so far and on the other hand, cannot be used to monitor the disease [103, 104].

1.4.2. Poser Criteria

Poser criteria [105] allowed the classification of MS patients according to the number of attacks, clinical and paraclinical evidence and CSF OB or increased IgG index. These guidelines define an attack or relapse as *“the occurrence of a symptom or symptoms of neurological dysfunction, with or without objective*

confirmation, lasting more than 24 hours". The Poser classification divided MS patients in two major groups: "definite" and "probable" and these two as "clinically" and "laboratory-supported" (Table 2).

Clinically definite MS	2 attacks and clinical evidence of 2 separate lesions.
	2 attacks; clinical evidence of 1 lesion and paraclinical of another separate lesion.
Laboratory-supported definite MS	2 attacks; clinical or paraclinical evidence of 1 lesion and CSF OB or increased IgG index.
	1 attack; clinical evidence of 2 separate lesions and CSF OB or increased IgG index.
	1 attack; clinical evidence of 1 lesion and paraclinical of another separate lesion; and CSF OB or increased IgG index.
Clinically probable MS	2 attacks and clinical evidence of 1 lesion.
	1 attack and clinical evidence of 2 separate lesions.
	1 attack; clinical evidence of 1 lesion and paraclinical of another separate lesion.
Laboratory-supported probable MS	2 attacks and CSF OB or increased IgG index.

Table 2 Poser diagnostic criteria for multiple sclerosis [105].

The two clinical attacks must involve different locations of the CNS, must be separated for at least one month and last a minimum of 24 hours. One of the attacks must involve a different CNS site than the one demonstrated by clinical or paraclinical evidence. Clinical or paraclinical evidence of lesions must be separated in time for at least one month.

These criteria enable a reliable but not very early diagnosis of the disease. Due to the importance of an early treatment of MS, another group of experts proposed new diagnostic guidelines based in the time and spacial dissemination of lesions provided by MRI [106].

1.4.3. McDonald Criteria

Poser criteria were updated by the McDonald criteria [106] which underline the importance of MRI in the diagnosis of MS that allows earlier diagnosis of patients at their first clinical episode (Table 3).

These criteria included a scheme for the diagnosis of PP-MS, which lacks relapses from disease-onset.

In the definition of attack or relapse, the authors added to the definition by Poser [105] that multiple episodes occurring over not less than 24 hours were considered also a relapse. In a prospective study of a cohort of patients with clinically isolated syndrome (CIS), McDonald's criteria showed to have higher sensitivity, specificity, positive and negative predictive value and accuracy after one year than Poser criteria. One year after disease onset, McDonald's criteria were able to detect half of the patients with MS, while only one fifth with Poser criteria [107]. So these McDonald's criteria (Table 3) have a higher predictive value than the former ones and help in establishing an early diagnosis of MS, without the need of waiting till the next clinical relapse occurs. The McDonald Criteria were revised in 2005 and the changes mainly focus on the use of T2-weighted lesions and spinal cord imaging [108].

MRI Evidence of Dissemination in Space

In these criteria, MRI provides evidence in the diagnosis of MS of dissemination in space if at least three of the four criteria [109, 110] for brain abnormality are involved: one Gd-enhancing lesion or nine T2 hyperintense lesions if there is no Gd-enhancing lesion; at least one infratentorial lesion; at least one juxtacortical lesion; at least three periventricular lesions.

MRI Evidence of Dissemination in Time

In order to assess the dissemination of lesions in time these criteria must be fulfilled: a Gd-enhancing lesion in a scan done at least 3 months after the onset of clinical symptoms at a different site from the relapse; if there is no Gd-enhancing lesion at 3 month scan, serial scans after additional 3 months must show Gd-enhancing lesions or new T2 lesions.

MRI informs about the histopathology of MS lesions. The pathological hallmark of MS is focal demyelination in the lesions, with variable degrees of inflammation, demyelination, gliosis and axonal injury [100]. The site of the lesion is very important for MS diagnosis, as MS lesions are commonly located in the brainstem, cerebellum and periventricular white matter [111]. Typical MRI include T1-weighted (T1) with and without Gd administration, T2-weighted (T2), proton-density (PD), and fluid-attenuated inversion recovery (FLAIR) [112]. T2-weighted

scans show hyperintense lesions while T1-weighted scans show hypointensive lesions. T2, PD, and FLAIR demonstrate well most demyelinated lesions [112]. In the acute phase, T1 hypointensity reflects edema and demyelination that disappears when inflammation attenuates. On the contrary, chronic foci of T1 hypointensity (known as *black holes*) reflect persistent axonal loss [113].

Clinical relapses	Objective lesions	Additional Data for MS Diagnosis
≥2	≥2	None; clinical evidence is sufficient. (Additional tests should be consistent with MS if performed).
≥2	1	Dissemination in *space* by MRI **or** ≥2 MRI MS lesions and positive CSF **or** further clinical relapse involving a different site.
1	≥2	Dissemination in *time* by MRI **or** second clinical relapse.
1 (monosymptomatic; CIS)	1	Dissemination in *space* by MRI **or** ≥2 MRI MS lesions and positive CSF[a]. **And** Dissemination in *time* by MRI **or** second clinical relapse.
0 (progression from onset*)	1	Positive CSF **And** Dissemination in space by MRI evidence of ≥9 T2 brain lesions **or** ≥2 lesions in the spinal cord **or** 4-8 brain plus 1 spinal cord lesion. **Or** Abnormal VEP[b] with 4-8 MRI lesions **or** with <4 brain lesions plus 1 spinal cord lesion. **And** Dissemination in time by MRI **or** continued progression for 1 year.

Table 3 McDonald Diagnostic Criteria for multiple sclerosis [106].
*Criteria proposed in the previous paper by Thompson *et al.* [114]
[a]Positive CSF means positive OB detected in CSF and not in the serum or by a raised IgG index.
[b]Abnormal visual evoked potential (VEP) seen in MS: delayed but well-preserved wave form.

1.4.4. Differential Diagnosis

Due to the clinical heterogeneity of MS, there are many different diseases that should be well differentiated from MS diagnosis. Monophasic disorders affecting multiple separate sites, as acute disseminated encephalomyelitis; disorders affecting one site but with a relapsing-remitting or progressive course as tumors; systemic diseases with CNS involvement and a relapsing-remitting

course as systemic vasculitis; diseases of the brain and spinal cord with a progressive course as hereditary cerebellar ataxia; somatization disorders as MS Münchausen [95]. CNS vasculitis, such as SLE, Sjögren's disease, polyarteritis nodosa, syphilis, retroviral diseases and Behcet disease, may all produce multifocal lesions with or without a relapsing-remitting course.

1.5. PATHOGENESIS OF MULTIPLE SCLEROSIS

MS has classically been considered a white matter disease with characteristic focal and multiple demyelinated plaques in brain, spinal cord and optic nerve.
Even if the "pioneers" in MS, Carswell and Charcot, already remarked the presence of lesions in the gray matter [6, 115, 116] little attention has been paid until recently to cerebral cortex gray matter, probably due to the lack of better immunohistochemistry methods and to better imaging techniques like double inversion recovery (DIR) that are now available [117, 118]. Recent advances in MS pathology show that cortical demyelinated lesions with an inflammatory nature are present in early MS and may preceed white matter damage [117, 119-122].
The demyelination observed in MS occurs through autoimmune inflammation mediated by T cells, B cells and macrophages. In many MS patients, there are meningeal follicles of B-cells with T-cells, plasma cells and follicular dendritic cells [121]. These follicles in the cerebral meninges indicate a locally continued humoral response with the maturation of B-cells to plasma cells and intrathecal production of immunoglobulins that compose characteristic OB [123, 124]. The cortical neurodegeneration associated with meningeal inflammation and lymphocyte infiltrates was evident in SP-MS patients and not in PP-MS patients [125, 126]. However, later studies have confirmed gray matter demyelination associated with meningeal inflammation at all stages of MS disease, including PP-MS patients [127, 128]. In older patients, inflammation fades away and the degree of axonal injury is similar to age-matched controls [128]. A trigger to B-cell proliferation has been attributed to EBV infection in the CNS, but this association remains in question because many groups have not found an excess of EBV-infected B-cells in MS CNS [121]. Cognitive impairment and epilepsy are more common in those MS patients with multiple cortical lesions and atrophy [129, 130]. In MS, the inflammation driven mainly by Th17 and Th1

lymphocytes and the defect of regulatory mechanisms induce the perivascular CD8+ cell infiltrates causing MS plaques that gather around the lateral ventricles, corpus callosum, cortex and subcortical white matter, optic nerves, brainstem and in the spinal cord [95]. Pro-inflammatory cytokines secreted by T, B lymphocytes and macrophages amplify the pathological response recruiting naïve microglia [95] that additionally upregulate proinflammatory pathways [131]. In demyelinated white matter lesions, oligodendrocytes upregulate anti-inflammatory genes [131] and oligodendrocyte precursors surround lesions [132], so these cells probably have a role in the remyelination process [131, 133]. The formation of remyelinated or shadow plaques has been found in a 20% of patients with early and progressive MS [134] and gray matter remyelinates more extensively than white matter [135]. In the progressive stages of the disease, apart from demyelination there is also axonal rupture, neuronal degeneration [95] and accumulation of hyperphosphorilated and insoluble tau [136, 137].

Antigen specificity is still not clear in MS. Myelin proteins such as myelin basic protein (MBP), proteolipoprotein (PLP) and myelin oligodendrocyte glycoprotein (MOG), are implicated but not totally specific to MS as autoreactive clones of lymphocytes are also detected in healthy individuals [95]. Antibodies to neurofascin, a neuronal protein in myelinated fibers at nodes of Ranvier, cause axonal injury and have been found in MS patients [138]. Other autoantibodies detected in MS patients' CSF, against αβ crystalline, have been found to suppress inflammatory pathways in the immune system and CNS [139].

1.6. IMMUNOPATHOPHYSIOLOGY OF MULTIPLE SCLEROSIS

MS is a clinically heterogeneous disease whose clinical manifestations can be explained by the pathological changes observed in axons and neurons of affected patients. Whether neuronal pathology of gray matter is primary or secondary to white matter axonal damage is unclear [140].

The RR-MS and PP-MS forms of MS present different clinical courses. RR-MS patients display a "two-stage" disease: a first stage with predominance of inflammation (relapses and remissions) and a second stage with more neurodegeneration and progression (demyelination and axonal loss) as SP-MS. However, neuroimaging studies have shown the coexistence of inflammation and neurodegeneration [141]. Consequently, therapy strategies differ depending

on the target phase of the disease: immunomodulatory therapies combat inflammation in the inflammatory phase and neuroprotective agents fight against myelin/neural degeneration in the progressive phase (Figure 4). By contrast, PP-MS patients present with a steady progression and degeneration from the disease onset.

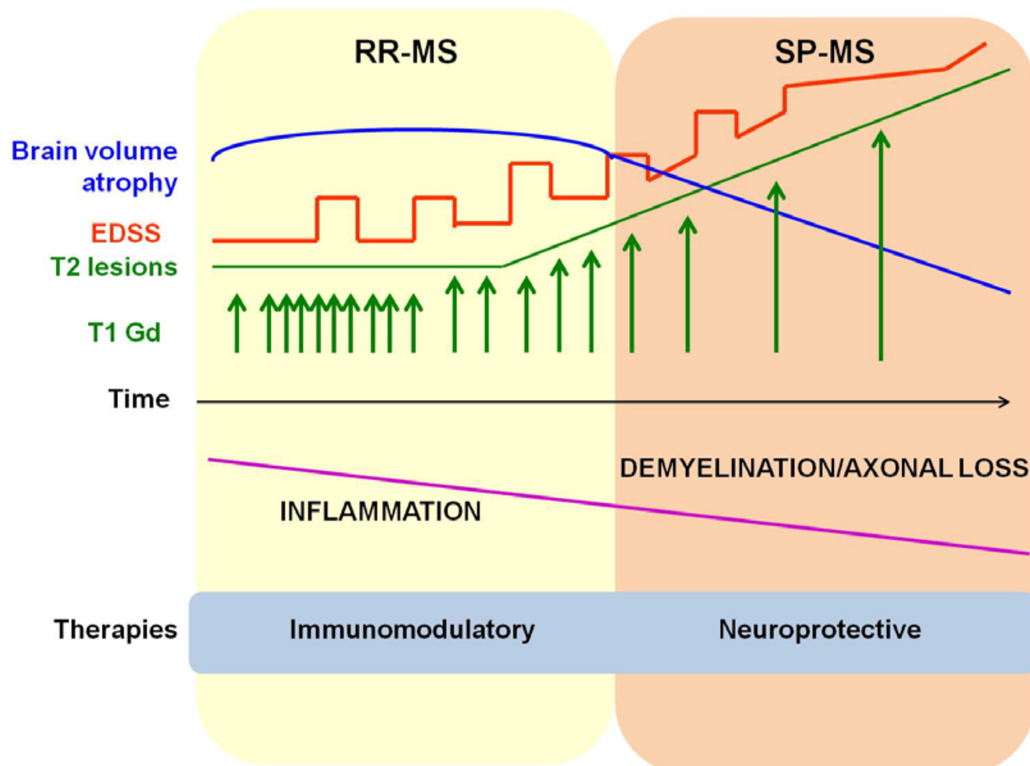

Figure 4 Clinical evolution of relapsing-remitting MS to secondary-progressive MS (RR-MS to SP-MS). The clinical scale is represented by EDSS (red line); the frequency of inflammatory events when studied by MRI (T1 lesions with gadolinium enchancement showing blood-brain barrier opening (green arrows); T2 lesions load reflecting tissue damage (green line) and loss of brain volume with brain atrophy (blue line). Adapted from [37].

Acute inflammation occurs during relapses and partial or complete remyelination happens during remissions, but the progressive degree of neurodegeneration leads to a higher brain volume loss and clinical disability. The etiology of MS is still unresolved and the nature of the disease has been proposed to be of autoimmune, infectious, genetic, metabolic, dietary or neurodegenerative origin. None of these hypotheses by itself can explain the clinical heterogeneity of the disease, so it is more probable that all of them may contribute to some extent to trigger and maintain the disease. Nonetheless, the

immune system plays an important role in MS pathophysiology. This is sustained by diverse facts:

- Susceptibility to MS disease is linked to important genes of the immunological response.
- MS plaques or lesions are crowed with inflammatory lymphocytes and macrophages.
- Oligoclonal bands of immunoglobulins are present in the CSF of most of the patients with MS.
- Immunomodulatory treatments modify the disease course and can stop inflammatory attacks but not progression once started.

The contribution of the target organ, the CNS, has been almost completely ignored [37]. Pathological and imaging studies [7, 142], as well as the research of the molecular aspects of the disease in the animal model of MS, EAE and in MS, provide now large evidence that CNS-specific factors are important [143, 144].

The most accepted theory for MS onset is that autoreactive T lymphocytes against myelin peptides reach the CNS crossing the BBB and trigger the pathological events that lead to demyelination and axonal damage. This insult to axons can be mild and reversible or severe and irreversible, with transection and likely loss of neuronal function. The failure of immunoregulatory mechanisms, such as defective suppressive function of Treg [33, 145, 146] and the overexpression of β-arrestin 1, promoter of $CD4^+$ T-cell survival [147], maintain autoreactive $CD4^+$ T lymphocytes and their ongoing inflammatory recruitment of other activated leukocyte subsets. Active lesions of MS patients show a perivascular infiltration of oligoclonal T cells ($CD8^+$>$CD4^+$) α/β and ɣ/δ, monocytes, occasional B cells and few plasma cells [5]. The degree of axonal transection is proportional to the number of macrophages and $CD8^+$ T lymphocytes present in MS lesions [148]. B lymphocytes are also relevant players in MS pathophysiology, as they are abnormally activated in meningeal follicles and differentiate to plasma cells that produce intrathecally immunoglobulins detected as OB in most of MS patients [124]. Programmed cell death, apoptosis of immune cells, is also an important pathophysiological mechanism in MS inflammation. It regulates elimination of autoreactive T and B cells and macrophages from the circulation and prevents their entry into the CNS.

Dysregulation of apoptosis and other immune functions may lead to inflammation within the CNS tissue and subsequent development of tissue damage [149, 150].

1.6.1. Important Players

1.6.1.1. Dendritic Cells

Dendritic cells (DCs) are one of the main actors of MS disease pathophysiology. They are antigen-presenting cells (APC) that link innate and adaptive immunity and thus presumably responsible of activating autoreactive T cell clones and of initiating the disease.

DCs are bone-marrow derived leukocytes that initiate and modulate the immune response by presenting endocytosed antigenic peptides on both MHC class I or class II molecules to naïve T lymphocytes. Small numbers of DCs are able to strongly stimulate T-cell responses with low levels of Ag, they are able to induce tolerogenic T cells to self-antigens and can capture Ag in the periphery and migrate to lymphoid organs to select optimal $CD4^+$ and $CD8^+$ T clones that clear the pathogenic Ag [151]. DCs have different degrees of maturation depending on the antigen and microenvironmental signals. Resident-immature DCs are located in peripheral tissues, where they are specialized in the capture and processing of Ag of invasive pathogens but have low antigen-presenting ability. After the pathogen encounter, they start a program of maturation where they change phenotypically, express higher quantities of MHC class II, co-stimulatory molecules and cytokines and migrate from peripheral tissues to secondary lymphoid organs, where they present processed Ags in the MHC complex to naïve T lymphocytes [151-153]. There are different subtypes of circulating DCs described in humans mainly: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).

DCs have been found in the CSF of MS patients at clinical relapse and their circulating levels in the periphery are increased with respect to healthy controls [154, 155]. DCs coexpressing the ligands for CCR7, CCL19 and CCL21 have been found in MS lesions [156-158]. In the periphery, the phenotype of mDCs and pDCs is altered. Nowadays, DCs are considered potential therapeutic targets in MS [159]. Specifically, tolerogenic DCs - induce T cell tolerance and secrete anti-

inflammatory cytokines: IL10, TGFβ, IDO and PD-1 that induce Treg - have been proposed as therapy for their ability in restoring peripheral tolerance [160]. Recently, Raïch-Regué and colleagues have generated semimature tolerogenic monocyte-derived DCs, in the presence of 1α,25-dihydroxyvitamin-D(3), loaded with myelin peptides that induced stable antigen-specific hyporesponsiveness in myelin-reactive T cells from RR-MS patients [161], what moves closer to the clinic the use of tolerogenic DCs.

MS patients seem to have higher titers of mature ($CD80^+$) proinflammatory (IL12 and TNFα producers) mDCs [162], while pDCs have also an altered phenotype that contributes to the immune regulation impairment seen in MS [163-165]. MS patients starting therapy with IFNβ-1a and low baseline circulating $BDCA1^+$ mDCs (≤0.4%) have a 7 fold-risk of developing clinical activity compared to patients with higher baseline $BDCA1^+$ mDCs levels. Furthermore, patients that responded to therapy with IFNβ-1a increased their proportions of mDCs while decreasing those of pDCs [166]. Other authors found that non-responder patients to IFNβ treatment had higher expression of CD86 on mDCs before initiation of treatment [167]. These studies point to mDCs rather than to pDCs, the interferon-secreting cells, as responsible for the clinical response to IFNβ in MS.

The humanized monoclonal antibody natalizumab, approved for MS therapy blocks the adhesion molecule very late activation antigen 4 (VLA-4) and reduces the number of DCs in the CNS [168] presumably by blocking the entrance of DCs in the CNS due to the gradually lower expression of the integrin VLA-4 on their surface [169].

1.6.1.2. $CD4^+$ T Lymphocytes

The activation of autoreactive T $CD4^+$ lymphocytes seems crucial in the induction of autoimmune disease [5]. These autoreactive lymphocytes to myelin peptides are modestly increased in MS patients with respect to normal subjects [170, 171]. Therefore, something “extra” must happens to unleash autoimmunity in some individuals. The T cell receptor (TCR) from these autoreactive cell clones appears to be highly specific for immunodominant p85–99 epitope of MBP [5] and $HLA\text{-}DR2^+$ restricted [172]. However, when this peptide ligand is altered, the TCR conformation is also modified causing T cell cross-reactivity [5, 173]. This would

partly explain why most MS patients have T cell reactivity to multiple myelin epitopes [172]. Moreover, viral epitopes can trigger autoreactive T cell clones [61, 174], evidence that would support the theory that microbial antigens cross-reactive with myelin can trigger MS disease [175]. MCH class II alleles (HLA-DR) specifically confer risk in MS [37], what reflects again the importance of antigen (Ag) recognition in the pathophysiology of MS disease. Windhagen *et al.* found an upregulation of B7.1 and IL12 in early white matter lesions of MS patients and in inflammatory cerebral infarcts [176], suggesting that inflammation in MS lesions has an important T cell activation component.

Leukocytes patrol continuously different organs including CNS for damaging insults that disrupt homeostasis [177]. Resident T cells found in the CSF of healthy individuals are predominantly central memory T cells ($CD4^+CD45RA^-CD27^+CD69^+$) that express high levels of CCR7, CXCR3, L-selectin and P-selectin [178, 179]. In response to a pathological or inflammatory insult, proinflammatory cytokines such as interleukin-1 (IL-1), tumoral necrosis factor-α (TNFα) and interferon-gamma (IFNɣ) induce an increase in the adhesion molecules, E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), and other adhesion molecules that facilitate the entrance to human brain have been found in the vessels and subarachnoid space of human brains [178, 180]. Once activated, T cells have enough surface molecules to cross the BBB and reach the CNS parenchyma, capture and adherence of Th1 T cells occurs thanks to the interaction of the integrin very late antigen-4 (VLA-4) present in Th1 T cell and the VCAM-1 present on endothelial cells [169, 177]. The transendothelial migration across the venule wall is mediated by the leukocyte function associated antigen (LFA-1) [181]. Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 that degrade extracellular matrix and help in the recruitment of autoreactive T cells are also present in MS lesions [182, 183]. Th17 migration is known from the animal models. In EAE, Th17 cell migration depends on the chemokine receptor CCR6 on Th17 cells that binds to CCL20 constitutively expressed by the choroid plexus epithelium [184] and others have reported that the earliest CNS entry of $CD4^+$ T cells in EAE occurs at the fifth lumbar level of the spinal cord with a high CCL20 chemokine expression of CCL20 in the dorsal root vessels [185].

1.6.1.3. CD8$^+$ T Lymphocytes

CD8$^+$ T lymphocytes have been less studied in MS than CD4$^+$ T lymphocytes [37]. However, they are more probably directly involved in the CNS tissue damage due to their effector functions than CD4$^+$ T lymphocytes [37]. Indeed, CD8$^+$ T cells are more abundant in the active lesions of MS patients [5, 186], oligoclonal expansion of memory CD8$^+$ T cells have been found in the CSF [187] and CD8$^+$ T cells clones can persist for years in the CSF and blood of MS patients [188]. Of note, oligodendrocytes and neurons can only express MHC class I, so they can only be recognized by CD8$^+$ T lymphocytes [189, 190]. Furthermore, cytokine production by CD8$^+$ T cells correlates with tissue destruction determined by MRI [191]. The CD8$^+$ cytotoxic response to MBP is increased in MS patients [192] and CD8$^+$ myelin-specific T lymphocytes secrete chemokines (CCL3/MIP-1α, CCL4/MIP-1β, IL-16 and CXCL10/IP10) that chemoattract CD4$^+$ myelin-specific T lymphocytes, being this chemoattraction mainly mediated by CXCL10/IP10, perpetuating the immune response [193]. All these pieces of data sustain the important role of CD8$^+$ T lymphocytes in MS pathophysiology.

1.6.1.4. B Lymphocytes

The intrathecal production of oligoclonal Igs in most MS patients is the most important evidence of the role of B lymphocytes in MS pathophysiology [124]. B cells cross the BBB once the inflammation process has started [37], the activation of B lymphocytes to plasma Ig producing cells can occur because of activation with self or foreign antigens, through a random effect during inflammation in MS plaques or by superantigen stimulation [37]. In addition, B lymphocytes can contribute to MS pathophysiology in different ways: they can act as APC for autoreactive T lymphocytes; they can provide costimulation to T lymphocytes and produce myelin-specific antibodies that mediate the myelin destruction with plaques [37].

1.6.1.5. Regulatory T Lymphocytes

The CD4$^+$ regulatory T lymphocytes or Treg are a minor subset (5-10% of total CD4$^+$ T cells) essential in maintaining immune tolerance to self-antigens and in avoiding harmful immune responses to the host. Functionally mature Treg can be produced in the thymus (naturally occurring or naïve: nTreg) or can be induced from naïve T lymphocytes in the periphery (induced: iTreg). nTreg are

specialized in the immune suppression of autoreactive T lymphocytes and the absence of this population cause severe autoimmune diseases in humans (IPEX syndrome: immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [194] and animals [195]. Treg can also be induced or converted during inflammatory processes by certain cytokines (IL 10 and TGFβ) [196, 197 198] or by the action of tolerogenic DCs [199, 200]. iTreg have a more limited TCR repertoire than nTreg, so they are more specific for particular cell types, tumors or foreign antigens [201].

In 1995, Sakaguchi and coworkers identified the molecule CD25 (the IL-2 receptor α chain) as a putative surface marker of Treg [202]. nTreg are memory lymphocytes ($CD45Ro^+$) that persist in the periphery controlling not only pathogenic self-reactive T cells but also immune responses to quasi-self-antigens (autologous tumor cells) and non-self-antigens (microbres and allografts) [203]. nTreg specifically express the nuclear transcription factor FoxP3 (forkhead box P3) and human mutations in this gene cause IPEX syndrome in humans [194]. FoxP3 is crucial in nTreg function as its transduction upregulates the expression of CD25, cytotoxic T cell associated antigen-4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related gene (GITR), and it suppresses the production of IL-2, IFNγ and IL4 [203], clue cytokines in Th1 and Th2 responses. In humans, FoxP3 is also detected in iTreg, whereas in mice it is detected only in nTreg [204]. As FoxP3 is expressed intracellulary, the isolation for investigations of $CD4^+$ nTreg was limited due to the lack of specificity of the other surface markers (CD25, CTLA-4, GITR). Nonetheless, nTreg express constitutively a higher density of CD25 [205] and low levels of the antigen CD127 (the IL-7 receptor α chain) [206-208]. This enabled a more specific isolation of nTreg characterized as $CD4^+CD25^+CD127^{low}$.

Treg lymphocytes from MS patients show a defective suppressive function compared to those from healthy controls [33, 145, 209]. In early MS patients the nTreg thymic output is diminished compared to those of healthy controls [208]. Similar Treg frequencies in the periphery have been found in RR-MS patients and healthy controls [145], while others have found decreased expression of FoxP3 levels in Tregs from MS patients and decreased function [210, 211]. In the CNS, $CD4^+$ Treg are found at higher frequencies than in peripheral blood during

relapses [212-214], what suggests an active recruitment of these cells to the central compartment in order to control the inflammation. Results from our group show that CD4$^+$ Treg cells express higher intracellular expression of perforin than their blood counterparts what points out a relevant role of this molecule in the suppressive effects CD4$^+$ Treg in MS [214]. Furthermore, CSF CD4$^+$ Treg cells were more abundant in women than in men reflecting the sexual dimorphism present in MS [214]. Treatment with IFNβ expands Treg frequencies and restores its suppressive function [208, 215].

Treg have different suppressive mechanisms of the immune response [216] that are constantly being investigated. The most important suppressive mechanisms are mediated by CTLA-4 [217-219]; by direct cell to cell contact suppressing the IL-2 production of responder T cells [220] or competing for IL-2 [221]; by perforin mediated suppression [222] that is influenced by the sexual hormone estradiol [36]; by apoptosis induction [223] or by the lymphocyte activation gene 3 (LAG-3 or CD223) mediated suppression [224].

1.6.2. The Innate Immune System

1.6.2.1. Toll-Like Receptors

Our innate immune cells are able to detect pathogens through pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) present on the surface of pathogens [225]. Toll-Like Receptors (TLRs) were the first PRRs to be described. The PAMPs recognized by the TLRs include pathogenic molecules from bacteria, parasites and virus [226]. TLR1, TLR2, TLR4, TLR5, TLR6 are on the cell surface, whereas TLR3, TLR4, TLR7, TLR8, TLR9 are intracellularly expressed [227]. There are five TLRs: TLR-3, TLR-4, TLR-7, TLR-8 and TLR-9 that induce type I interferon production (Figure 5). TLR3 is the only TLR that does not require the adaptor molecule myeloid differentiation primary response protein 88 (MyD88) for the recruitment of the IRAK family protein kinases and to the activation of TRAF, NF-κb and final production of inflammatory cytokines. TLR4 and TLR3 use the adaptor protein TRIF for the activation of the transcription factor IRF3 and subsequent production of type I interferons [227]. The main producers of type I IFN are monocytes (via TLR-4 and TLR-8), plasmacytoid dendritic cells (pDCs) (via

TLR-7 and TLR-9) and myeloid dendritic cells (mDCs) (via TLR-3). TLRs are also expressed by microglia and astrocytes playing an important role in the central surveillance and homeostasis [177, 228]. Microglia express a wide range of different TLR family members, while astrocytes and oligodendrocytes express TLR-2 and TLR-3 [229]. Neurons express TLR-2, -3, -4 and -8 [230] and in the peripheral nerves, Schwann cells express high levels of TLR-3 and TLR-4 [231]. Although most of these TLRs are redundant in host defenses against most microbes, some of them are vital for natural immunity within the CNS. For instance, TLR-3 deficient patients suffer from Herpes Simplex Encephalitis, a potentially fatal manifestation of herpes simplex virus-1 (HSV-1) infection [232-234]. These monogenic primary immunodeficiencies have provided new insights on the relevance of the production of IFN-α/β and IFN-λ after infection by neurotropic viruses of the CNS. A large body of evidence highlights the importance of TLRs in the modulation of CNS responses to inflammation or injury.

In MS, there is evidence of viral infections triggering CNS autoimmunity both in the animal model and in the clinical setting. In MS patients, it is frequently observed that after infections or fever, patients suffer changes in their neurological function that seem to be related to a clinical relapse called pseudoexacerbations [235]. Some patients with urinary tract infections caused by bacterial agents as *Escherichia coli*, require steroids to recover from their clinical symptoms and sometimes do not recover completely. It has been suggested that these bacterial agents may stimulate the innate immune response via TLRs and activate autoreactive T cells that provoke MS exacerbations [236]. Microglia and astrocytes may be implicated in the pathogenesis of MS when they are activated by pathogenic molecules that bind and activate continuously their TLRs, leading to an abnormal activation of effector myelin-self T cell clones in the CNS [237, 238]. In EAE, MyD88 knockout mice do not develop active EAE, reflecting the importance of the TLRs-dependent pathways in EAE pathophysiology [239]. Stimulation of TLR1, TLR2, TLR4 and TLR9 in the presence of myelin peptides is important in the induction and modulation of EAE [236], whereas TLR3 stimulation through the MyD88-independent pathway ameliorates the disease. For instance, the synthetic TLR3

ligand polyinosinic:polycytidylic acid (poly I:C) added as an adjuvant with myelin peptides does not induce active EAE [240]. Treatment with poly(I:C) suppresses the development of EAE, increasing the production of endogenous IFNβ [241]. TLR3 and TLR4 type I IFN production pathways seem to play an important role in EAE, as type I IFN receptor and TRIF knockout mice develop a more severe disease, with an increased endogenous IL17 production and Th17 driven inflammation [242]. Moreover, the synthetic cannabinoid R(+)WIN55,212-2 has recently been identified as a novel regulator of TLR3 signaling via IRF3 activation and IFNβ endogenous production [243]. All this body of evidence indicates that TLRs play an important role in the modulation of EAE disease and need to be further investigated in MS.

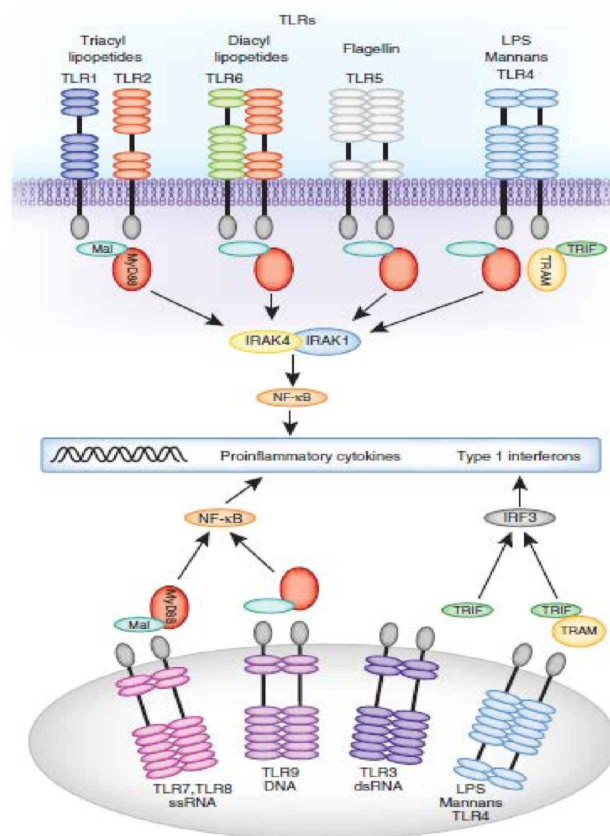

Figure 5 Cellular localization and main ligands of TLRs with intracellular pathways that lead to the stimulation of type I IFN and proinflammatory cytokines. Taken from the review by Netea *et al.* [227].

1.6.2.2. Mast Cells

Elevated numbers of mast cells have been found in MS plaques [244] and the chemokine Rantes/CCL5 is a potent chemoattractor for mast cells that is elevated in MS lesions [37]. Mast cells can augment leukocyte infiltration in the CNS increasing their recruitment, adhesion and rolling through their mediators (histamine and tryptase) and cytokines (lymphotactin, IL-16, TNFα and IL-1) [37].

1.6.2.3. NK Cells

Circulating frequencies of NK cells are significantly reduced in MS [245]. The subset $CD56^{bright}$ which is expanded *in vivo* in MS patients under therapy with daclizumab (humanized monoclonal antibody against the IL-2 receptor α chain), has immunoregulatory functions, as their increased perforin expression correlates with a reduction in the inflammatory activity and the direct lysis *in vitro* of $CD4^+$ T lymphocytes via perforin and grazyme K [246].

1.6.2.4. Nitric Oxide Synthase

Granulocytes and macrophages present the enzyme inducible nitric oxide synthase (iNOS) that generates nitric oxide (NO), a short-lived and bioactive free radical with antimicrobial properties [37]. NOS has been found in MS lesions [247] but its role in MS lesions is uncertain [37].

1.7. BIOMARKERS IN MULTIPE SCLEROSIS DISEASE

MS is a heterogeneous disease not only in its clinical manifestations and forms, but also in the disease course and response to therapy. The search for reliable biomarkers that help in the diagnosis, stratification, treatment response and prediction of MS clinical disability has increased greatly in the last years and is strongly needed. Putative biomarkers need to be validated for clinical use and this is the main obstacle in biomarker discovery for MS, as very few candidate biomarkers have been validated for its clinical use [248] except for the oligoclonal bands (OB), the neutralizing antibodies to IFNβ in non-responder patients to IFNβ and the antibody response to the JC virus as a predictor of those patients who would develop progressive multifocal leukoencephalopathy (PML) when receiving natalizumab (anti-α4 integrin monoclonal antibody) [249].

The definition of biomarker or biological marker encompasses “a characteristic that is objectively measured and evaluated as an indicator of either normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention [250] (Figure 6).

In MS, biomarkers can be classified depending on their proposed use [251] as:

- Predictive for the risk of presenting the disease
- Diagnostic
- Classifiers of the staging and clinical form of the disease
- Predictive for the natural history of the disease or treatment response

IDEAL MS BIOMARKER	
Measures clinically relevant MS outcomes	Preferably reflects a causal association
The assay for its identification is simple, reliable, affordable, stable and can be independently validated and standardized.	
Detected in an easily accessible biological simple with minimal pre-analytical perturbations	

Figure 6 Characteristics of the ideal biomarker for MS. Adapted from [251].

1.7.1. Cytokines as Biomarkers in Multiple Sclerosis

Cytokines are very important in the regulation of the activity and function of immune cells; they have a clear role in MS pathogenesis and have been thoroughly studied as potential biomarkers.

Cytokines as Biomarkers for Disease Activity

Hagman *et al.* have recently found in a prospective study that serum Fas and MIF (macrophage migration inhibitory factor) are candidate biomarkers of neurological worsening in progressive neurodegeneration, while serum TNFα and MCP-1/CCL2 could be for PPMS patients [252]. Increased levels of the proinflammatory cytokines TNFα, IL-12, IL-17 and IFNɣ and decreased of the anti-inflammatory cytokine IL-10 are found in the CSF and blood of MS patients

at relapse [248], giving an idea of the nature Th1 and Th17 of the immune attack. Indeed, systemic IL-12 production, that stimulates the production of other proinflammatory cytokines, is prior to MS relapses and correlates with clinical activity [248].

Cytokines as Biomarkers for Treatment Response

Several cytokines have been studied for monitoring the treatment efficacy to disease modifying therapies (DMTs) and prediction of its response. For instance, IFNβ responder patients had lower serum IL-10 [253] and lower baseline IL-12p35 mRNA in blood than non-responders [254], while serum IL-17F levels were higher in non-responder patients to IFNβ [255].

1.7.2. Chemokines as Biomarkers in Multiple Sclerosis

Chemokines and their receptors have an important role in the pathophysiology of MS given the organ-specific nature of MS. They can act as chemoattractants through the recruitment of autoreactive cells from the periphery to the CNS and induce the secretion of proinflammatory cytokines, which promote the final demyelination and neuronal loss [256, 257]. The chemokines CXCL8, Rantes/ CCL5 are increased in the CSF and serum of those patients at relapse [258] and the levels of CXCL13 in those patients with clinical disease activity [259, 260]. The interferon gamma-induced protein 10 (IP10) or CXCL10, whose secretion is enhanced after exposure to IFNs [261], and its receptor CXCR3 are expressed widely by a variety of cells (including immune cells and astrocytes). The levels of IP10 have been reported to be elevated in the CSF and serum of MS patients during the active phase of the disease compared to NIND controls [262-264], possibly due to its production by astrocytes in demyelinating lesions [265]. On the other hand, MCP-1/CCL2 levels are decreased at relapse [258, 262]. CXCR3 and CCR5 expression on peripheral blood lymphocytes increases during relapses [266] and in progressive patients [265]. The frequency of $CD8^{+}CXCR3^{+}$ correlates with MRI measures of MS inflammatory activity and tissue destruction [267]. Additionally, polymorphisms in Rantes and CCR5 have been found to be associated with axonal loss and MRI measures of disease severity [268]. Most of the β-chemokines or CC chemokine ligands (CCL) genes are clustered in chromosome 17q11.2-12, a location that has been associated with MS in

different studies [269-271]. In a model of EAE, increased levels of Eotaxin/CCL11 were associated with milder disease phenotype, tighter blood brain barrier, reduced antigenic specific response and an anti-inflammatory Th2 phenotype [272].

1.7.2.1. sDPP4 and Multiple Sclerosis

Dipeptidylpeptidase 4 (DPP4, also known as CD26) is a multifunctional molecule that has been implicated in autoimmune pathophysiology with a putative immunoregulatory role. DPP4 is the prototypic member of a family of X-prolyl dipeptidylpeptidases, a family of multifunctional serine proteases whose role depend on the cell type and local conditions in which it is expressed [273]. DPP4 is a 110-kD transmembrane glycoprotein constitutively expressed as a dimer on epithelial cells of the liver, hepatocytes, kidney and intestinal tissues, as well as in some endothelial cells, fibroblasts and lymphocytes [274]. It also exists in a soluble form (sDPP4), and is present in different biological fluids such as serum, plasma, seminal fluid and in low amounts in the CSF [274, 275]. DPP4, also known as adenosin-deaminase (ADA) binding-2 protein, binds ADA on the surface of T cells [276], and provides a co-stimulatory signal [277, 278] that increases T cell proliferation and induces the production of Th1 cytokines [278]. DPP4 is involved in the regulation and migration of T cells [279] and thymocytes [280]. The $DPP4^{hi}$ T cell subset shows an effector-memory phenotype ($CD45RO^{+}CCR7^{low}$) [281, 282], induces a Th1 and Th17 profile of cytokines and their DPP4 expression is upregulated under inflammatory conditions [274]. Indeed, Th17 cells that can be found in sites of inflammation (e.g., active lesions of inflammatory bowel disease) show the highest expression of DPP4, which is enzymatically active and may regulate Th17 cell migration to inflamed tissues by modulating chemokine gradients [283]. DPP4 cleaves NH_2-terminal dipeptides from polypeptides with either L-proline or L-alanine at the penultimate position [284]. In many instances, this results in regulation of the substrate, inactivating the ligand activity or altering its function (e.g., generating dominant negative forms of the protein). Biologically important substrates for DPP4 include several chemokines, gastrointestinal hormones and neuropeptides [275, 285]. Chemokines regulated by DPP4 (reviewed by [275, 285], include but are not limited to: MIP-1α/ CCL3, MIP-1β/CCL4, Rantes/CCL5, Eotaxin/CCL11, Mig/CXCL9,

IP10/CXCL10, I-TAC/CXCL11 and SDF-1α/CXCL12. Recent findings by Casrouge *et al.* that IP10 cleavage by DPP4 generates a truncated form with negative function have yielded a new understanding of the processes governing leukocyte migration and a changing paradigm of the regulatory circuit of inflammatory responses [1].

Prior studies in MS patients have focused on the role of DPP4 expression on T cells. Hafler *et al.* were the first to describe an increase in the subset of $DPP4^+$ T (named as $Ta1^+$) cells in the peripheral blood and in the cerebrospinal fluid (CSF) of MS patients with progressive forms of the disease [286]. Subsequent studies presented conflicting results [287, 288], but it was eventually confirmed that circulating $DPP4^+$ T cells correlate with clinical and magnetic resonance imaging (MRI) disease activity scores in MS [289-291]. Krakauer *et al.* demonstrated that the memory $CD4^+CD45RO^+DPP4^{hi}$ cells in MS patients contained the largest fraction of Th1 cells coexpressing markers linked with MS disease (e.g. CCR5, CXCR3 and VLA-4^{hi}) and correlated with clinical MS disease severity [281]. The frequency of circulating DPP4- and CD71-expressing $CD8^+$ T cells decreases after interferon-β (IFNβ) immunomodulatory therapy, whereas interleukin IL-10 and IL-13 $CD8^+$ producing T cells increased in frequency [292]. Indeed, IFNβ treatment as well as endogenous type I IFN activity reduced the expression of CD49d integrin (subunit of VLA-4) on $CD4^+DPP4^{hi}$ T cells [293]. Narikawa *et al.* found increased levels of soluble DPP4 (sDPP4) in the CSF of a small series of relapsing neuromyelitis optica and MS patients compared to patients with non-inflammatory neurological diseases (NIND) [294].

All this body of evidence suggests that DPP4 expression may have an important role in MS pathophysiology that has been scarcely explored.

1.7.3. Growth Factors as Putative Biomarkers in Multiple Sclerosis

Different growth factors have relevance in the CNS disorders. The hepatocyte growth factor (HGF) is a multifunctional protein in the CNS that is produced by microglial cells, oligodendroglial progenitor cells, astrocytes and neurons [295-298]. The receptor for HGF, c-Met, is the tyrosine kinase product of the c-Met proto-oncogene [299, 300] and both HGF and its receptor c-Met are present during brain

development and persist in adulthood with important neurotrophic functions [301, 302]. Besides, peripheral HGF shows immunomodulatory effects: promoting the adhesion of B cells [303], migration of T cells [304] and recruitment of dendritic cells (DCs) [305, 306]. HGF has been found to be increased in CNS diseases, including MS and in prototypic neurodegenerative diseases, such as Alzheimer's disease [307]. However, other studies have found decreased HGF CSF levels in patients with MS [308, 309] or unaltered levels of plasmatic HGF between MS patients and healthy controls [308]. The epidermal growth factor (EGF) has positive effects in the proliferation and differentiation of neurons, astrocytes and oligodendrocytes [310, 311] and its co-administration with growth hormone (GH) in EAE improved the clinical score and survival rate of mild and severe EAE forms [312]. In MS patients, EGF in CSF has been found to be significantly lower than in other non-inflammatory neurological diseases [313]. Also, the basic fibroblast growth factor (FGFb or FGF-2), is a key regulator of the growth, differentiation, migration and survival of CNS glial progenitors [310], and its expression is enhanced in active MS lesions [314]. FGFb has a controversial role in MS. It has been associated with demyelination and inhibition of myelin production by oligodendrocytes *in vivo* [315, 316] while with the induction of myelination in oligodendrocytes *in vitro* [317]. Recently, the knockout mouse of FGFb revealed rather a neuroprotective role for this growth factor in the animal model of MS [318].

1.7.4. Humoral and Antibody Biomarkers

Detection of two or more IgG **oligoclonal bands** (OB) in the CSF and not in the serum of a patient with clinical signs of MS provide very useful diagnostic value, as more than 95% of MS patients present with positive OB [319]. OB are the most commonly used MS biomarker [248]. They are detected since disease onset and persist during the disease regardless of disease activity. However, they are not specific of MS, as they are also detected in other inflammatory disorders and infections affecting the CNS [319].

The presence of **IFNβ neutralizing antibodies** (NAbs) has been found in patients at relapse. When there are persistent high titres of IFNβ NAbs, the physician might consider stopping treatment with IFNβ as it may not be effective [320] in a high percentage of patients who do not have a satisfactory clinical

response to this therapy. In patients with low or middle titers of NAbs, the induction by IFNβ of the protein myxovirus resistance protein A (MxA), encoded by the MX1 gene can provide additional information of IFNβ response to therapy [320].

Finally, elevated **kappa free light chains** in the CSF may correlate with disability prognosis [321] and elevated Epstein-Barr virus nuclear antigen 1 (**EBNA-1**) IgG correlates with gadolinium-enhancing lesions [322].

1.7.5. Biomarkers for Interferon-β Therapeutical Response

IFNβ remains the most widely prescribed treatment for RR-MS, with positive effects in reducing disease activity. However, there is still a high proportion of patients (up to 40%) who do not respond to this therapy [323]. IFNβ is a type I interferon that binds to the heterodimeric receptor IFNAR1 IFNAR 2 and activates the JAK-STAT pathway with phosphorilation of STAT1 and STAT2, which bind IRF9 and finally translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate the transcription of type I IFN-stimulated genes (ISGs) [324]. There has been an intensive search for specific biomarkers predictors of therapeutic response to IFNβ. Baranzini *et al.* found nine blood sets of gene triplets whose expression at baseline (before starting IFNβ therapy) could predict the response to IFNβ with an accuracy > 80% [325]. Another group described that nonresponder patients to IFNβ had a baseline overexpression of some type I IFN genes and that their expression did not vary significantly during IFNβ treatment, while responder patients strongly upregulated these genes [167]. Recently, Bustamante *et al.* have found that nonresponder patients to IFNβ have decreased expression levels of endogenous IFNβ in their PBMCs and increased IFN receptor 1 (IFNAR1) expression in monocytes compared to responder patients and controls. Moreover, they found that the baseline expression of interleukin-1 receptor-associated kinase 3 (IRAK3), a negative regulator of TLR4 signaling, was significantly decreased in responder compared to nonresponder patients. This study links TLR4 and endogenous type I IFN signaling pathways and endogenous production of IFN with the clinical response to IFNβ therapy [326]. Finally, the chemokines IP10/CXCL10 and MCP-1/CCL2 and have also been

described as biomarkers for the positive clinical response to IFNβ treatment [327]. IP10/CXCL10 levels are higher in patients under treatment with IFNβ [327-329] and IP10/CXCL10 and MCP-1/CCL2 have been proposed as biomarkers for IFNβ response [327, 329].

1.8. MULTIPLE SCLEROSIS TREATMENTS

Available first-line MS treatments act in the RR stage of the disease (IFNβ, glatiramer acetate and Natalizumab) and partially in the SP stage of the disease (Natalizumab), but not in the PP-MS. Fingolimod or FTY720 is the first oral treatment for RR-MS that has recently been approved and has greater efficacy compared to IFNβ-1a [330]. Up to now, PP-MS continues with no effective treatment to stop disease progression.

The main MS treatments are summarized below according to their mechanisms of action:

Immunomodulatory Properties

In the 1990s, IFNβ and glatiramer acetate (GA) were the first disease-modifying therapies (DMTs) to be approved. Nowadays, they are still the most used first line treatments for RR-MS.

IFNβ has different formulations: IFNβ-1a (Avonex® and Rebif®) and IFNβ-1b (Betaseron® and Extavia®). IFNβ has multiple immunomodulatory properties: It increases Th2 cytokines (IL4 and IL10) while decreases Th1 cytokines (IL12, IFNɣ and TNFα) [331, 332]; diminishes T-cell expansion and differentiation; increases T-cell apoptosis [333, 334]; downregulates MHC-II expression [335] and antigen presentation; inhibits MMPs secretion and migration of immune cells across the BBB [336]; expands blood Treg and enhances Treg function [146, 166] and normalizes ex vivo T lymphocyte apoptosis that is increased in active MS patients [337]. IFNβ is usually well tolerated, with some patients experiencing flu-like symptoms, myalgia and less frequently depression, anemia, leukopenia, and thrombocytopenia [338]. The main problem with IFNβ is its immunogenicity (IFNβ-1a is more immunogenic than IFNβ-1b and Rebif® more than Avonex®). A mean of 25% of patients develop neutralizing antibodies (NAbs) to IFNβ, and this is a factor that contributes to non-responsiveness to this treatment [338].

Glatiramer acetate (Copaxone®) was discovered in the 1960s relatively by chance. Scientists were looking for encephalitogenic copolymers to induce EAE

and they found the opposite with the copolymer randomly composed of L-alanine, L-lysine, L-glutamic acid and L-tyrosine, in a molar ratio of 4.2:3.4:1.4:1.0 [339]. In humans, GA is administered daily as a subcutaneous dose of 20 mg and is able to reduce relapse rate and disability in MS. GA modulates peripherally autoreactive T cells as it competes for the MHC-II with myelin peptides and induces a Th2 antiinflammatory profile (IL4, IL5, IL10 and TGFβ) while reducing Th17 cells. Moreover, GA ameliorates the function and frequencies of regulatory T cells in the CNS and in the periphery and has neuroprotective properties [339].

Inhibition of lymphocyte migration

Natalizumab (Tysabri®) is a humanized monoclonal antibody (mAb) that binds to the adhesion molecule VLA-4 or CD49d and diminishes greatly lymphocyte migration through the BBB. VLA-4 is expressed by all white blood cells except neutrophils. Tysabri's more severe adverse event is progressive multifocal leukoencephalopathy (PML), a life-threatening brain infection caused by JC virus that is extremely severe in immunodeficient subjects [340]. Natalizumab is administered every four weeks in IV perfusion of 300 mg.

Fingolimod (Gilenya®) or FTY720 is an immunosuppressive metabolite obtained from the fungus *Isaria sinclairii* [341]. It is a sphingosin-1 phosphate receptor agonist and the first oral treatment approved for MS treatment. During fingolimod administration, lymphocytes are recruited within the lymph organs, inhibiting their migration to the CNS but without impairing their activation or proliferation [330]. The lymphopenia observed with fingolimod is reversible once the treatment is stopped. Fingolimod is a lipophilic molecule able to directly enter the CNS. Direct neurobiological effects are also under investigation [330].

Immunosuppressive properties

Alemtuzumab (Campath-1H) is a humanized mAb against CD52, antigen expressed on T and B lymphocytes, NK cells, DCs, most monocytes and thymocytes [342]. This treatment is also approved to treat leukemia as it rapidly depletes $CD52^+$ cells [343]. Alemtuzumab is administered intravenously and reduces relapse rate and accumulation of disability greater than IFNβ-1a in untreated patients and in patients that relapsed after IFNβ or GA [344, 345]. Patients under treatment with Alemtuzumab are more susceptible to herpes

infections, thyroid disorders than with IFNβ-1a and a few (1%) had immune thrombocytopenia [344, 345].

Azathioprine is an off-label therapy for MS in most countries, except in Germany [346]. Azathioprine is a purine antimetabolite immunosuppressor that affects DNA replication and impairs correct T lymphocyte function [347]. It has been used for decades but nowadays it is only recommended as prophylactic for those patients who do not tolerate first-line treatments. A continued use increases the risk of developing malignancies.

Depletion of T lymphocytes

Daclizumab is a humanized mAb against the IL-2 receptor alpha chain (IL-2RA or CD25). The main mechanism of action of daclizumab is the enhancement of regulatory NK cells $CD56^{high}$ rather than the restraint of the expansion of autoreactive T $CD25^{+}$ cells [348]. Several small phase II clinical trials have tested daclizumab in patients with RR-MS, SP-MS and in RR-MS patients that did not respond to IFNβ. Although the number of patients in these trials is low, the efficacy seen is very promising and there are no serious adverse effects reported [348].

Depletion of B lymphocytes

Several mAbs that bind to the antigen CD20, present on pre-B and mature B lymphocytes but not in plasma cells have been tested for MS treatment. *Rituximab (Mabthera ®)*, *ocrelizumab* and *ofatumumab* bind to different epitopes of CD20 and have been tested in small phase I and II clinical trials for MS therapy. There are reported rapid beneficial effects of these drugs, but larger phase III clinical trials are warranted to determine the best efficacy/safety dose [349].

Promising new drugs

Cladribine is a purine nucleoside analog that produces lymphospecific toxicity and depletion through the inhibition of DNA synthesis and cell death. Phase III studies with oral cladribine have been completed and it has been authorized for the treatment of RR-MS in Russia and Australia [350].

Laquinimod is an immunomodulatory quinolone, orally administered, that is believed to favor Th2/Th3 cytokine production, interferes with VLA-4 binding,

and inhibits immune cell transmigration to the CNS (Phase III clinical trials ongoing) [351].

Teriflunomide is an inhibitor of the enzyme dihydroorate dehydrogenase (DHODH), key enzyme in pyrimidine synthesis. Teriflunomide inhibits de novo synthesis of pyrimidine, which is required for the proliferation of activated lymphocytes, thus preventing clonal expansion of T and B lymphocytes, but does not induce lymphopenia (Phase III completed) [352].

Cannabinoids have neuroprotective and immunomodulatory properties that have been shown to be beneficial in the animal models of MS and are now being evaluated to assess their impact in slowing the development of disability in progressive patients with MS (SP-MS and PP-MS) (Cannabinoid use in progressive inflammatory brain disease-CUPID-trial) [353].

2. AIMS

Multiple sclerosis is characterized by a variable clinical course and response to therapy that may represent divergent etiologies, given the distinct pathological patterns that the different clinical forms exhibit. To date, there are no reliable serum biomarkers for MS, which would be necessary for an improved diagnosis and evaluation of disease activity and treatment response. Early identification of the clinical forms of MS, especially the primary progressive form, remains a challenge for clinicians. The fact that progressive forms of the disease have still no available therapies to stop neurodegeneration and clinical disability makes more urgent the need for biomarkers that can anticipate the onset of progression. Identification of such biomarkers of MS in the individual patient is an important issue and would allow more personalized therapies for MS.

The purpose of this thesis work was to identify reliable blood biomarkers that could distinguish between MS patients and healthy controls and reflect pathophysiological differences in MS clinical forms.

<u>Specific Aims:</u>

I. Identify blood biomarkers that discriminate between MS patients and healthy controls.

II. Characterize the expression of DPP4 in MS to test the hypothesis that this enzyme is an important feature in the pathophysiology of the disease, given the role of DPP4 as key chemokines immunoregulator and the organ-specific nature of MS.

III. Identify blood biomarkers that stratify MS patients according to their clinical form.

IV. Identify biomarkers related with type I interferon signaling pathways that could help to develop personalized strategies in MS and provide mechanistic insight of MS pathophysiology.

3. POPULATION OF STUDY AND METHODS

3.1. STUDY DESIGN

A cross-sectional and observational study was carried out in 182 MS patients and healthy controls. Two independent cohorts of MS patients diagnosed by McDonald's revised criteria [106, 108] by the same neurologist at the Neurology Service in the University General Hospital Gregorio Marañón were included. Patients were informed about the study and recruited by their hospital neurologist. The only patients who were hospitalized at the moment of sampling were those undergoing a clinical relapse, the rest were consecutively appointed in the Neurology Service to participate in this observational study. The majority of patients with a progressive and more disabling stage of the disease were recruited from the Center Alicia Koplowitz for Multiple Sclerosis of the Community of Madrid, where they and their families were informed and gave written consent to participate in the study. The group of sex- and age-matched healthy controls came from the Blood Donor Bank of the University General Hospital Gregorio Marañón. This project was approved by University Hospital Gregorio Marañón Ethics Committee in Madrid and all subjects gave written informed consent.

There were two independent cohorts of patients and healthy controls studied, with the objective to find reproducible differences which correlate with a defined clinical outcome and to minimise the influence of experimental bias, overfitting and stastitical chance:

- Cohort 1: the Pilot cohort was recruited from November 2010 to February 2011 and consisted of 81 subjects: 65 MS patients (47 women and 18 men) and 16 HC (9 women and 7 men), adjusted by age and sex.
- Cohort 2: the Validation cohort was recruited from June 2011 to June 2012 and consisted of 101 subjects: 64 MS patients (38 women and 26 men) and 37 HC (20 women and 17 men), adjusted by age and sex.

The Validation cohort was designed and used to confirm the findings in the cohort 1 and to increase the number of patients in the different clinical MS groups, incrementing the statistical power of the results.

The total number of subjects included in this study was of 182 subjects: 129 MS patients (85 women and 44 men) and 53 healthy controls (29 women and 24 men).

The experiments were divided in three main parts:

1) Screening of plasma analytes that are differentially expressed in all MS patients as a whole with respect to healthy controls.
2) Screening of plasma analytes that are differentially expressed in the different clinical forms of MS and that can discriminate between inflammatory and neurodegenerative.
3) Study of the genetic expression of a vast array of different genes related with MS pathogenesis and the interferon signature in the different clinical forms of MS.

Functional experiments with freshly isolated peripheral blood mononuclear cells (PBMCs) and additional flow cytometry analysis were performed so as to go further in the research of the biomarkers described.

Due to the number of putative biomarkers proposed in each section of the results, the results of each biomarker and its known role in MS pathogenesis will be shortly introduced in the results section so as to facilitate the comprehension of the study.

3.2. SUBJECTS AND PERIOD OF STUDY

The study included 182 subjects: a total of 129 (85 women and 44 men) patients with MS diagnosis defined by McDonald's revised criteria [106, 108] were consecutively recruited for this study from November 2010 to June 2012, at the Unit of Multiple Sclerosis of the University Hospital Gregorio Marañón and from Center Alicia Koplowitz for Multiple Sclerosis of the Community of Madrid. A group of 53 sex- and age-matched healthy controls (HC) (29 women/ 24 men; age: 37 (31-43) (median years: 25th-75th percentiles) from the Blood Donor Bank of the University Hospital Gregorio Marañón was also included.

Patients were characterized based on the 7 defined groups of MS patients:

- Recurrent-remitting MS patients in remission (RR-MS Remission).
- RR-MS patients with active disease (RR-MS Active).

- Patients at clinical exacerbation or relapse (RELAPSES).
- RR-MS patients under treatment with IFNβ for a minimum of 6 months defined as long-term IFNβ responders (RESPONDERS).
- RR-MS patients that had not previously responded to IFNβ (NON-RESPONDERS).
- Secondary-progressive MS patients (SP-MS).
- Primary-progressive MS patients (PP-MS).

In the RESPONDERS group, response to IFNβ treatment was defined as the absence of relapses or progression (increase in EDSS score) during the time of treatment with IFNβ. All the patients included in this study as RESPONDERS have been followed-up in our institution and they continue with IFNβ therapy and clinically stable. Samples were drawn at 36-48 hours after injection of IFNβ. NON RESPONDERS were defined as RR-MS patients that suffered relapses or increased their progression (EDSS) during the period when they were treated with IFNβ and and were going to start other disease-modifying therapy (DMT).

In order to evaluate the effects of IFNβ ex vivo after the first injection of IFNβ, a small subset of four RR-MS Active patients who started therapy with IFNβ, were studied before, after 10 hours, after 32 hours and after 1 month (post 10 hours from injection).

Except for the long-term IFNβ RESPONDERS group and the group of four patients previously mentioned, the rest of the patients had not received any glucocorticoid treatment, immunosuppressive or DMT before blood sampling for this study in the three months prior to study entry.

Most of the patients of the RR-MS Remission group were benign forms of MS (17 out of 23) that had been symptom-free for years.

Relapse was defined as the appearance or reappearance of one or more neurological abnormalities that persisted for at least 24h and which had been preceded by at least 30 days of stable or improved neurological state, without any underlying infectious disease, blood samples were drawn from relapse patients before initiation of glucocorticoids treatment. RR-MS Active patients

were defined as patients diagnosed with RR-MS that were eligible to start a DMT because they had clinical and paraclinical activity of the disease and they were studied at least 1 month after the end of a clinical exacerbation. Clinical disease severity and disability were scored according to the Kurtzke Expanded Disability Status Scale (EDSS). For detailed patient characteristics, see Table 6 of the results section.

3.3. CLINICAL VARIABLES STUDIED

Clinical variables included from each patient were the following (Table 6): Epidemiological variables such as age, sex, disease duration and age at onset of the disease.

Clinical disease progression was evaluated by the Expanded Disability Status Scale (EDSS) [99] at the study point. The progression index was determined as disability degree divided by the duration of the disease (EDSS/disease duration in years).

Clinical disease activity was determined by calculating the number of relapses in the preceding two years.

The patients in the groups of RESPONDERS to IFNβ (n=20) were the only group under treatment with disease modifying therapies (DMT) and their treatment duration when samples were drawn are depicted in Table 6. Eleven patients were under treatment with IFNβ-1b (Betaseron®) and nine with IFNβ-1a (4 with Avonex® and 5 with Rebif®).

3.4. LABORATORY TECHNIQUES

3.4.1. Extraction and Blood Samples Processing

Peripheral blood samples were taken by venous puncture and collected in sterile EDTA Vacutainers, heparin and serum dried tubes between 8:00 and 10:00 a.m., and they were processed within 2 hours after extraction. The collected blood was allowed to clot for 30 minutes before separation. Serum (from dry tubes) and plasma samples (from EDTA tubes) were obtained after high speed centrifugation for 10 minutes at 3500-4000 rpm. Plasma samples were immediately aliquoted and frozen at -80ºC for its conservation. Anticoagulated blood from the EDTA tubes was used for flow-cytometry and for RNA extraction of their peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from MS patients and healthy donors by density gradient centrifugation using Ficoll- Paque (Amersham Biosciences, RU) and then washed with phosphate buffered saline (PBS).

3.4.2. Extraction and Processing of CSF

The extraction of 1 to 3 ml of CSF was performed by lumbar puncture in those patients undergoing clinical relapse. CSF was processed immediately after spinal tap, centrifuged after collection and the CSF supernatant was stored at -80ºC until it was used.

3.4.3. Multi-analyte Profiling

The Luminex xMAP® technology platform was used for the simultaneous measurement of multiple plasma analytes in our population study. Plasma samples were previously clarified by high-speed centrifugation and the commercially available Human cytokine 30-Plex panel (Invitrogen) was performed. The current investigation required the assembly of an extensive multiplex array consisting of cytokines, chemokines, soluble receptors, growth and angiogenic factors, which were evaluated using bead-based immunoassays. The following cytokines, chemokines and growth factors were studied: IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-

13, IL-15, IL-17, TNFα, IFNα, IFNγ, GM-CSF, MIP-1α, MIP-1β, IP10, Mig, Eotaxin, Rantes, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF.

This analysis of a broad spectrum of biomarkers enables a comprehensive analysis of an individual's immunological state. The xMAP® technology combines the efficiency of the simultaneous analysis of diverse proteins with a similar reproducibility to ELISA. In this technology each analyte-specific assay is assigned a microsphere set labeled with a specific fluorescence signature. Beads, with defined spectral properties, conjugated to protein-specific capture antibodies, are added along with samples and standards into wells of a filter-bottom plate. Washings are performed by aspiration with gentle vacuum. Protein present in samples binds to the capture antibodies conjugated to the beads; later protein specific biotinylated detector antibodies bind to the appropriate proteins and finally streptavidin conjugated with a fluorochrome (R-Phycoerithrin) binds to the biotinylated detector. The analysis with the Luminex detection system reveals an amount of fluorescence signature that allows the determination of the concentration of the different proteins studied. Human samples were diluted by half in this technique. The 96 well plates analyzed included samples from all clinical groups of MS and healthy controls to minimize inter-assay variation. The inter-assay and intra-assay values are reported to be less than 15% by the manufacturer. For data analysis and presentation, values that were below the lowest concentration detected in the standard curve were replaced with a value that was half of the lowest value measured or detection limit. On the other hand, values that their mean relative fluorescence was higher than the highest point of the standard curve were replaced by a value that was two-fold the measurement of the highest concentration detected in the standard curve. The lower detection limits expressed in (pg/mL) were the following: VEGF (3.8); IL-1b (20.1); GCSF (71.2); EGF (23.7); IL-10 (17.03); HGF (119.5); FGFb (9.4); IFNα (16.2); IL-6 (7.4); IL-12 (10.4); Rantes (14.2); Eotaxin (4.9); IL-13 (38.4); IL-15 (33.9); IL-17 (76.1); MIP-1α (15.4); GM-CSF (61.6): MIP-1β (13.4); MCP-1 (6.5); IL-5 (6.4); IFNɣ (25.2); TNFα (5.8); IL-1RA (36.5); IL-2 (13.2); IL-7 (26.4); IP10 (6.5); IL-2R (48.8); Mig (44.8); IL-4 (41.2) and IL-8 (8.2).

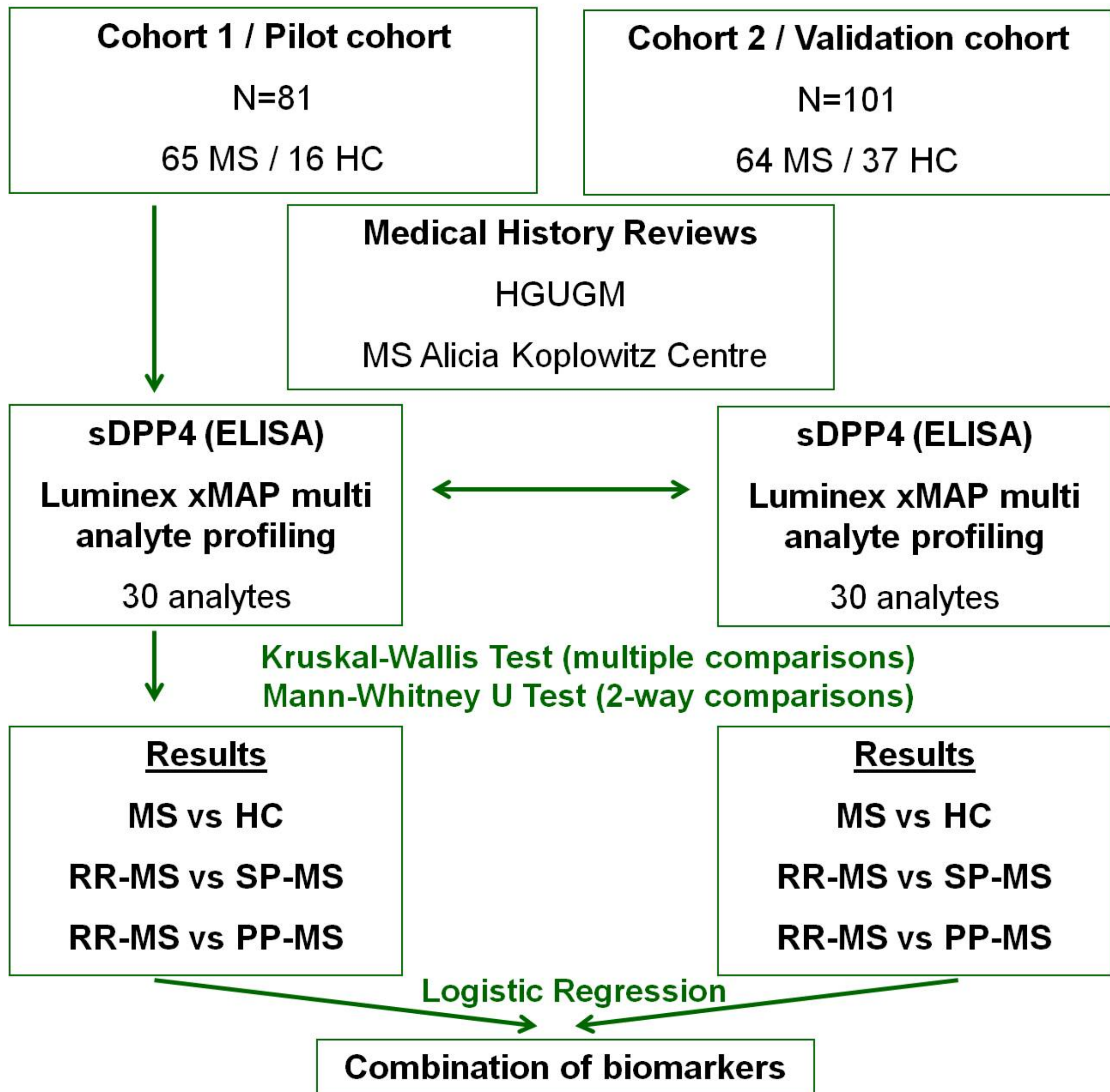

Figure 7 Working plan for the evaluation of plasma biomarkers and elaboration of a combination of biomarkers with the results of the subjects included in the study. HGUGM: Hospital General Universitario Gregorio Marañón.

3.4.4. Enzyme-linked Immunosorbent Assays

3.4.4.1. ELISA-sDPP4

The levels of sDPP4 were measured with the commercial sandwich ELISA (human DPPIV/CD26 Duo Set ELISA. R&D Systems) following the supplier's instructions. Briefly, Maxisorp plates (Nunc) were coated with 2 µg/ml of the commercial capture antibody in PBS overnight at room temperature. The wells were saturated with 300 µl of 1% in PBS for 1 hour. A seven-point standard curve was performed with the highest concentration at 2,000 pg/ml. Human plasma samples were used at dilution 1/800 while cell culture supernatants at dilution 1/2 in 1% of bovine serum albumin (BSA) in PBS. Plates were read in a Labsystems Multiskan MS (Thermo) device at a wavelength of 450 nm.

3.4.4.2. ELISA-IP10/ CXCL10

The levels of IP10/CXCL10 present in different samples were measured with a homemade sandwich ELISA. Briefly, Maxisorp plates (Nunc) were coated with 2 µg/mL of the capture antibody in PBS overnight at room temperature. Plates were washed twice with PBS. The wells were saturated with 200 µL of saturation buffer (PBS-Tween 0.05%-1% BSA) for 1 hour. The rest of the washings were done with PBS-Tween 0.05%. The secondary antibody was utilized at a concentration of 0.5 µg/mL. Detection limit was 31.25 pg/mL. Samples were diluted to 1/3 in saturation buffer PBS-Tween 0.05%-1% BSA. Plates were read in a Labsystems Multiskan MS (Thermo) device at a wavelength of 450 nm.

3.4.4.3. ELISA-IFNα

The levels of human IFN-α present in cell culture supernatants after 24 hours were detected with the commercial sandwich immunoassay Verikine^{TM} Human IFN-α ELISA Kit (PBL Interferon Source, NJ, USA) following the manufacters instructions. Two different six-point standard curves were performed. The high sensitivity standard curve: 12.5–500 pg/mL and the Extended Range curve: 156–5,000 pg/mL. Cell cultures were used undiluted and plates were read in an Opsys MR^{TM} Dynex technologies device at a wavelength of 450 nm.

3.4.5. Analysis of DPP Activity

The dipeptidylpeptidase (DPP) activity present in patient plasma was measured using a luciferase-based assay (DPP4-Glo™ protease Assay; Promega), following the manufacter's instructions. This assay provides a luminogenic DPP substrate, Gly-Pro-aminoluciferin. After cleavage of the proximal two amino acids from the substrate, the aminoluciferin is free to engage luciferase. Relative luminescent units (RLU) are proportional to the DPP activity. This assay was performed in all plasma samples, serially diluted between 0.025%, 0.25% and 2.5% in 10 mm Tris–HCL pH 8 with 0.1% prionex stabilizer. Plates were read using the Tristar LB941 device (Berthold Technologies, Oak Ridge, TN, USA). The DPP activity is expressed as relative luminescent units (RLU) as results were relative to the background of the technique. A dilution of 0.025% gave values that were within the linear range of the assay.

3.4.6. Sex Hormones Detection

Serum cortisol was quantified by competitive immunoassay (IMMULITE 2000, Diagnostic Products Corporation, Los Angeles, CA, USA). Serum testosterone, estradiol and progesterone were determined by a chemiluminescent immunoassay (Immuno I, Bayer Leverkusen, Germany), as previously described [35].

3.4.7. 25-Hydroxi Vitamin D Quantitation

The concentration of 25-hydroxi vitamin D present in serum was measured by chemiluminescent immunoassay (LIAISON® 25 OH vitamin D TOTAL Assay, DiaSorin Inc, MN, USA), following manufacturer's instructions.

3.4.8. RNA Extraction

PBMCs were lysed in 1 ml of Trizol (TRIzol® Reagent, Invitrogen), very well resuspended with pipette, and stored at -80ºC until use. They were thawed once, vortexed and centrifuged to obtain the aqueous phase. Most of the RNA samples were extracted with columns of the commercial kit RNeasy Mini Kit (QIAGEN, CA, USA) and some of the samples with a number of PBMCs < 1 million were extracted with RNeasy Micro Kit (QIAGEN, CA, USA). Most RNA

concentrations were superior to 100 ng/µL (median [25th-75th] 186.9 [129.6-246.5]) and the absorbance coefficient 260/280 was between 1.8 and 2. RNA in nuclease-free water was stored at -80ºC.

12 µL of RNA (>1µg) was reversed transcribed to complementary DNA (cDNA) with the reverse transcription enzyme RevertAid™ Premium Reverse Transcriptase (Fermentas Life Sciences) following the manufacter's instructions with random hexamer primers. cDNA samples were stored at -30ºC until use.

3.4.9. Gene Expression by Biomark™ HD System

In our study population, gene expression studies were performed with the BioMark™ System (Fluidigm) that enables microarray-like studies using high-throughput real-time qPCR by performing more than 9,000 qPCR reactions in a single assay. This technology allows the performance of 96 different primers * 96 different samples or 48 different primers * 48 different samples generating a large number of data within one technique. Advantages over conventional array based transcriptional profiling profiling are that the Biomark has a CV <5% as compared to 25% to 35% for microarrays.

Samples and primers distribute across 48 or 96 reaction chambers in 9 or 6 nL aliquots, respectively. The primers utilized in these techniques were related with type I interferon signaling pathway and multiple sclerosis pathogenesis (list of genes in Appendix 1). In each Biomark experiment, there were several references or housekeeping genes employed: GUS (β-glucuronidase), GADPH (glyceraldehyde-3-phosphate dehydrogenase), ACTB (beta-actin), HPRT (hypoxanthine-guanine phosphoribosyltransferase), and TBP (TATA box-binding protein). cDNA samples were diluted to 1/10 in TE Buffer and 2.7 µL of cDNA were added to a Sample pre-mix of 0.3 µL GE Sample loading reagent (20x) (Fluidigm, PN 85000746) and 3 µL of TaqMan® Universal PCR Master Mix (2x) (Applied Biosystems, PN 4304437). Biomark arrays were loaded as follows. The assay mix consisted of 3 µL of the primer TaqMan® Gene expression assay (20x) and 3 µL of Assay Loading Reagent (2x) (Fluidigm, PN 85000736). After the loading of the chip assay inlets were loaded with 5 µL of assay mix and sample inlets with 5 µL of sample mix.

In order to dissect the unique molecular signature of type I interferon response in MS patients using high throughput microarray technology, we focused on up to 96 top up- and down-regulated genes to discriminate among MS clinical forms.

Primers and probes of the assays were designed by Thermo Scientific Solaris (Solaris qPCR Gene Expression Assays). An image of the 96*96 Biomark chip with an amplification of the reaction chambers is shown below (Figure 8).

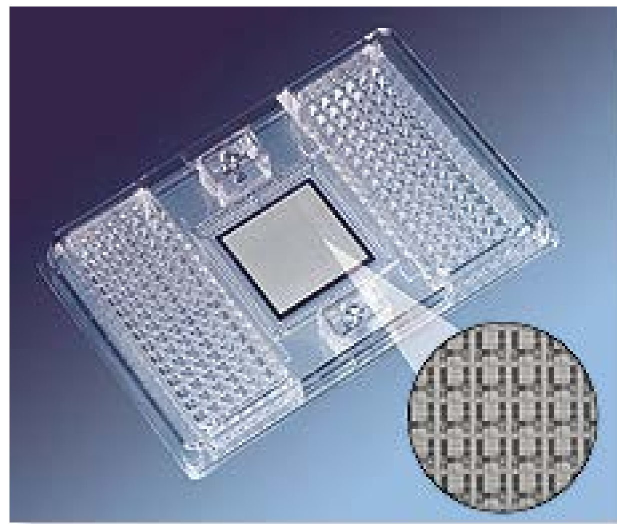

Figure 8 Dynamic Array^{TM} 96*96 IFC © Fluidigm Corporation

The relative level of gene expression for each sample was calculated using the $2^{-\Delta\Delta CT}$ method [354].

$$\Delta\Delta Ct = (C_t \text{ target gene} - C_t \text{ reference gene})_{sample} - (C_t \text{ target gene} - C_t \text{ reference gene})_{calibrator}$$

In the experiments with stimulated cultured PBMCs, an endogenous calibrator (unstimulated control) and a reference gene (normally GUS or ACTB) were used to normalize the amounts of target gene. In the experiments with untreated baseline samples from patients, these were used as calibrators and the gene ACTB was used as reference gene to normalize the amounts of target gene. Finally, those assays with ex vivo PBMCs, the reference gene to normalize the results was ACTB and the calibrator was the mean of the threshold cycles (C_t) of the target and reference genes in the group of healthy controls, similarly as described in other studies [355]. The relative quantitative value for each sample was expressed as $2^{-\Delta\Delta CT}$ method, representing a fold

change in gene expression normalized to the endogenous reference gene and relative to its respective calibrator.

3.4.10. Vitamin D Stimulation

In a subet of MS patients (n=8) and healthy donors (n=8), $2*10^6$ PBMCs were cultured in complete medium (RPMI 1640 containing 10% fetal calf serum (FCS), streptomycin 50 U/ml, penicillin 50 U/ml and L-glutamine 2 mM) supplemented with $1,25(OH)_2$ vitamin D_3 (Sigma-Aldrich) (100nM), in 6 well flat bottom plates. Cells were cultured for 24 hours and 5 days. At 24 hours PBMCs were lysed in 1 ml of trizol. After 5 days, supernatants were collected. As the reagent $1,25(OH)_2$ vitamin D_3 was diluted in ethanol 99%, in those wells without $1,25(OH)_2$ vitamin D_3, the same volume of ethanol 99% was added.

3.4.11. Whole Blood Experiments

Whole blood experiments were performed to evaluate the *in vitro* production of IP10, in response to the stimulation with IFN, in two RR-MS male patients, two male PP-MS patients and 4 male healthy controls. Blood from heparinized tubes was diluted 1:1 with RPMI supplemented with glutamine. Blood was distributed in 24 well flat plates (1ml/well). The following reagents were added to whole blood: IFN-α: 1000 IU/ml and 250 IU/ml; IFNβ-1a: 1000 IU/ml and 250 IU/ml; IFN-ɣ: 1000 IU/ml; TNF-α: 20 ng/ml and no stimulus. TNF-α was used as negative control of the assay and IFN-ɣ as positive control. 100 µl of blood from each well were collected in tubes for posterior flow cytometry. Tubes and plates were incubated overnight at 37ºC and 5% of CO_2. The following day supernatants were collected from the plates for later ELISA experiments. The protein transport inhibitor, containing Brefeldin A, GolgiPlug™ (BD Biosciences) was added to the tubes, according to the manufacter's instructions and left in the incubator for 5 hours. The staining for flow cytometry is explained in the next section.

3.4.12. Multiparametric Flow-Cytometry

We studied different relevant marker expression antigens on the surface of cells from whole blood in order to preserve the original composition of mononuclear cells.

3.4.12.1. Characterization and Quantitative Analysis of IP10 Intracellular Expression

Whole blood (100 µL) was directly stained with the fluorochrome-conjugated monoclonal antibodies (mAbs): CD3 FITC, CD16/CD56PerCP and CD14APC (BD Immunocytometry Systems, San Jose, CA, USA). A tube of blood without staining was used to establish appropriate settings. Blood directly stained with the described mAbs was for 20 min at room temperature in the dark, lysed for 10 minutes and then washed with the solution Perm/Wash^{TM} (BD Biosciences) (1600 rpm for 5 minutes), for permeabilization. Cells were stained intracellulary with the fluorochrome-conjugated mAB IP10 PE and with the human FcR Blocking Reagent (Miltenyi Biotec) in order to increase specificity of the staining. After an incubation of 20 minutes at 4ºC in the dark, and two washes with Perm/Wash^{TM} solution, cells were acquired in a FACSort flow cytometer (Becton Dickinson) and analysis was performed with the flow cytometry analysis Software, FlowJo (TreeStar, Inc, Ashland, OR, USA). The intracellular expression of IP10 was restricted to the population of monocites ($CD14^{+}$). Briefly, lymphocytes and monocytes were gated according to forward and side scatter. 20,000 events were acquired. Monocytes were characterized as $CD14^{+}CD3^{-}$ and the frequency of monocytes with intracellular expression of IP10 was measured. The condition of “no stimulus” was utilized to set the markers.

3.4.12.2. Characterization and Quantitative analysis of $DPP4^{hi}$ and $CXCR3^{+}$ T cell and NK Subsets

Whole blood (100 µL) samples from EDTA Vacutainers were labeled by direct staining with the appropriate fluorochrome-conjugated monoclonal antibodies (mAbs) and isotopic controls for 20 min at room temperature and then lysed and washed, as previously described [146]. Direct conjugated mAbs: CD3-FITC, CD26-PE (DPP4), CD4, CD8, CD16 and CD56 PerCP and CXCR3 APC from

Becton Dickinson (BD Immunocytometry Systems, San Jose, CA, USA). Acquisition was performed in a FACSort flow cytometer (Becton Dickinson), and analysis was performed with the flow cytometry analysis Software, FlowJo (TreeStar, Inc, Ashland, OR, USA).

Lymphocytes were gated according to forward and side scatter and a total of 20,000 events were acquired. We observed three subsets of $CD3^{+}DPP4^{+}$ cells, with low, medium and high expression of DPP4 (Figure 9).

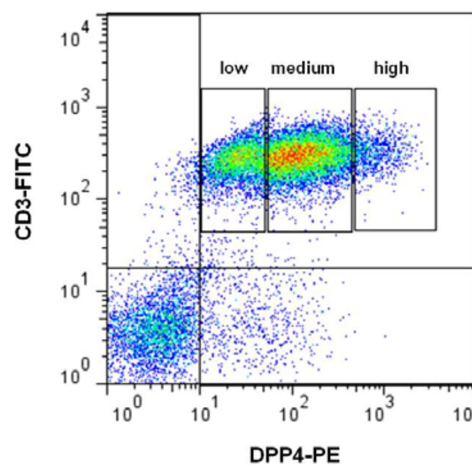

Figure 9 Subsets of $CD3^{+}DPP4^{+}$ with low, medium and high expression of DPP4.

Krakauer *et al.* [281] described the subset of $CD4^{+}DPP4^{hi}$ as memory T cells ($CD45RO^{+}$), with high expression of Th1 chemokines and cytokines, including CXCR3. We checked that our subsets of T cells $DPP4^{hi}$ were $CD45RO^{+}$ (Figure 10A), and as described by Krakauer *et al.* we observed that $DPP4^{hi}$ subsets coexpressed largely (>80%) CXCR3 (Figure 10B).

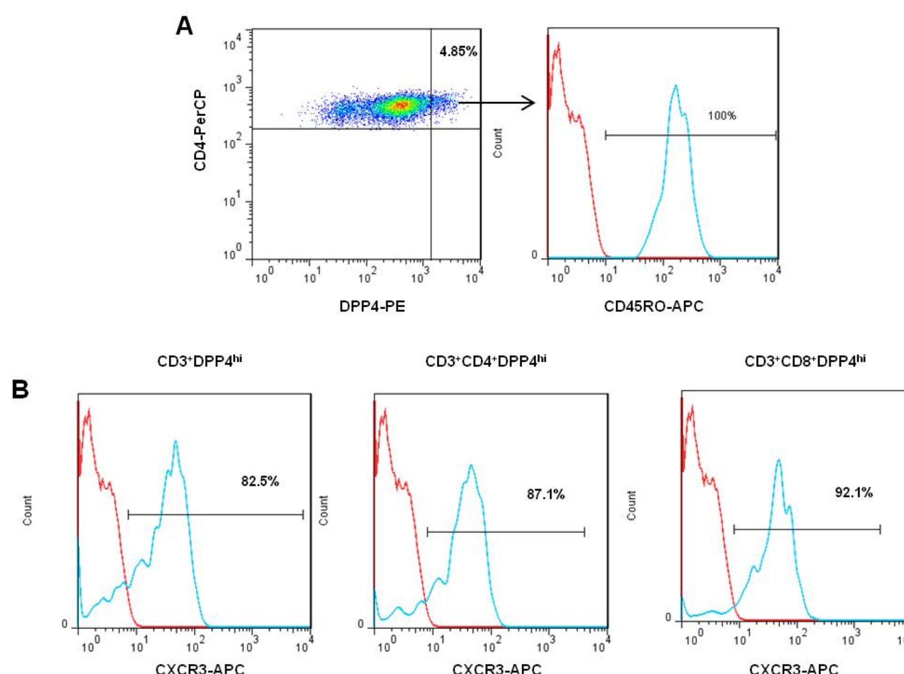

Figure 10 **(A)** Expression of CD45RO in the subset $CD3^{+}CD4^{+}DPP4^{hi}$. **(B)** Expression of CXCR3 in the different T cell subsets studied.

Therefore we focused our attention on $DPP4^{hi}$ T cells, a memory T cell subset with Th1 characteristic molecules [281]. Briefly, lymphocytes were gated according to forward and side scatter. CD3 positive lymphocytes were selected and a gate was set according to DPP4 high ($DPP4^{hi}$) expression on the $CD3^{+}$ population, and we measured the frequency as a percentage of $CD3^{+}$ lymphocytes and the mean intensity fluorescence (MFI) of DPP4 in this subset. Similarly, $DPP4^{hi}$ subsets of $CD4^{+}$, $CD8^{+}$ T cells and $CD16^{+}CD56^{+}$ Natural Killer (NK) cells were analyzed as described on $CD3^{+}$ lymphocytes. An alternative plot to DPP4 was done with CXCR3, to analyze $CXCR3^{+}$ $CD3^{+}$, $CD4^{+}$, $CD8^{+}$ T cells and $CD16^{+}CD56^{+}$ NK cells (Figure 11).

Tubes	FITC	PE	PerCP	APC	Subsets
1	anti-IgG_1	anti-IgG_{1k}	anti-IgG_1	anti-IgG_1	Isotipic control
2	anti-CD3	anti-DPP4	anti-CD4	anti-CXCR3	T $CD4^{+}$
3	anti-CD3	anti- DPP4	anti-CD8	anti-CXCR3	T $CD8^{+}$
4	anti-CD3	anti- DPP4	anti-CD16+ anti-CD56	anti-CXCR3	NK cells

Table 4 Monoclonal antibodies employed for the immunophenotyping of $DPP4^{hi}$ and $CXCR3^{+}$ T cell and NK subsets.

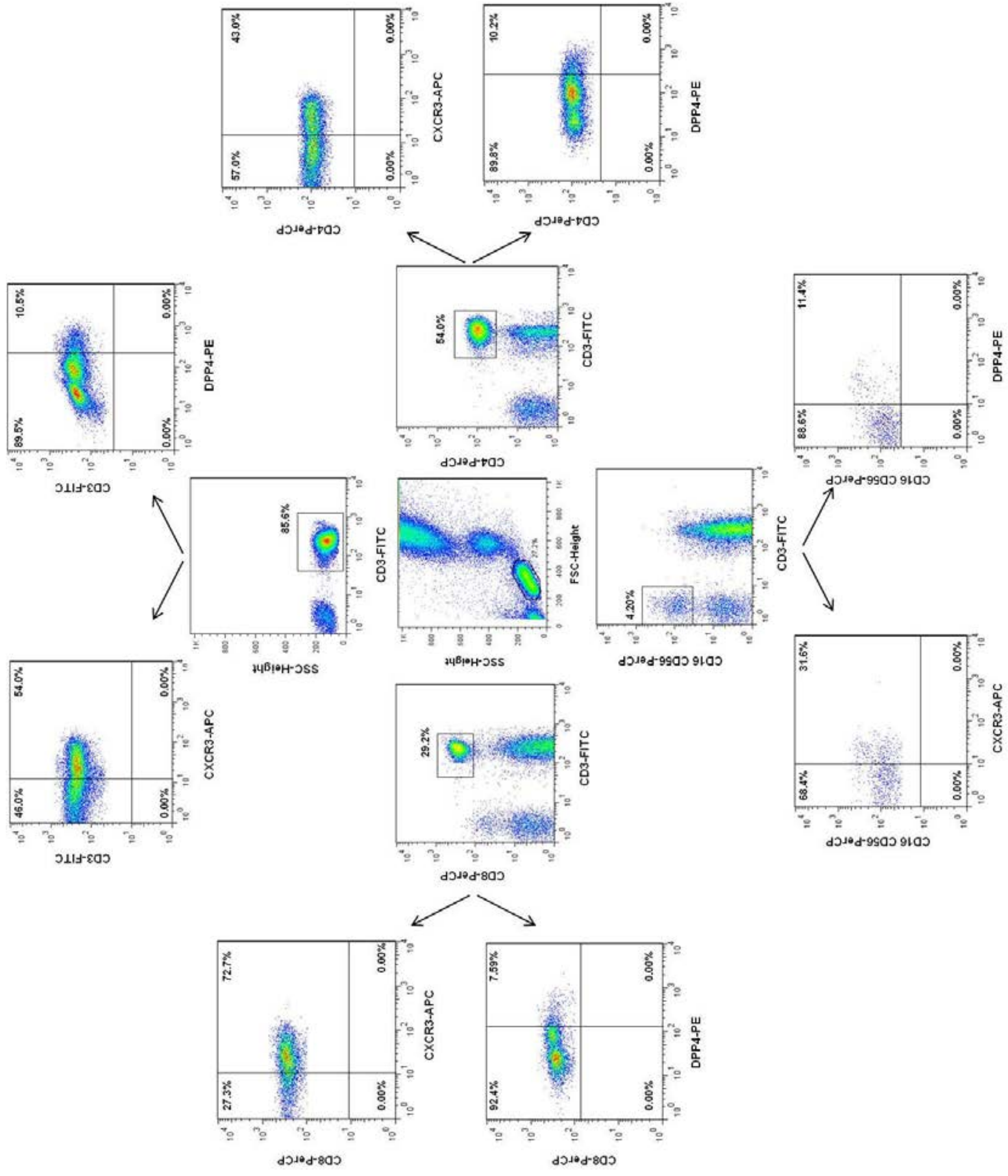

Figure 11 Gating strategy for the characterization of DPP4^{hi} and CXCR3^{+} positive subsets of CD3^{+}, CD4^{+}, CD8^{+} and CD16^{+}CD56^{+} cells.

3.4.13. Proliferation Assays

PBMCs were incubated with different stimuli and their proliferations were evaluated by [^{3}H] thymidine incorporation assays and by flow cytometry by Carboxyfluorescein succinimidyl ester (CFSE). A total of 100,000 PBMCs were seeded per well in 96*96 round bottom plates.

[^{3}H] Thymidine Incorporation

After 5 days of cell culture, cells were pulsed with 1µCi/well of tritiated thymidine (^{3}H-thymidine) and were incubated overnight. Afterwards, cells were filtered by an automatic Filter (Tomtec) and incorporated radioactivity was measured in a scintillation counter (Microbeta Trilux, Wallac, Finland). Proliferation was calculated as the geometric mean of the counts per minute (c.p.m).

Carboxyfluorescein Succinimidyl Ester

PBMCs were resuspended in PBS for the staining with CFSE. They were incubated with CFSE (1µM) for 10 minutes at 37ºC with continuous shaking every minute. PBMCs stained with CFSE were cultured *in vitro* with the appropriate stimulus (Table 5) and after 5 days of culture they were stained with mAbs (CD25 PE) and (CD4, CD8 PerCP). The proliferation of different cell subsets was observed by flow-cytometry with this method.

Stimulus	Concentration	Company
soluble anti-CD3	1 µg/ml	Becton Dickinson Co, NJ, USA
soluble anti-CD28	1 µg/ml	Becton Dickinson Co, NJ, USA
phytohemagglutinin (PHA)	2 µg/ml	Sigma-Aldrich®
PMA	40 ng/ml	Sigma-Aldrich®
Ionomycin	2,000 ng/ml	Sigma-Aldrich®
Poly (I:C)	25 µg/ml	Invivogen
Poly (A:U)	50 µg/ml	Invivogen
IFNβ-1a	1,000 IU/ml or 250 IU/ml	Rebif®, Merck Co
IFNα	1,000 IU/ml or 250 IU/ml	Intron® A, Merck Co
IFNɣ	1,000 IU/ml	Immukin®, Boehringer Ingelheim
1,25(OH)$_2$ vitamin D$_3$	100 nM	Sigma-Aldrich®

Table 5 Reagents and concentrations employed in functional assays.

3.5. STATISTICS

3.5.1. Variable Distribution

3.5.1.1. Kolmogorov-Smirnov Test

This test was employed in order to test the goodness of fit for testing the normality of the samples distribution. This test compares the cumulative distribution function observed in a variable with a theoretical normal distribution. The Z of the Kolmogorov-Smirnov test quantifies the distance (in absolute value) between the empirical and the theoretical cumulative distribution functions [356].

3.5.2. Contingency Tables

3.5.2.1. Pearson's chi-squared Test

Pearson's chi-squared test (χ2) measures the association (contingency) between two variables qualitative or categorical. In this test, it is contrasted if the variables are independent or not [357].

3.5.2.2. Fisher's Exact Test

This test is a variant of Pearson's chi-squared test for 2*2 contingency tables that include the Yates' continuity correction. It is used as an alternative to Pearson's chi-squared test when sample sizes are small. This test measures the association (contingency) between two variables qualitative or categorical when simple sizes are small [357].

3.5.3. Survival Analysis

It is a group of statistical techniques that study a subject for a certain time when it is observed the presence or absence of a determined event. The objective is to describe the probabilities of that event to happen and the evolution of the incidence of that event along time [358].

3.5.4. Parametric Tests

3.5.4.1. Student's t-Test

The Student's t-test is used to contrast hypothesis about means in populations with a normal distribution. It also gives approximate results for the contrasts in means in samples large enough, when these samples are not normally distributed (though in this case, it is preferable to choose a non-parametric test). [356].

3.5.4.2. Pearson Linear Correlation Coefficient (r)

This coefficient allows the measurement of the strength of a linear association between two variables. This strength will be null (inexistent linear association), if $r=0$, increasing the strength of the association as the value of r is more close to -1 or to 1. The sign of the coefficient indicates if the correlation is direct (positive) or inverse (negative) [356].

3.5.5. Logistic Regression

The logistic regression model allows the association of quantitative and categorical data with binary responses. The dependent variable (Y) is a dichotomic variable (positive/negative) and the independent variables (X_i), one or several, can be quantitative and/or dichotomical with values 0 and 1. The parameters of the model are estimated by the method of maximal likeliness. The result of the logistic regression for each independent variable is expressed in Odds Ratio (O.R.), which is the factor that multiplies the ratio between the probability of a positive response of Y and the probability of a negative response of Y [359].

An OR value greater than 1, called *n,* and statistically significant ($p<0.05$) indicates that the probability that the response Y appears, is *n* times higher among those who present factor X, than among those who do not present it. When the value of OR is lower than 1 and statistically significant ($p<0.05$), the interpretation is the contrary and the probability is 1/*n* lower.

3.5.6. Non-parametric Tests

Non parametric statistical tests were used when the simple size was small or the distribution of the data in the population was free (data were not from normal populations and with the same variations) [356].

3.5.6.1. Kruskal-Wallis

The Kruskal- Wallis test is the non-parametric analogous method to the parametric test one-way analysis of variance (ANOVA). This method tests whether independent samples originate from the same population compares globally more than two samples or groups studied. If the Kruskal-Wallis is significant, at least one of the samples differs from the others. To identify the statistical differences between sample pairs or groups, the data were analysed by Mann-Whitney [356].

3.5.6.2. Mann-Whitney U Test

This test compares if two samples of two populations have the same or different distribution. This test is the non-parametric alternative to the Student's t-test. Observations from both samples are combined and classified with respect to the mean Rank assigned if values are equal [356].

3.5.6.3. Wilcoxon Rank-sum Test

This test, as Mann-Whitney test, compares the distribution of two samples. It is another alternative to the Student's t-test when the sample data do not follow a normal distribution [356].

3.5.6.4. Spearman's Rank Correlation Coefficient

This coefficient is a variant of Pearson linear correlation coefficient. It is a measure of the association between two variables and it is calculated from the assignation of ranks to ordered values. The association or correlation will be null if R=0, increasing if the value of R is closer to 1 or -1. The sign of the R coefficient indicates the sense of the association. It is direct is the sign is positive and inverse if the sign in negative [356].

4. RESULTS

4.1. DEMOGRAPHIC AND CLINICAL CHARACTERSITICS OF MS PATIENTS

MS patients were recruited during two consecutive years, as detailed in the materials and methods. In the total MS study population, 80 out of 129 (62%) patients had RR-MS distributed as follows: RR-MS Remission n=23, RR-MS Active n=13, RESPONDERS n=20, NON RESPONDERS n=13 and RELAPSES n=11 (for 6 was their first clinical episode (CIS), and they were later diagnosed as RR-MS), 31 were diagnosed as SP-MS and 18 as PP-MS. RR-MS patients and patients at relapse had similar ages, while progressive patients SP-MS and PP-MS were around 20 years older (Table 6). The lowest disease duration (median 1 year) was in the groups of RR-MS with active disease and patients at relapse. In RR-MS at remission and responders and non-responders to IFNβ, mean disease duration was about a decade and in the progressive forms (SP-MS and PP-MS) two decades. SP-MS and PP-MS had the highest EDSS compared to the other groups. EDSS in RR-MS Remission and in IFNβ RESPONDERS were the lowest (median, 0 and 1, respectively), as expected. The progression index (EDSS/Disease duration) was the highest in RR-MS Active patients, as they had been recently diagnosed and presented with considerable disability. As depicted in Table 6, the patients with highest number of relapses in the preceding two years where those under treatment with IFNβ (RESPONDERS) about to start therapy with a DMT (RR-MS Active and NON RESPONDERS).

Table 6 Epidemiological and clinical characteristics of multiple sclerosis patients included in the study.

Characteristics	CD-MS	RR-MS Remission	RR-MS Active	RELAPSES	RESPONDERS	NON RESPONDERS	SP-MS	PP-MS
No. of patients[a]	129	23	13	11	20	13	31	18
Gender F/M[a]	85/44	17/6	8/5	8/3	10/10	11/2	22/9	9/9
Age (years)[b]	42 (33-53)	38 (32-46)	29 (26-37)	30 (26-42)	37 (33-41)	38 (29-43)	53 (46-58)	58 (45-64)
Disease duration (yrs)[b]	11 (3-17.5)	9 (5-16)	1 (1-4.5)	1 (0-9)	9 (1.8-16.3)	11 (5-15.5)	19 (13-27)	20.5 (8.8-26.3)
Age at onset (yrs)[b]	28 (24-36)	28 (22-32)	26 (25-35)	28 (25-40)	26 (23-35)	26 (24-38)	32 (26-41)	40 (33-50)
EDSS[b]	2.5 (1.0-5.5)	0.0 (0.0-1.5)	2.5 (1.0-3.0)	3.0 (2.0-4.0)	1.0 (0.0-2.0)	3.5 (2.3-4.5)	8.0 (7.5-8.5)	8.0 (7.3-8.0)
Progression Index[c]	0.3 (0.1-0.6)	0.0 (0.0-0.1)	1.5 (0.2-2.9)	0.8 (0.2-5.4)	0.1 (0.0-0.3)	0.3 (0.2-0.5)	0.4 (0.3-0.6)	0.4 (0.3-0.9)
No. of relapses[d]								
0	73	17	0	6	2	0	30	18
1	18	4	3	3	4	3	1	0
2-5	38	2	10	2	14	10	0	0
Treatment, IFNβ[a]	20	–	–	–	20	–	–	–
Treatment duration (mo)[b]	18 (6-24)	–	–	–	18 (6-24)	–	–	–

CD-MS clinically definite MS group, RR-MS Remission: relapsing remitting MS in remission, RR-MS Active: relapsing remitting MS with active disease, RESPONDERS: RR-MS under treatment with IFNβ, NON-RESPONDERS: relapsing remitting MS that did not respond to Interferon-β, SP-MS: secondary progressive MS, PP-MS: primary progressive MS.
EDSS expanded disability status scale, IFNβ: interferon-β.
[a] Number of patients
[b] Median (25th-75th percentiles)
[c] EDSS/Disease duration (years)
[d] Number of relapses during preceding 2 years

4.2. CANDIDATE IMMUNOLOGICAL BIOMARKERS FOR MULTIPLE SCLEROSIS

4.2.1. Specific Biomarkers in Multiple Sclerosis Patients

In our cohort of MS patients, we found that the circulating plama levels of the analytes: sDPP4, IL-7, IL-1RA and Mig were significantly decreased in MS patients with respect to healthy controls. The circulating levels of the different analytes and the PBMCs collected for gene expression assays were measured in all patients at stable clinical conditions, except in those patients undergoing a clinical relapse (n=11). Only RR-MS patients under treatment with IFNβ (RESPONDERS) were receiving immunomodulatory treatment, the rest of patients included in the study were non-treated. Blood samples from MS patients and healthy donors included in this study were drawn at the same time point (between 8 and 10 a.m.).

4.2.1.1. DPP4 Expression and Activity in Multiple Sclerosis

Dipeptidylpeptidase 4 (DPP4, also known as CD26) is a multifunctional molecule that has been implicated in autoimmune pathophysiology with a putative immunoregulatory role. Membrane expression of DPP4 on T cells has been associated with MS clinical activity, but there is limited information about soluble (sDPP4) in MS. We sought to integrate disparate pieces of data and analyze the plasma levels of sDPP4, DPP activity and DPP4 surface expression on $CD3^+$, $CD4^+$, $CD8^+$ T-cells and $CD16^+CD56^+$ NK-cells in our 129 MS patients with different clinical forms and 53 healthy controls from two independent cohorts.

4.2.1.1.1 Validation of sDPP4 as MS Biomarker

The circulating levels of sDPP4 were measured in all patients at stable clinical conditions, except in those patients undergoing a clinical relapse (n=11).

The Pilot cohort of MS patients (cohort 1) presented significantly lower circulating levels of sDPP4 than healthy controls (p=0.01, Figure 12A). This finding was reproduced in the Validation cohort (cohort 2) (p=0.01, Figure 12B). As the only group of patients under treatment was the long-term IFNβ responders (RESPONDERS), this group was excluded from the analysis and

we compared the circulating levels of sDPP4 in the rest of MS patients without medication with the group of HC. In the Pilot cohort untreated MS patients presented lower levels of sDPP4 than HC (p=0.002, Fig. 12A), and in the Validation cohort MS patients presented a trend of lower levels of sDPP4 than HC (p=0.09, Fig. 12B). In the Validation cohort, most of the RR-MS patients at remission had higher levels of sDPP4 (77%: 7 out of 9, where 5 were benign forms of RR-MS) than the median in the group of healthy donors, which accounts for not achieving statistical significance in this comparison, indeed when RR-MS in remission were excluded, differences were statistically significant (p=0.02).

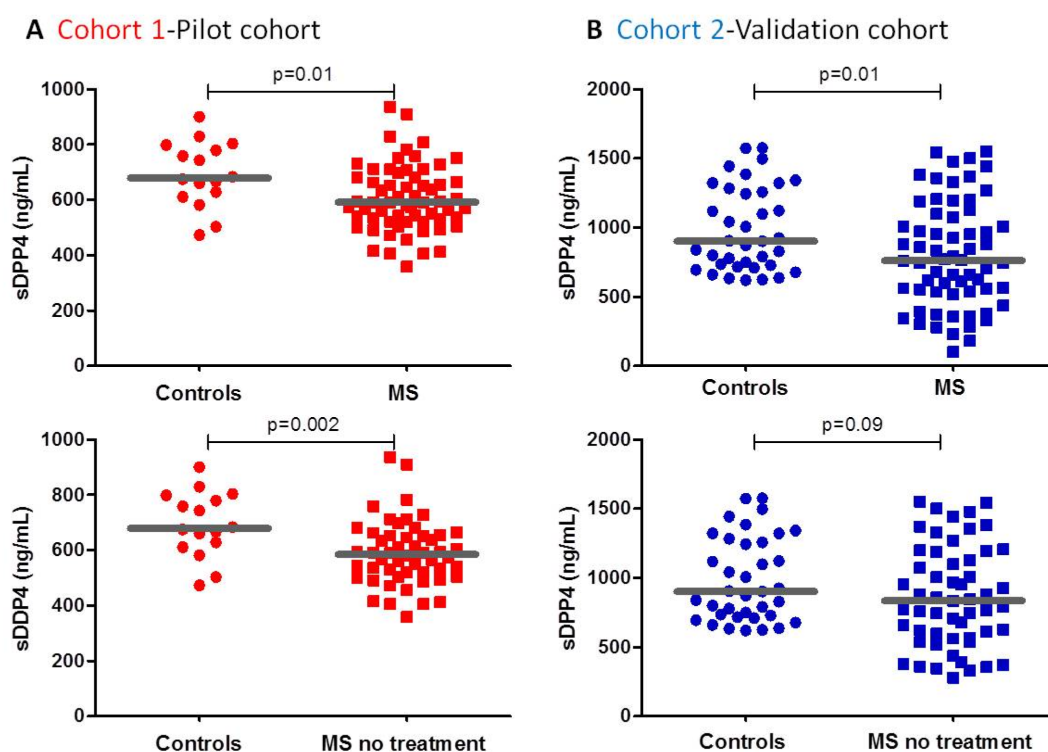

Figure 12 Comparison of sDPP4 between healthy controls (Controls, n=16) and multiple sclerosis patients (MS, n=65) or between healthy controls and MS patients when the group of patients undergoing treatment with Interferon-β was excluded (MS no treatment, n=54) in the Pilot cohort **(red, A)**. The analogous comparison was performed with the Validation cohort (Controls, n=37; multiple sclerosis patients, n=64; multiple sclerosis patients without treatment, n=55) **(blue, B)**. Mann- Whitney *U* test comparison, median and individual dots shown.

Given the intrinsic heterogeneity of MS, the two cohorts were pooled to increase the number of cases and controls. Again circulating levels of sDPP4 were

significantly lower in MS patients (705±300 vs 898±292 ng/ml, p<0.0001) and in untreated MS patients (731±304 vs 898±292 ng/ml, p<0.0001) as compared to healthy donors. The Receiver Operating Characteristic (ROC) curves indicated a good performance of plasma sDPP4 to discriminate between MS patients and controls (p<0.0001; area under the curve (AUC): 0.713) (Figure 13). A cut-off value of 622 ng/mL for plasma sDPP4 showed a sensivity of 48.0% and a specifity of 90.5%; positive predictive value (PPV) of 92.5% and a negative predictive value (NPV) of 41.7%. Concentrations of sDPP4 <622 ng/mL was a classifier for individuals with MS (p<0.0001 Fisher's Exact Test, 2-sided) with an Odds Ratio of 8.8 (95% confidence interval: 3.3 - 23.7). Importantly, this cut-off value was developed in the Pilot cohort (cohort 1) and could be accurately applied to the Validation cohort (cohort 2) (Table 7).

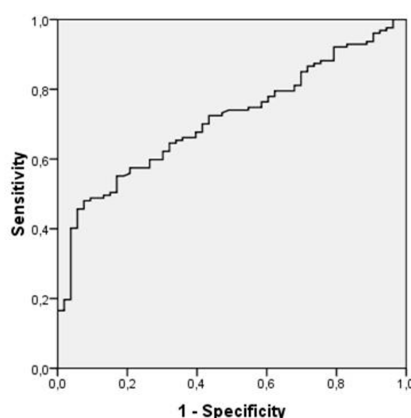

Figure 13 Receiver Operating Characteristic curve of plasma sDPP4 to discriminate between multiple sclerosis patients and healthy controls. AUC: 0.713 p<0.0001 (95% confidence interval: 0.638-0.788).

sDPP4 (ng/mL)	AUC	95% CI	p value	Cut-off	Sensitivity	Specificity	PPV	NPV
Pilot cohort	0.709	0.566-0.853	0.01	622	60.0%	75.0%	90.7%	31.6%
Validation cohort	0.648	0.542-0.753	0.01	622	37.5%	97.3%	96.0%	47.4%

Table 7 Clinical utility of sDPP4 as plasma MS biomarker.
AUC: Area under the curve, 95% CI: confidence interval, PPV: positive predictive value and NPV: negative predictive value.

Among the different MS clinical forms studied, there were differences in the sDPP4 levels (Figure 14A Kruskal-Wallis test, $p<0.0003$). In particular, we found that the groups of patients under treatment with IFNβ (RESPONDERS), RR-MS patients with active disease and SP-MS patients had the lowest levels of sDPP4 (RESPONDERS: M-W $p<0.0001$; RR-MS Act.: $p<0.001$; and SP-MS: $p<0.001$, respectively; comparisons made to healthy donors). Patients at relapse (RELAPSES, $p<0.02$) and those with PP-MS ($p<0.02$) also had significantly lower levels of sDPP4 than healthy controls (Figure 14A). By contrast, RR-MS patients in remission had similar levels of circulating sDPP4 to those of healthy controls. This was also observed if the two independent cohorts were analyzed separately. If the groups of RR-MS patients in remission and those with active disease, and recent clinical activity, that were about to start DMTs (RR-MS Rem. vs RR-MS Act.) were compared; RR-MS patients with active disease had significantly lower levels of sDPP4 than RR-MS patients in remission ($p=0.03$) and healthy controls ($p=0.0004$) (Figure 14B).

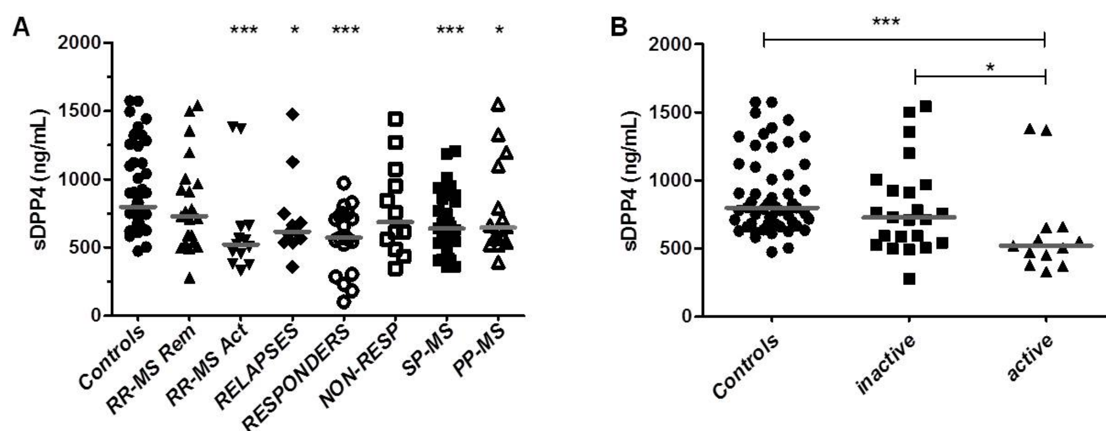

Figure 14 **(A)** Representation of the circulating levels of sDPP4 in the healthy control group (Controls, n=53) and in multiple sclerosis (MS) patients split by clinical forms: Recurrent-remitting MS patients in remission (RR-MS Rem, n=23), recurrent-remitting MS patients with active disease (RR-MS Act, n=13) patients at relapse (RELAPSES, n=11), long time Interferon-β responders (RESPONDERS, n=20), non responders to Interferon-β (NON-RESP., n=13), Secondary-progressive MS patients (SP-MS, n= 31), Primary-progressive MS patients (PP-MS, n=18) (Kruskal-Wallis test $p<0.0003$). The groups of RR-MS Act, RESPONDERS and SP-MS had significantly lower levels of sDPP4 than the healthy control group (***p=<0.001). The groups of RELAPSES and PP-MS had also significantly lower levels of sDPP4 than the healthy control group (*p=<0.02).
(B) Comparison between healthy controls (Controls, n=53) and the group of RR-MS split by inactive: those in continued clinical remission and non treated (inactive, n=23) and active: those with recent clinical activity and about to start disease modifying therapies (active, n=13). RR-MS active patients had significantly lower circulating levels of sDPP4 than healthy controls (***p=0.0004) and than RR-MS inactive (*p=0.03).

When MS patients and HC were analyzed according to sex, we observed a higher concentration of sDPP4 in healthy men than in healthy women (p=0.01). However, these sex differences in healthy controls were lost in MS patients, what favors its use as a biomarker. This lack of differences in the levels of sDPP4 between MS men and women persisted if the group of patients on treatment was excluded. Healthy men had statistically higher levels of sDPP4 than MS men (p<0.001) and similarly, healthy women showed higher sDPP4 than MS women (p<0.01) (Figure 15). These differences were also seen if the group on treatment with IFNβ was excluded from the group of MS patients.

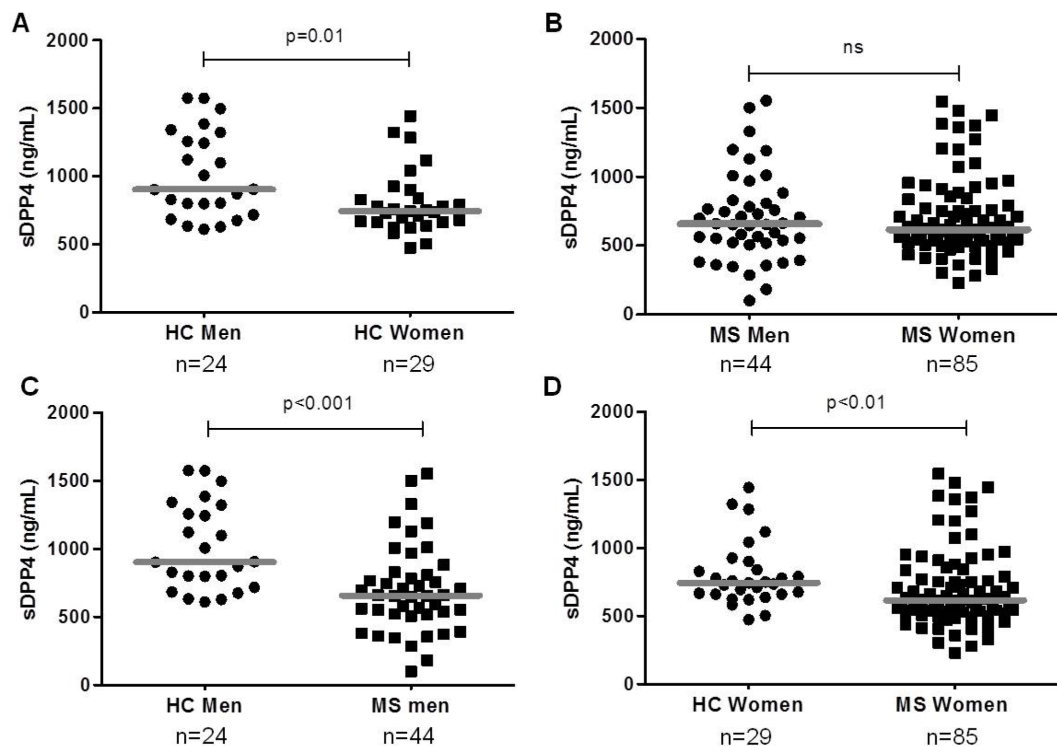

Figure 15 Comparison of men and women levels of sDPP4 in the group of healthy controls (n=53, F/M= 29/24) and in the group of MS patients (n=129, F/M= 85/44). Levels of sDPP4 were significantly higher in men compared to women in the group of healthy controls **(A)** but not in MS patients **(B)**. When comparing men **(C)** and women with or without the disease, the differences were also significant (C: p<0.001 and D: p<0.01 Mann-Whitney *U* test). Medians and individual dots shown.

In order to analyze whether sex differences between healthy men and women on DPP4 concentration could be due to sex hormonal variations, we analyzed a group of 18 healthy women (age: 36 (32-40) years), evaluating the

concentrations of sDPP4 at day 1 of their menstruation cycle and at day 14, the time of ovulation. We observed no differences in sDPP4 between these two interval time points (Figure 16A). When the sDPP4 levels of healthy men and women at day 1 or at day 14 were compared, we found significantly higher sDPP4 levels in healthy men than in healthy women (p<0.01, Table 8). No correlation between sDPP4 and the serum levels of progesterone, estradiol, testosterone and cortisol was found for the population included in this study (p>0.05).

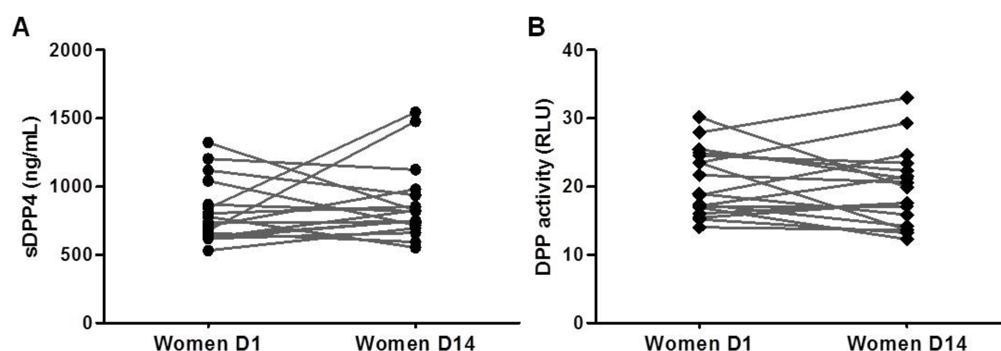

Figure 16 **(A)** Levels of sDPP4 and of **(B)** dipeptidyl peptidase (DPP) activity in healthy women at day 1 and at day 14 of the menstrual cycle (Two-tailed Paired t tests, not significant).

	Men[a]	Women D1[b]	Women D14[c]	p value
sDPP4 (ng/mL)	1133±297.4	808.1±223.1	866.2±270.8	< 0.01[a vs b]
	1122 (476.6)	738.7 (277.7)	785 (236.4)	< 0.01[a vs c]
DPP activity (RLU)	24.18±4.22	20.49±4.81	19.51±5.69	< 0.05[a vs b]
	24.47 (7.27)	18.84 (7.9)	18.77 (8.53)	< 0.01[a vs c]

Table 8 Levels of plasma sDPP4 levels and dipeptidyl peptidase (DPP) activity in healthy men (n=17) and healthy women (n=18) at D1 (menstruation) and D14 (ovulation) of their menstrual cycle. Results expressed as mean±SD and median (interquartile range). Significant differences were observed when healthy men were compared with healthy women at both time points of the menstrual cycle (Mann-Whitney *U* test).

4.2.1.1.2 Lower Dipeptidyl Peptidase Activity in MS patients

Two of the six DPPs are extracellular – DPP4 and FAP – but the majority of plasma DPP activity is due to sDPP4 [360]. The DPP activity present in the plasma of our samples was analyzed in the two independent cohorts of MS and healthy controls. We could not pool together the results from different years as

the assay technique is relative (measuring enzymatic activity as a function of the release of a dipeptide-luciferin molecule), and assays were performed at different times.

MS patients had significantly lower DPP activity than healthy controls (p=0.01, Figure 17A) and again, the median value was lower when the group of RESPONDERS was excluded (p=0.003 Figure 17A below). In the Pilot cohort, differences the same trend was observed (Figure 17A).

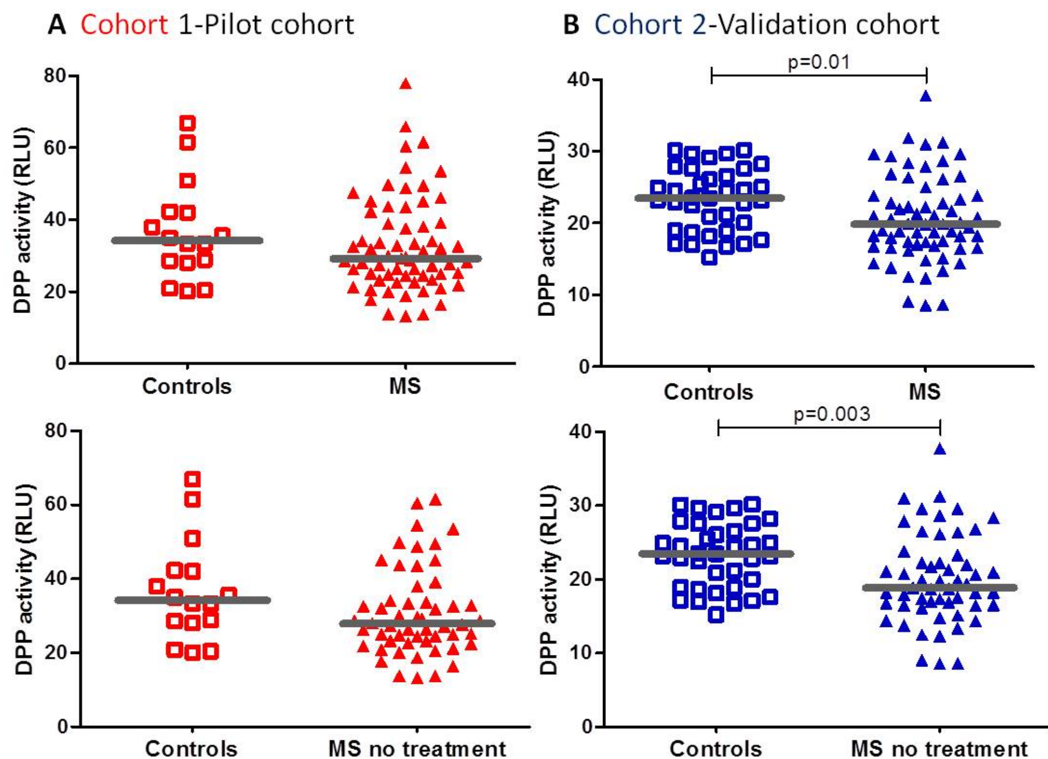

Figure 17 Comparison of DPP activity measured in relative luminescent units (RLU) in the Pilot and Validation cohorts. **(A)** Comparison of DPP activity between healthy controls (Controls, n=16) and multiple sclerosis patients (MS, n=65) or between healthy controls and MS patients without treatment (MS no treatment, n=54) in the Pilot cohort (red). **(B)** Analogous comparison in the Validation cohort (Controls, n=37; multiple sclerosis patients, n=64; multiple sclerosis patients without treatment, n=55) Validation cohort (blue) Differences were statistically significant (p=0.01 and p=0.003, respectively Mann-Whitney *U* test comparison). Medians and individual dots shown.

Then, we sought to determine whether DPP activity correlated with clinical MS evolution. Similarly to sDPP4, all the medians of DPP activity varied significantly among different MS clinical forms (Kruskal-Wallis test, p=0.003) (Figure 18). Interestingly, when analyzing the different clinical groups separately, the group of long-term responders to IFNβ (RESPONDERS) showed similar DPP activity

to the group of healthy controls in both cohorts. This may be a reflection of response to therapy, or alternatively the lower levels may account for a patients' responsiveness. Both in the Pilot and Validation cohorts, SP-MS patients showed the lowest DPP activity among all MS clinical forms (Figure 18). SP-MS patients displayed significantly lower DPP activity compared to healthy controls (Pilot cohort: $p<0.02$, Validation cohort: $p<0.001$) and to long-term responders to IFNβ (Pilot cohort: $p<0.005$, Validation cohort: $p<0.004$).

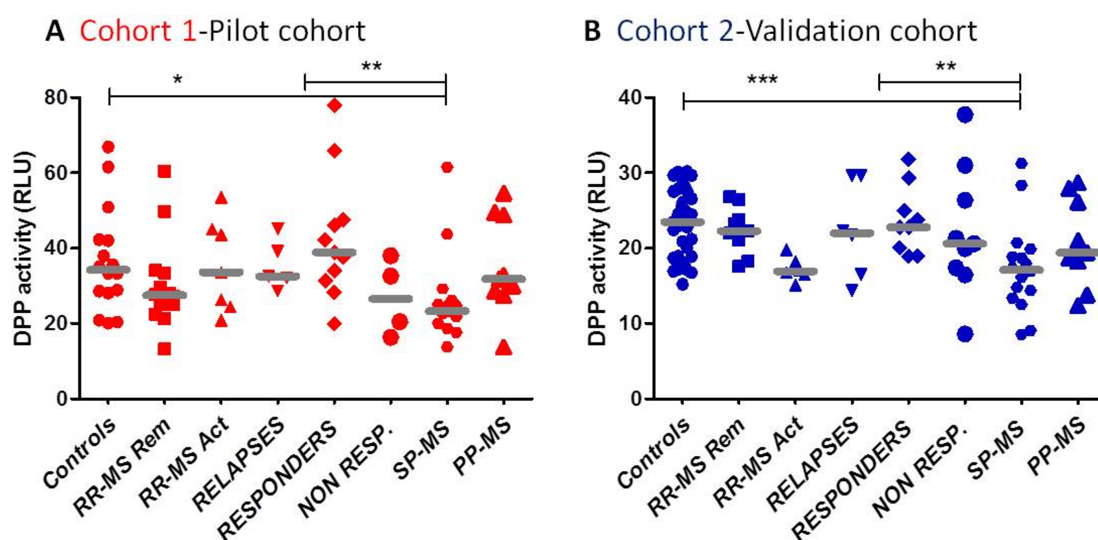

Figure 18 Representation of the DPP activity measured in relative luminescent units (RLU) in the Pilot and Validation cohorts with the healthy control group (Controls, Cohort 1-n=16; Cohort 2-n=37) and the MS patients split by clinical forms: Recurrent-remitting MS patients in remission (RR-MS Rem, Cohort 1-n=14; Cohort 2-n=9), recurrent-remitting MS patients with active disease (RR-MS Act, Cohort 1-n=7; Cohort 2-n=6), at relapse (RELAPSES, Cohort 1-n=5; Cohort 2-n=6), long time Interferon beta responders (RESPONDERS, Cohort 1-n=11; Cohort 2-n=9), non responders to Interferon beta (NON RESP., Cohort 1-n=4; Cohort 2-n=9), Secondary- progressive MS patients (SP-MS, Cohort 1-n=15; Cohort 2-n=16), Primary- progressive MS patients (PP-MS, Cohort 1-n=9; Cohort 2-n=9). The group of SP-MS patients had significantly lower DPP activity than the healthy control group and the long-term responders to IFNβ in both cohorts (*$p<0.02$; **$p<0.005$; ***$p<0.001$). Medians and individual dots shown.

Of note, DPP activity correlated negatively with individual clinical disease activity score EDSS (r_s= -0.315, p=0.01) and with disease duration in years (r_s= -0.255, p=0.04).

In order to establish that DPP activity is related to sDPP4 concentrations, but also to examine potential differences in the activity per molar concentration, we plotted DPP activity as a function of sDPP plasma concentration. The DPP activity and plasma concentration of sDPP4 positively correlated both in HC

(Pilot cohort: r_s=0.638, p=0.007 and Validation cohort: r_s=0.383, p=0.01) and MS patients without treatment (Pilot cohort: r_s=0.385, p=0.004; and Validation cohort: r_s=0.359, p=0.007). Additionally, the two populations showed a similar slope, arguing against the presence of allosteric regulators or genetic variants of DPP4 in MS patients (Figure 19).

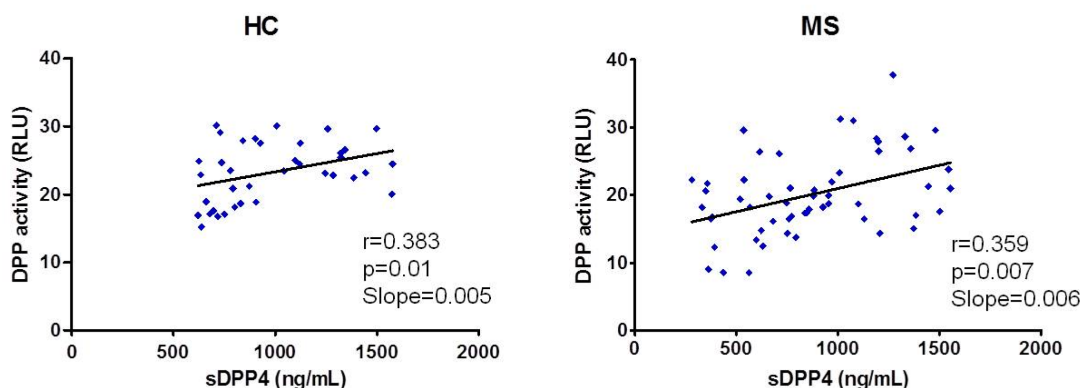

Figure 19 Correlation between plasmatic sDPP4 and DPP enzymatic activity in the Validation cohort (Spearman correlation).

Examining other correlations, we found an inverse relationship between circulating levels of 25(OH)-vitamin D and the activity of DPP in MS patients (r_s= -0.262, p=0.05). This inverse correlation has not been previously described and was exclusive found in female patients with MS (r_s= -0.444, p=0.009). In contrast, male patients showed no correlation (Figure 20). Of note, circulating levels of 25(OH)-vitamin D were only measured in the Validation cohort.

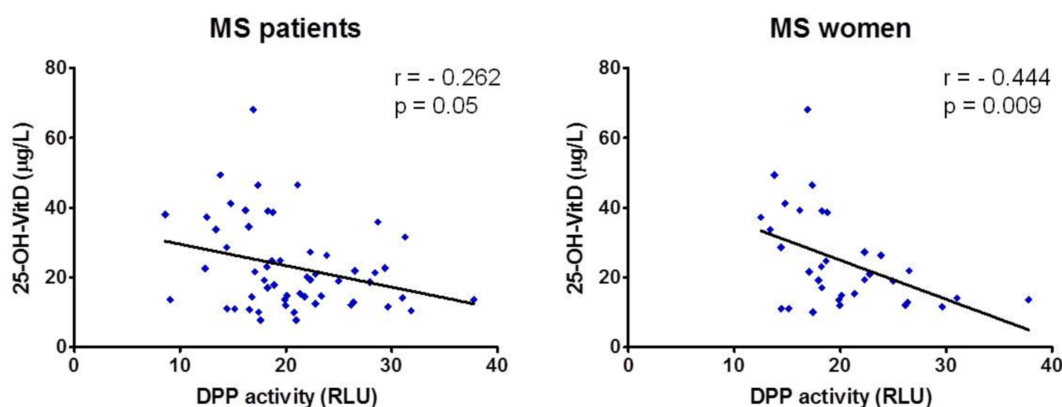

Figure 20 Inverse correlation between circulating 25(OH)-vitamin D (µg/L) circulating levels and DPP enzymatic activity in multiple sclerosis patients **(A)** in multiple sclerosis women **(B)** (Spearman correlation).

We observed no differences in DPP activity between women at day 1 (menstruation cycle) and women at day 14 (ovulation) (Figure 16B). When the DPP activity between healthy men and healthy women at these two time points of the menstrual cycle were compared, we found significantly higher DPP activity in healthy men than in healthy women at day 1 ($p<0.05$) and at day 14 ($p<0.01$) (Table 8).

As an additional exploratory study, we evaluated sDPP4 concentration and DPP activity in the CSF of five patients at a time of relapse. Both measures were undetectable, and therefore this line of investigation was not continued (data not shown).

4.2.1.1.3 DPP4 cell surface expression on circulating T cell subsets and NK cells

In order to obtain a more comprehensive and informative picture of the role of DPP4 in MS, we studied the DPP4 expression on T cell subsets and NK cells ($CD3^{-}CD16^{+}CD56^{+}$) in our Validation cohort of MS patients.

Significantly higher frequencies of circulating $CD8^{+}DPP4^{hi}$ T lymphocytes in MS patients with respect to healthy controls were noted ($p=0.01$) (Figure 21A). The mean fluorescence intensity (MFI) at the single cell level of DPP4 in $CD3^{+}DPP4^{hi}$ and $CD4^{+}DPP4^{hi}$ lymphocytes was also significantly higher in the group of MS patients than in healthy controls ($p=0.02$ and $p=0.03$, respectively) (Figures 21B and C). Among the different MS clinical forms, patients undergoing a clinical relapse (RELAPSE), RR-MS Active patients (RR-MS Act.) and RR-MS patients that had not previously responded to IFNβ (NON RESPONDERS) presented higher frequencies of circulating $CD4^{+}DPP4^{hi}$ T lymphocytes compared to RR-MS patients in remission, although not statistically significant. In the groups of patients with RR-MS form (RR-MS Remission, RR-MS Active, RESPONDERS, NON RESPONDERS and RELAPSES) the frequencies of circulating $CD4^{+}DPP4^{hi}$ T lymphocytes correlated positively with the clinical disease activity score EDSS ($r_s= 0.504$, $p=0.008$).

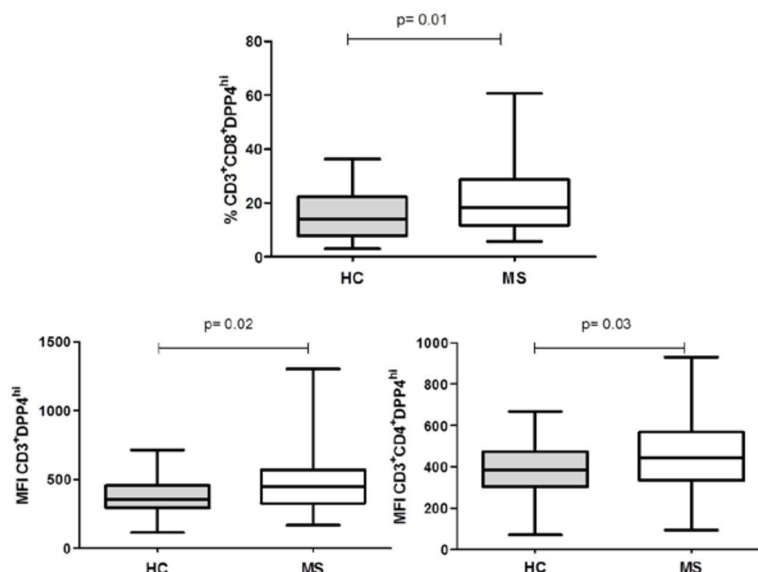

Figure 21 Significant differences (p=0.01) between the frequencies of circulating $CD3^{+}CD8^{+}DPP4^{hi}$ lymphocytes in healthy controls (HC, n=30) and in multiple sclerosis patients (MS, n=48) **(A)**. The below figures represent the mean fluorescence intensity (MFI) of DPP4 in $CD3^{+}DPP4^{hi}$ (p=0.02) **(B)** and $CD3^{+}CD4^{+}DPP4^{hi}$ (p=0.03) lymphocytes **(C)**.

The expression of DPP4 was also measured on NK cells characterized as $CD3^{-}CD16^{+}CD56^{+}$ cells. As described in the literature [361] the constitutive expression of DPP4 on NK cells was low. Nevertheless, we found significant higher circulating levels (6.7±5.6 vs 4.8±5.2, p=0.05) and MFI of $CD3^{-}CD16^{+}CD56^{+}DPP4^{+}$ (35.8±13.1 vs 31.6±10.4, p=0.03) in MS patients with respect to healthy controls.

4.2.1.1.4 T-cell lymphoproliferation in MS patients after TCR stimulation. Association with DPP4.

In a subgroup of 4 MS patients and 4 healthy controls, PBMCs were stimulated *in vitro* with different mitogens and antigens: soluble phytohemagglutinin (PHA) and soluble anti-CD3 and anti-CD28. sDPP4 was quantified from the supernatants at day 6. Two out of the 4 patients (P1 and P4, see table below) showed a marked proliferative defect to soluble anti-CD3 and anti-CD28 with normal proliferation to PHA with respect to the healthy controls included in the study. These data reflect a proliferative defect in a subgroup of MS patients associated with lower (undetectable) levels of DPP4, while sDPP4 is restored by a stronger T-cell proliferation stimulus, and thus discarding an intrinsic defect of DPP4 expression and shedding. As depicted in Table 9, low cell proliferation was associated with values below the least detectable dose (LDD) of sDPP4 in

the cell culture supernatants. This subgroup was enlarged to 13 MS patients and 11 healthy controls and their proliferation under different stimuli was also studied by CFSE (see section 4.3).

	NS		Anti-CD3+Anti-CD28		PHA	
	[3H] Thymidine incorporation, cpm	sDPP4 (pg/mL)	[3H]Thymidine incorporation, cpm	sDPP4 (pg/mL)	[3H]Thymidine incorporation, cpm	sDPP4 (pg/mL)
P1	1,012	<LDD	583	<LDD	84,977	885
P2	1,392	178	62,422	439	36,486	770
P3	2,887	<LDD	55,212	332	79,911	637
P4	1,706	<LDD	2,309	<LDD	45,514	470
C1	487	<LDD	27,165	360	57,840	701
C2	3,183	<LDD	32,887	462	51,463	918
C3	1,269	122	74,197	440	80,244	653
C4	3,404	<LDD	70,101	387	46,026	594

Table 9 Comparison between the proliferation of stimulated or non stimulated PBMCs and measured concentration of sDPP4 in the cell culture supernatants after 6 days. Note that sDDP4 concentrations are in pg/mL. (LDD: least detectable dose).

4.2.1.1.5 sDPP4 and DPP activity correlate with chemokines in MS

Among chemokines that are cleaved by DPP4, there are some proinflammatory chemokines produced by immune cells and CNS glia that are expressed in the CNS in MS and EAE initiating immune cells influx into the CNS and the inflammatory cascade, such as IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, Rantes/CCL5, MCP-3/CCL7 and Mig/CXCL9 [362-373].

In our study, in untreated MS patients (n=105), sDPP4 directly correlated with the chemokines MIP-1α/CCL3 (r_s=0.277, p=0.004) and MIP-1β/CCL4 (r_s=0.272,

p=0.004). In the subgroup of patients with MS relapse there was also a positive correlation with DPP activity and IP10/CXCL10 (r_s=0.828, p=0.04).

4.2.1.1.6 Vitamin D (1α,25-Dihydroxivitamin D_3) stimulates *in vitro* sDPP4 expression

In order to investigate whether *in vitro* addition of 1,25$(OH)_2$ vitamin D_3 (100 nM) had any effect in the expression of DPP4 by PBMCs, we stimulated $2*10^6$ PBMCs of a small subset of 4 healthy donors and 2 MS patients for 24 hours and quantified the mRNA DPP4 expression. We observed a remarkable increase of the expression of DPP4 in five of the six subjects analyzed (Figure 22A). We utilized an endogenous positive control gene that is induced by Vitamin D: CYP24A1 gene that encodes the mitochondrial enzyme 1,25-dihydroxyvitamin D3 24-hydroxylase; to check that the effect observed in PBMCs was due to the Vitamin D. Those PBMCs stimulated by 1,25$(OH)_2$ vitamin D_3 had an enhanced expression of CYP24A1.

Our genetic expression findings were confirmed with the increased levels of the protein sDPP4 detected in the cell culture supernatants by ELISA after 5 days (time point when the highest increase of sDPP4 was detected). Even in the healthy control (C3) where there was no increase of genetic expression after 24 hours (Figure 22B).

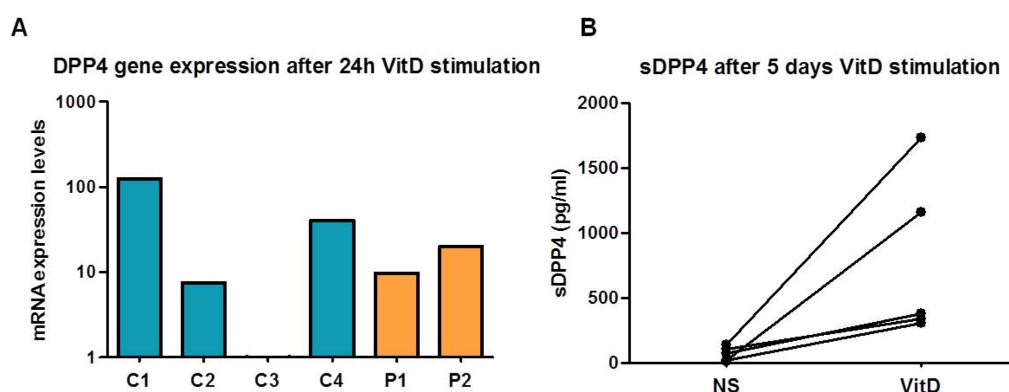

Figure 22 **(A)** The *in vitro* stimulated PBMCs of five out of the six subjects studied increased presented a remarkably increased genetic expression of DPP4 after 24 hours stimulation with 1,25$(OH)_2$ vitamin D_3 100 nM. **(B)** The levels of sDPP4 increased in the cell culture supernatants of PBMCs stimulated with 1,25$(OH)_2$ vitamin D_3 after 5 days. NS: no stimulus, VitD: 1,25$(OH)_2$ vitamin D_3.

We stimulated PBMCs with 1,25$(OH)_2$ vitamin D_3 for 5 days, of 6 additional MS patients and 4 more healthy controls and analyzed sDPP4 levels in cell culture supernatants. We observed an increase of sDPP4 levels in all subjects studied without differences of sDPP4 expression in MS patients or healthy controls (Figure 23).

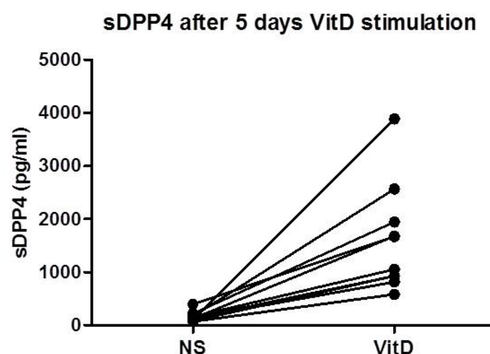

Figure 23 The levels of sDPP4 increased in the cell culture supernatants of PBMCs stimulated with 1,25$(OH)_2$ vitamin D_3 after 5 days in six additional MS patients and four healthy controls. NS: no stimulus, VitD: 1,25$(OH)_2$ vitamin D_3.

To the best of our knowledge these results confirm for the first time that after *in vitro* stimulation of PBMCs with 1,25$(OH)_2$ vitamin D_3 100nM, the expression of DPP4 in PBMCs is enhanced.

4.2.1.2. Interleukin 7 in Multiple Sclerosis

Interleukin 7 (IL-7) is a non-redundant cytokine with an essential regulatory role in lymphopoiesis and in peripheral T cell homeostasis [374]. Recently, decreased systemic levels of IL-7 and of sIL-7RA and increased ratio of membrane-bound to soluble IL-7RA have been found in MS patients [375] and MS. Serum levels of IL-7 predict clinical response to IFNβ therapy in patients with RR-MS and IL-7 favors Th1 differentiation of human T cells *in vitro* and in the mouse model *in vivo* [376].

4.2.1.2.1 Interleukin 7 is decreased in Multiple Sclerosis

In our cohort of MS patients, we found that circulating levels of IL-7 were uniformly decreased in all the groups of MS patients studied, with respect to healthy controls (60±55 vs 102±88 pg/mL, p<0.0005) (Figure 24). If the Pilot and Validation cohorts were analyzed separately, this diminished concentration plasma levels of IL-7 was statistically significant in the Validation cohort (p=0.0004).

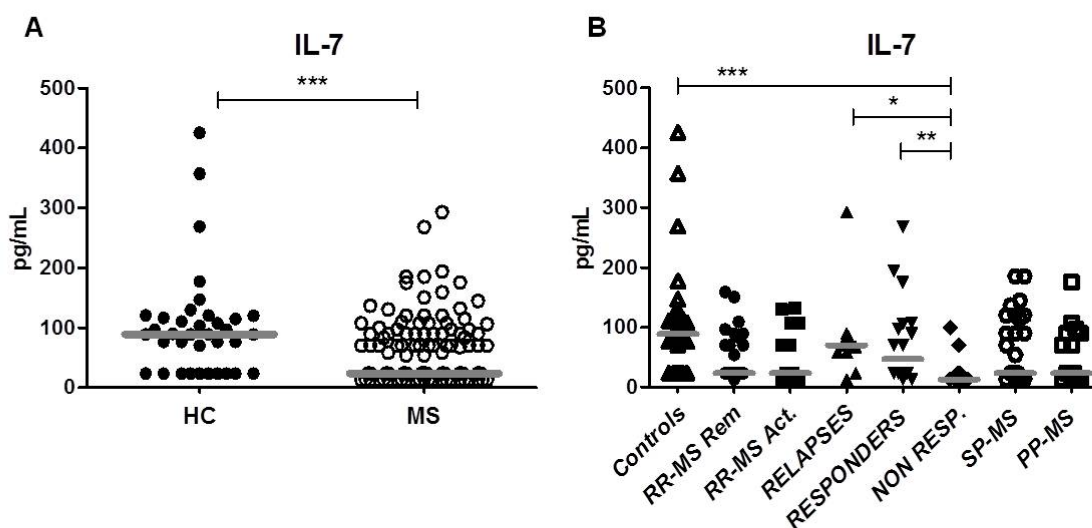

Figure 24 **(A)** Circulating levels of IL-7 where higher in the healthy control group (HC, n=36) than in multiple sclerosis patients (MS, n=129) (***p<0.0005, Mann-Whitney U test). **(B)** Clinical MS groups differed in IL-7 levels (Kruskal-Wallis test p<0.002). MS patients split by clinical forms: Recurrent-remitting MS patients in remission (RR-MS Rem, n=23), recurrent-remitting MS patients with active disease (RR-MS Act, n=13) patients at relapse (RELAPSES, n=11), long time Interferon beta responders (RESPONDERS, n=20), non responders to Interferon beta (NON-RESP., n=13), Secondary-progressive MS patients (SP-MS, n= 31), Primary-progressive MS patients (PP-MS, n=18). The group of NON-RESP. had significantly lower levels of IL-7 than the groups of Controls, RESPONDERS and RELAPSES (***p<0.0001, **p=0.007, *p=0.01; Mann-Whitney U test. Medians and individual dots shown).

All the medians of IL-7 varied significantly among different MS clinical forms (Figure 24B; Kruskal-Wallis test, $p<0.002$). Those patients who had not previously responded to IFNβ treatment (NON RESP.) presented significantly lower levels than healthy controls (28±29 vs 102±88 pg/mL, $p<0.0001$), than patients under long-term treatment with IFNβ (28±29 vs 76±73 pg/mL, $p=0.007$) and than patients who were undergoing a clinical relapse (28±29 vs 82±74 pg/mL, $p=0.01$) (Figure 24B). The groups of patients of RR-MS in remission and active phase of the disease and the progressive forms of MS (SP-MS and PP-MS) had also significantly lower concentration in plasma of IL-7 ($p<0.05$) than healthy controls (Figure 24B).

The ROC curves indicate a good performance of plasma IL-7 for the discrimination of MS (AUC: 0.692, $p=0.0004$). The cut-off value of 73 pg/mL of circulating IL7 showed a sensitivity and specificity of 69.4% both; PPV of 88.7% and NPV of 39.7%. Concentrations of IL-7 <73 pg/mL was a classifier for individuals with MS ($p<0.0005$ Fisher's Exact Test, 2-sided) with an Odds Ratio of 5.1 (95% confidence interval: 2.3 – 11.5).

4.2.1.3. Interleukin 1 Receptor Antagonist in Multiple Sclerosis

The interleukin-1 receptor antagonist (IL-1RA) is a protein encoded in the gene IL-1RN (2q) that binds to the IL-1 receptor; inhibiting the binding of the proinflammatory cytokines IL-1alpha (IL-1α) and IL-1beta (IL-1β) [377].

IL-1RA is produced as different isoforms by alternatine mRNA splicing; there is a secreted isoform of IL-1RA (sIL-1RA) and other three intracellular isoforms [378]. The IL-1RA blood levels are altered in different immune based diseases and the recombinant human IL1R blockers are successfully employed in IL-1 driven diseases[378, 379]. In EAE, the animal model of MS, sIL-1RA prevents the induction of the disease [331]. In MS patients sIL-1RA serum levels increase after treatment with IFNβ [380, 381], glatiramer acetate [382] or steroids [383], indicating that these drugs mediate their anti-inflammatory effect by the increase of the sIL-1RA systemic concentration.

4.2.1.3.1 Interleukin-1 Receptor Antagonist is decreased in Multiple Sclerosis

In our cohort of MS patients, we found that all the different clinical groups of patients had significantly decreased plasma levels of sIL-1RA compared to the healthy controls (403±356 vs 712±884 pg/mL, p=0.02) (Figure 25A). When the Pilot and Validation cohorts were analyzed separately, this lower expression of sIL-1RA was statistically significant in the Validation cohort (p=0.004). We did not observe significant differences of IL-1RA expression among the groups studied (Figure 25B). However, the group of RESPONDERS had slightly higher levels than the rest of groups.

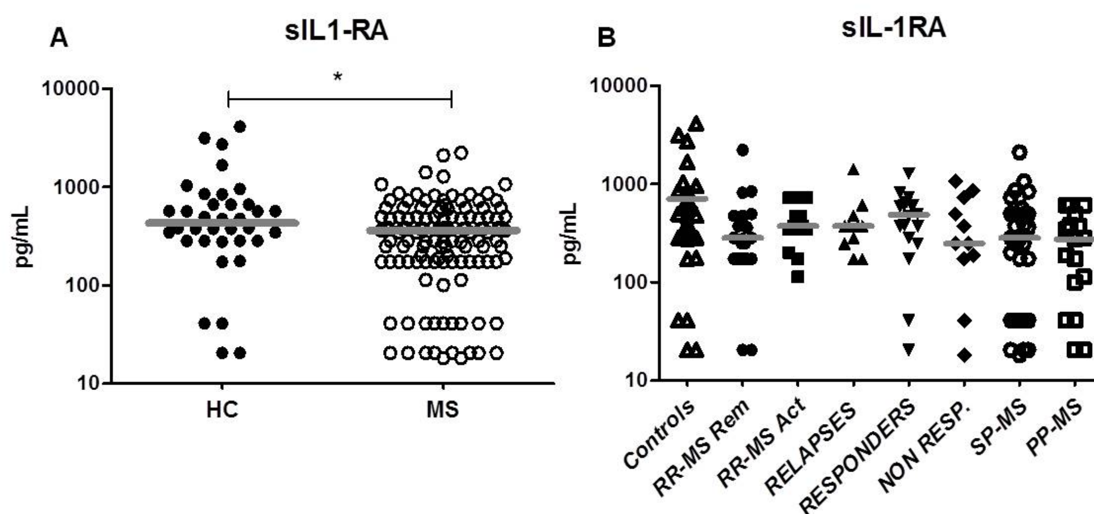

Figure 25 **(A)** Circulating levels of sIL-1RA where higher in the healthy control group (HC, n=36) than in multiple sclerosis patients (MS, n=129) (*p=0.02, Mann-Whitney U test). **(B)** MS patients split by clinical forms: Recurrent-remitting MS patients in remission (RR-MS Rem, n=23), recurrent-remitting MS patients with active disease (RR-MS Act, n=13) patients at relapse (RELAPSES, n=11), long time Interferon beta responders (RESPONDERS, n=20), non responders to Interferon beta (NON-RESP., n=13), Secondary-progressive MS patients (SP-MS, n= 31), Primary-progressive MS patients (PP-MS, n=18). No significant differences were observed among groups (Medians and individual dots shown).

The ROC curve indicated a good performance of sIL-1RA for the discrimination of MS (AUC: 0.625; p=0.023). The cut-off value of 377 pg/mL of circulating sIL-1RA showed a sensivity of 61.3%, specificity of 63.9%; PPV of 85.4% and a NPV of 32.4%. The concentration of sIL-1RA <377 pg/mL was a classifier for individuals with MS (p = 0.013 Fisher's Exact Test, 2-sided) with an Odds Ratio of 2.8 (95% confidence interval: 1.3 – 6.1).

4.2.1.4. Monokine Induced by Gamma Interferon in Multiple Sclerosis

The Monokine induced by gamma Interferon (Mig), also known as CXCL9, is a chemokine of the α/CXC subfamily that attracts neutrophils and activated T and NK cells [384]. Mig is induced by IFNɣ and shares the receptor CXCR3 with the related chemokines IP10/CXCL10 and I-TAC/CXCL11. In MS active demyelinating lesions, lymphocytes express CXCR3 and Mig is detected focally in microglia [263, 265]. Human astrocytes and brain endothelial cells express constitutively Mig and their protein secretion is enhanced after IFNɣ stimulation alone or synergistically with the combination of IFNɣ and IL-1β or IFNɣ and TNFα [385]. The CSF levels of Mig/CXCL9 and IP10/CXCL10 are increased in MS and in other neurological diseases [263] and the circulating frequencies of $CD4^{+}CXCR3^{+}$ T cells are also augmented in the intathecal compartment with respect to peripheral blood [263]. This may be reflecting an active recruitment of these $CXCR3^{+}$ T cells into the CNS guided by their chemokine ligands.

4.2.1.4.1 Monokine induced by Gamma Interferon is decreased in Multiple Sclerosis

Circulating Mig was found in significantly lower levels in MS patients than in healthy controls (210±237 vs 302±239 pg/mL, $p<0.002$) (Figure 26A). This finding was also observed when the Pilot cohort and the Validation cohort were analyzed separately ($p=0.04$ and $p=0.002$, respectively).

Among the different clinical MS groups there were significant differences in Mig circulating levels (Figure 26B, Kruskal-Wallis test, $p=0.01$). The group of patients under treatment with IFNβ had similar circulating levels of Mig than healthy controls and significantly higher levels than patients at relapse ($p=0.04$) (Figure 26B).

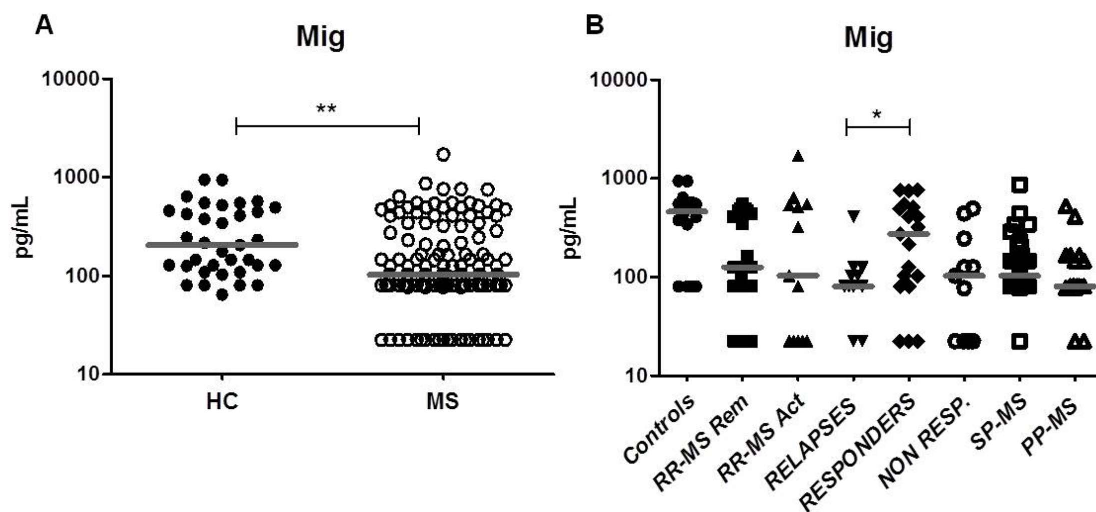

Figure 26 **(A)** Circulating levels of Mig were higher in the healthy control group (HC, n=36) than in multiple sclerosis patients (MS, n=129)(A)(**p<0.002, Mann-Whitney U test). **(B)** Significant differences were observed among groups (Kruskal-Wallis test, p=0.01). MS patients split by clinical forms: Recurrent-remitting MS patients in remission (RR-MS Rem, n=23), recurrent-remitting MS patients with active disease (RR-MS Act, n=13) patients at relapse (RELAPSES, n=11), long time Interferon-β responders (RESPONDERS, n=20), non responders to Interferon-β (NON-RESP., n=13), Secondary-progressive MS patients (SP-MS, n= 31), Primary-progressive MS patients (PP-MS, n=18). RESPONDERS to Interferon-β had significantly higher levels of Mig than patients at RELAPSE (*p=0.04). Medians and individual dots shown.

ROC curves analysis was used to assess the accuracy of Mig to identify MS patients (AUC: 0.673; p<0.002). The cut-off value of 106 pg/mL of circulating Mig showed a sensivity of 52.4%, specificity of 83.3%, PPV of 91.5% and a NPV of 33.7%. The concentration of Mig <106 pg/mL was a classifier for individuals with MS (p<0.0005 Fisher's Exact Test, 2-sided) with an Odds Ratio of 5.5 (95% confidence interval: 2.1 – 14.1).

4.2.1.5. Multivariate Logistic Regression Model for MS patients and Healthy Controls

A logistic regression model was carried out including independent selected plasma analytes that had survived to the variable selection process between MS patients and healthy controls taken as independent variables and the conditions (MS disease or non MS) as the dichotomic target variable. The model obtained included the three analytes listed in Table 10 as predictors for the condition of having MS.

	p value	O.R.	95% C.I.
IL-7 (pg/mL)	0.0107	0.9920	0.9860- 0.9981
Mig (pg/mL)	0.0193	0.9978	0.9960-0.9996
sDPP4 (ng/mL)	0.0012	0.9978	0.9964-0.9991

Table 10 Logistic regression model for MS. (O.R. >/< greater/less probability of MS)

The odds for a specific subject, i.e. the probability P of developing MS divided by the probability of not developing the disease 1-P, according to the model are given by the product of 62.9415 and the three odds ratios each elevated by the respective predictors' values measured in this subject. E.g., for a subject with values of IL-7: 60 pg/mL; Mig: 95 pg/mL and sDPP4: 605 ng/mL, the odds are $62.9415 \bullet 0.9920^{60} \bullet 0.9978^{95} \bullet 0.9978^{605} = 8.3$. Hence the model assigns to this subject a probability of being a patient with MS of 8.3 / (1+8.3) = 89% (classification performance).

The analytes IL-7, Mig and sDPP4 are protective factors for MS disease and the higher their levels in plasma, the lower the probability of having MS.

The model gives a sensibility of 96.5%, a specificity of 23.5%, a PPV of 80.9%, a NPV of 66.7% and an accuracy of 79.7% for the sample.

4.2.2. Biomarkers Discriminating among Clinical Forms of Multiple Sclerosis

In this part of the study, a multifaceted evaluation of circulating analytes was performed, in order to identify specific biomarkers and/or their combinations which might serve as effective tools in the diagnosis and therapeutic targeting of MS patients.

4.2.2.1. Panel of Plasma Biomarkers that Discriminate between Relapsing-Remitting and Progressive Clinical Forms of Multiple Sclerosis

HGF, Eotaxin and MCP-1 were present at significantly lower circulating levels in all the groups of RR-MS patients (RR-MS Remission, RR-MS Active, RESPONDERS, NON RESPONDERS and RELAPSES) than in SP-MS and

PP-MS patients, while Rantes was significantly higher in the progressive clinical forms of MS.

The analysis of the Pilot cohort revealed that HGF, Eotaxin/CCL11, MCP-1/CCL2, Rantes/CCL5 were present in significantly higher concentrations in the progressive MS clinical forms with respect to the inflammatory (RR-MS) phase, findings hat were confirmed in the Validation cohort (Table 11). HGF, Rantes and Eotaxin/CCL11 differences between these two groups were statistically significant in both cohorts analyzed independently. Plasma MCP-1 levels showed a trend to be higher in the Pilot cohort (p=0.06), while in the Validation cohort, this difference was statistically significant (p=0.0002).

	RR-MS	Progressive	HC
Pilot cohort			
HGF	218±181[b]	387±250	116±130[a,b]
Eotaxin	80±52[b]	119±65	81±26[b]
MCP-1	382±198[c]	483±212	382±170
Rantes	3,480±2,941[b]	6,366±2,527	3,898±5,949[b]
EGF	62±58[b]	16±19	73±38[b]
MIP-1β	138±70[b]	75±76	132±64[b]
Validation cohort			
HGF	364±234[b]	577±339	771±753[a]
Eotaxin	95±60[b]	252±167	199±167[a]
MCP-1	369±254[b]	689±357	654±351[a]
Rantes	4,865±6,176[b]	16,167±9,986	6,803±7,594[b]
EGF	109±76[c]	80±58	146±107[b]
MIP-1β	144±89[c]	110±53	198±138[b]

Table 11 Comparisons of the plasma levels of HGF, Eotaxin, MCP-1, Rantes, EGF and MIP-1β comparison in the Pilot and Validation cohorts. Note that the Relapsing-remitting group (RR-MS) comprises the clinical groups: RR-MS Remission, RR-MS Active, RESPONDERS, NON RESPONDERS and RELAPSES. The Progressive group comprises Secondary (SP-MS) and Primary-progressive patients. [a]p<0.05 vs RR-MS, [b]p<0.05 PROGRESSIVE vs, [c]p=0.06.

Given the intrinsic heterogeneity of MS, the two cohorts were pooled to increase the number of cases and controls. Again, in MS patients globally considered, we found that plasma levels of HGF, Eotaxin/CCL11, MCP-1/CCL2 were significantly diminished in the inflammatory (RR-MS patients) than in the progressive forms (SP-MS and PP-MS) (HGF: 294±221 vs 484±310, p= 0.0002; Eotaxin: 87±56 vs 187±143, p<0.0001; MCP-1: 375±227 vs 588±309, p<0.0001), while Rantes/CCL5 was significantly higher between both forms (Rantes: 11,371±8,800 vs 4,173±4,855, p<0.0001) (Figure 27). Interestingly, the levels of HGF, MCP-1/CCL2 and Eotaxin/CCL11 in the progressive MS patients were similar to those observed in healthy controls (HGF: 476±652; Eotaxin: 147±138; MCP-1: 538±315), while Rantes/CCL5 was more elevated in the progressive forms with respect to healthy controls (5,518±6,796) and to the rest of MS patients. RR-MS patients had significantly lower levels of Eotaxin, MCP-1 and Rantes than healthy controls (p=0.001, p=0.001 and p=0.0002, respectively).

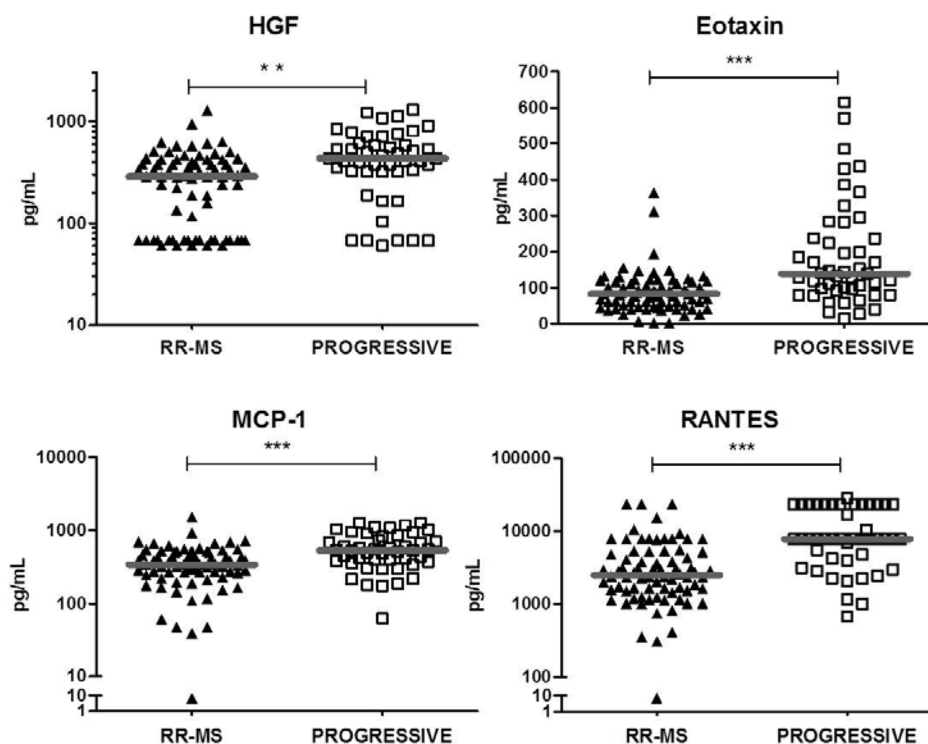

Figure 27 Comparison of the plasma levels of HGF, Eotaxin, MCP-1 and RANTES in relapsing-remitting (RR-MS, n= 80) and in the group of progressive (SP-MS and PP-MS, n=49) patients. *** p<0.0001, ** p=0.0002, Mann-Whitney U test. Medians and individual dots shown.

The ROC analyses indicated a good performance of plasma HGF, Eotaxin, MCP-1 and Rantes for the discrimination between RR-MS from progressive clinical forms (Table 12).

	AUC	(95% CI)	p value
HGF	0.702	(0.604-0.799)	<0.0002
Eotaxin	0.755	(0.662-0.849)	<0.0001
MCP-1	0.721	(0.626-0.816)	<0.0001
Rantes	0.792	(0.707-0.876)	<0.0001
EGF	0.689	(0.592-0.786)	0.0005
MIP-1β	0.702	(0.607-0.798)	<0.0002

Table 12 Areas under the curve (AUC), 95% Confidence Interval (95% CI) and p value for the analytes HGF, Eotaxin, MCP-1, Rantes, EGF, MIP-1β.

HGF correlated strongly with MCP-1/CCL2 both in MS patients and in healthy controls (r_s=0.4674, p<0.0001), whilst HGF correlated with eotaxin/CCL11 (r_s=0.5574, p<0.0001) and Rantes/CCL5 (r_s=0.3317, p=0.0002) only in MS patients. These proteins correlated also with neurological disability measured by the EDSS: HGF (r_s=0.3254, p=0.0003), MCP-1/CCL2 (r_s=0.2714, p=0.003), Eotaxin/CCL11 (r_s=0.3610, p<0.0001), Rantes (r_s=0.4386, p<0.0001).

EGF and MIP-1β are diminished in Secondary and Primary Progressive clinical forms

Both in the Pilot cohort and confirmed in the Validation cohort, EGF and MIP-1β plasma levels were lower in the progressive forms with respect to RR-MS patients. In the Pilot cohort, EGF and MIP-1β statistically differed in both classification groups (p=0.0001 and p=0.0002, respectively); in the Validation cohort, both analytes showed a trend (p=0.06) to be at lower concentrations in the plasma of progressive patients (SP-MS and PP-MS) than in RR-MS patients (Table 11).

The pool of both cohorts displayed the same distribution with significantly diminished circulating levels of EGF and of the chemokine MIP-1β/CCL4 than in RR-MS patients (EGF: 50±54 vs 87±71, p=0.0005; MIP-1β: 93±67 vs 141±79, p=0.0002) (Figure 28). RR-MS patients and HC showed similar circulating

levels of EGF and MIP-1β/CCL4 (in HC, EGF: 115±92; MIP-1β: 170±116) and progressive patients had significantly lower levels of EGF and MIP-1β than healthy controls ($p<0.0001$ and $p=0.0002$, respectively). The ROC curves of plasma EGF and MIP-1β were used to assess the identification potential between RR-MS and progressive (SP-MS or PP-MS) clinical forms (Table 12). As expected, EGF and MIP-1β correlated negatively with the disability score EDSS: EGF (r_s= -0.2906, $p<0.002$, MIP-1β (r_s= -0.355, $p<0.0001$).

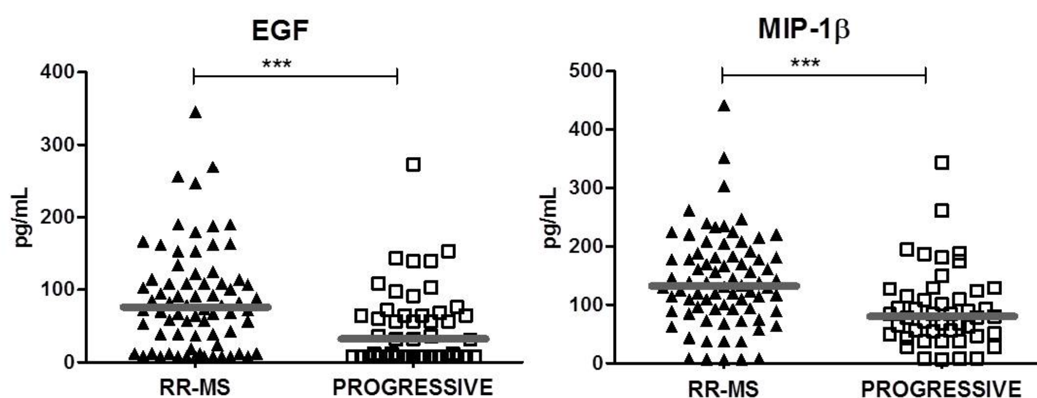

Figure 28 Comparison of the plasma levels of EGF and MIP-1β between patients with relapsing-remitting (RR-MS, n=80) and progressive (SP-MS and PP-MS,n=49) MS. EGF ***p=0.0005; MIP-1β ***p=0.0002, Mann-Whitney U test. Medians and individual dots shown.

We carried out a logistic regression with all these six factors (HGF, Eotaxin, MCP-1, Rantes, EGF and MIP-1β) as independent variables and the clinical MS-forms (Relapsing-Remitting vs. Progressive) as the dichotomous target variable. We obtained a model that included the factors HGF, EGF, Eotaxin and MIP-1β as predictors for the clinical form, while Rantes and MCP-1 did not reach statistical significance to be included in the model.

The odds for a specific patient, i.e. the probability P of being classified as progressive clinical form of MS divided by the probability of developing a relapsing-remitting form 1-P, according to the model are given by the product of 0.4021 and the four odds ratios each elevated by the respective predictors' values (in pg/mL) measured in an individual patient. For instance, for a patient with values [pg/mL] of Eotaxin: 200; HGF: 323; EGF: 17; MIP-1β: 54, the odds are $0.4021 \cdot 1.0147^{200} \cdot 1.0038^{323} \cdot 0.9808^{17} \cdot 0.9873^{54} = 9.14$. Hence, the

model assigns to this patient a probability of having a progressive form of 9.14 / (1+9.14) = 90.1%.

HGF and Eotaxin with O.R. > 1 are risk factors for developing a progressive clinical form of MS, while EGF and MIP-1β with O.R. < 1 are protective factors for developing a progressive clinical form of MS.

Considering odds >/< 1, i.e. a greater/less probability of having a progressive versus RR-MS clinical form according to the model, as a positive/negative prognosis, the model gives a sensitiviy of 71.7%, a specificity of 89.9%, a PPV of 82.5%, a NPV of 82.7% and an accuracy of 82.6% for our cohort of MS patients.

4.2.2.2. Progressive Clinical Forms of Multiple Sclerosis present High Gene Expression Levels of Clusterin and Low Gene Expression Levels of Interferon Regulatory Factor 2

In order to evaluate whether there were differences in the genetic expression of selected genes related with MS pathogenesis and type I Interferon signature in the different clinical MS groups, we analyzed the mRNA levels from PBMCs in patients at stable conditions (except for those undergoing a clinical relapse). The expression levels of the gene clusterin (CLU), also known as apolipoprotein-J, were significantly higher in progressive patients (SP-MS and PP-MS) compared with controls ($p<0.0001$) and with all the patients with RR-MS ($p=0.0002$), if the group under treatment with IFNβ (RESPONDERS) was excluded from the analysis, the differences between untreated RR-MS and progressive clinical MS forms remained significant ($p=0.003$) (Figure 29).

On the other hand, the interferon regulatory factor 2 (IRF2) was expressed at lower mRNA levels in the progressive patients compared to patients with RR-MS ($p=0.007$). Again this difference was significant when the group of RESPONDERS was not included in the RR-MS patients ($p=0.004$). Progressive patients had lower mRNA expression of IRF2 than healthy controls but this difference did not achieve statistical significance ($p=0.08$) (Figure 29).

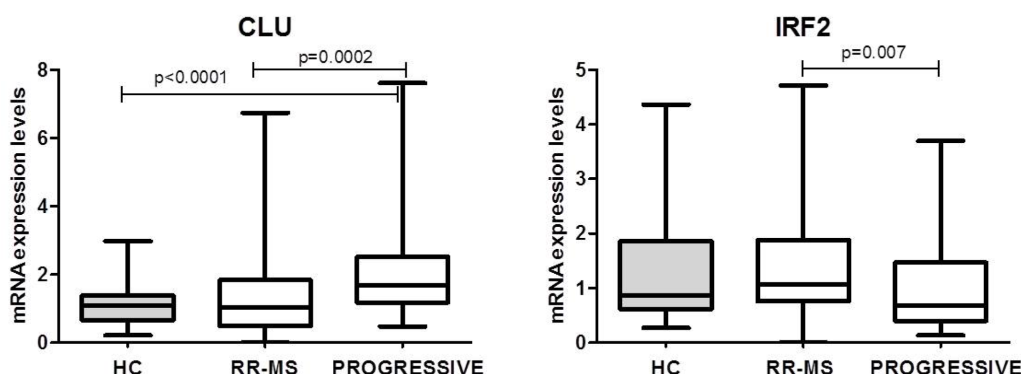

Figure 29 Comparison of gene expression levels of clusterin (CLU) and interferon regulatory factor 2 (IRF2) in the PBMCs from healthy controls (HC, n=46), RR-MS patients (n=76) and Progressive MS patients (n=48) (Mann-Whitney U test).

4.2.2.3. Plasma FGFb can discriminate between Primary and Secondary Progressive patients and between Primary Progressive patients and Relapsing-Remitting patients undergoing clinical relapse

Plasma FGFb was markedly diminished in PP-MS patients when compared to SP-MS (11±10 vs 26±27, p=0.01), patients undergoing a clinical relapse (11±10 vs 21±11, p=0.01), or healthy controls (11±10 vs 28±31, p=0.02) (Figure 30). This distinction is important because MS at onset can be divided into two main forms: relapsing-remitting (all relapses were RR-MS patients) and primary progressive.

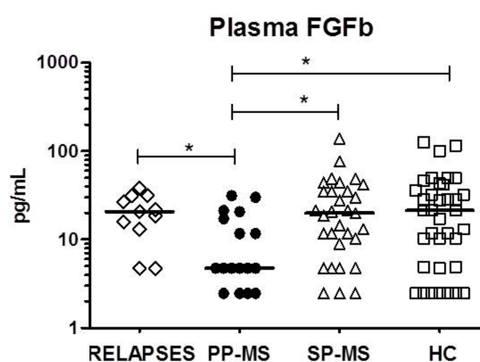

Figure 30 FGFb levels were significantly decreased in primary progressive patients (PP-MS) with respect to healthy controls (HC: * p =0.02) and to secondary progressive (SP-MS) and patients undergoing clinical relapse (RELAPSES) (* p=0.01) Mann-Whitney U test. Medians and individual dots shown.

4.2.2.4. Low Density Lipoprotein Receptor Gene Expression is increased in Primary Progressive patients

Patients with PP-MS presented higher mRNA expression levels of the Low Density Lipoprotein Receptor (LDLR) in their PBMCs than RR-MS patients without treatment (including RR-MS patients in remission, with active disease, relapses and non responders to IFNβ) (p=0.0003), SP-MS patients (p=0.01) and healthy controls (p=0.006) (Figure 31). RR-MS patients with IFNβ treatment (RESPONDERS) had similar mRNA expression levels than PP-MS patients, probably due to the effect of therapy in the expression of LDLR.

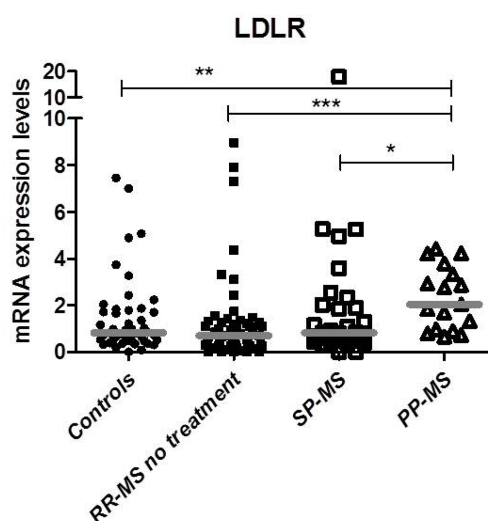

Figure 31 Comparison of gene expression levels of low density lipoprotein receptor (LDLR) in the PBMCs from healthy controls (HC, n=46), RR-MS patients without treatment (n=56), SP-MS patients (n=31) and PP-MS patients (n=17). (*p=0.01; **p=0.006,***p=0.0003 Mann-Whitney U test)

4.2.2.5. VEGF Levels were higher in Secondary Progressive than in Relapsing-Remitting MS

Patients with SP-MS had significantly higher circulating levels of the vascular epidermal growth factor (VEGF) than all the patients with RR-MS (32±28 vs 15±9, p=0.0004) (Figure 32). Patients with PP-MS had also lower circulating levels of VEGF than SP-MS, but this difference was not statistically significant.

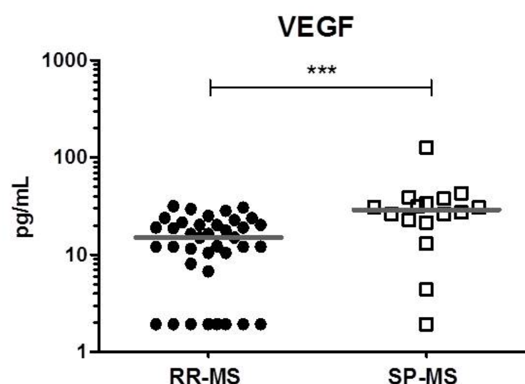

Figure 32 Plasma levels of the vascular growth factor (VEGF) in the Validation cohort, were higher in secondary progressive patients (SP-MS, n= 16) than in patients with the relapsing-remitting form (RR-MS, n=38) (*** p=0.0004). Mann-Whitney U test. Medians and individual dots shown.

The plasma biomarkers significant across MS clinical forms are summarized below (Figure 33).

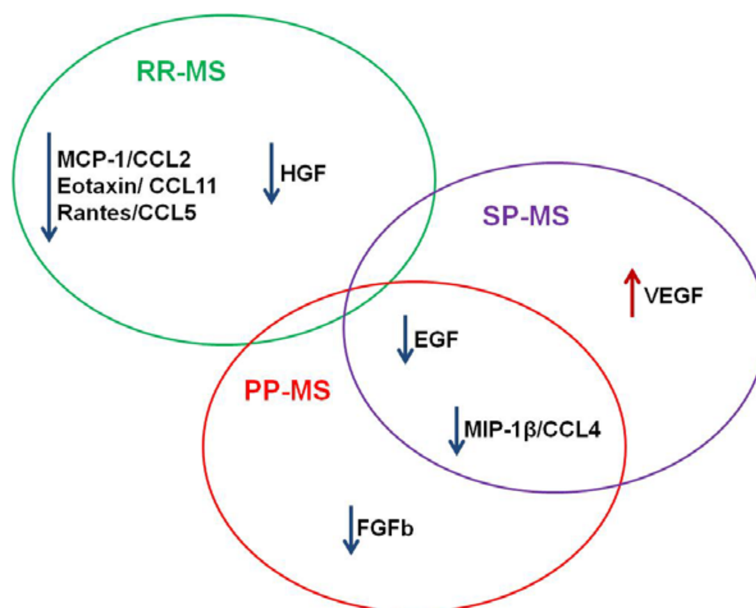

Figure 33 Plasma biomarkers significant across MS clinical forms. Findings are summarized. Listed biomarkers were found to differ significantly between comparison groups. Arrows preceding each biomarker name indicate increased or decreased plasma concentrations in MS groups.

4.2.2.6. IP10 and MCP-1 circulating levels are significantly higher in Relapsing-Remitting patients that respond to IFN-β treatment than in Non Responders

Significant among the chemokines tested were IP10/CXCL10 and MCP-1/CCL2, which were significantly higher in RR-MS patients under treatment with IFN-β (RESPONDERS) than in those patients who had been previously treated with IFN-β and did not respond to therapy and had clinical activity (NON RESPONDERS) (IP10: 111±60 vs 96±136, p=0.04 and MCP-1: 446±158 vs 272±152, p=0.008) (Figure 34). These two cytokines are induced by type I IFN, but only IP10/CXCL10 levels were significantly higher in the RESPONDERS group when compared to the levels of the group of HC (111±60 vs 86±86, p=0.005) and to those levels of the rest of RR-MS patients without treament (RR-MS, RELAPSES and NON RESPONDERS) (111±60 vs 75±78, p=0.0003). IP10 levels were also increased in the progressive forms of MS (SP-MS and PP-MS) than in the rest of RR-MS patients studied: In PP-MS patients, there was a modest increase (96±75 vs 75±78, p=0.05), whereas in SP-MS patients these differences were more remarkable (102±52 vs 75±78, p=0.0001). SP-MS had also higher circulating levels of IP10 than the group of healthy controls (102±52 vs 86±86, p=0.01) (Figure 35).

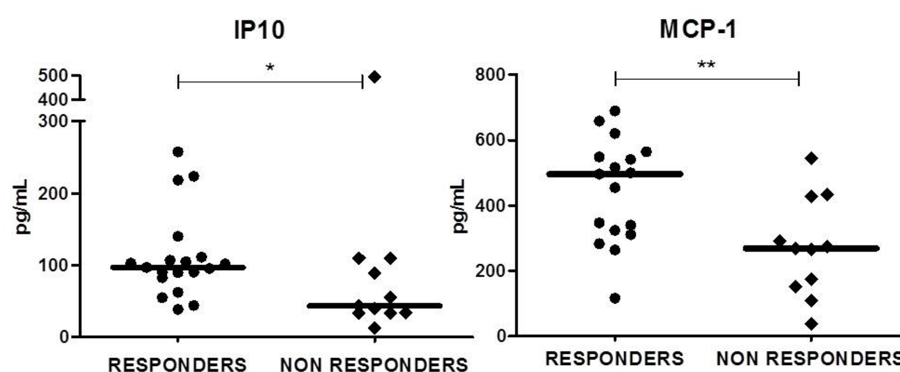

Figure 34 Long-term IFN-β treated RR-MS patients presented with higher circulating levels of IP10 (*p=0.04) and MCP-1 (**p=0.008). Mann-Whitney U test. Medians and individual dots shown.

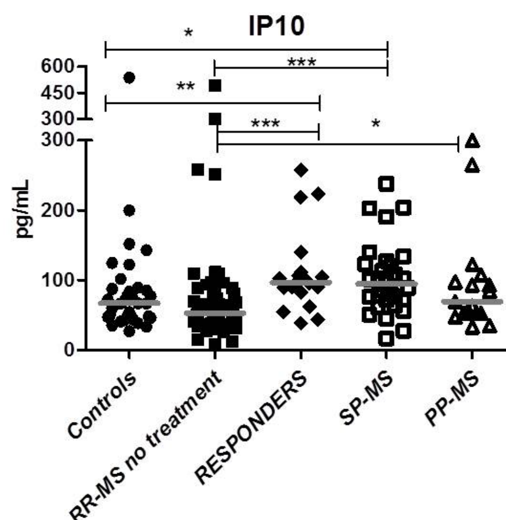

Figure 35 Plasma levels of IP10/CXCL10 in the different clinical groups and healthy controls (n=36). The groups of RR-MS patients without treatment (n=58) had significantly lower levels of IP10 than long time Interferon-β responders (RESPONDERS, n=19) (***p=0.0003), than Secondary-progressive MS patients (SP-MS, n= 31) (***p=0.0001) and than Primary-progressive MS patients (PP-MS, n=18) (*p=0.05). Levels of IP10 were more increased in RESPONDERS compared to healthy controls (**p=0.005) and in SP-MS with respect to healthy controls (*p=0.01). Mann-Whitney U test. Medians and individual dots shown.

In order to analize the ex vivo production of IP10 to IFNβ in MS patients we observed the plasma levels of IP10 in a subgroup of patients with RR-MS in the active disease that were about to start with IFNβ. Blood was drawn before the first injection, after 10 hours, after 32 hours, and after 1 month (10 hours post injection). Results are shown in Figure 36.

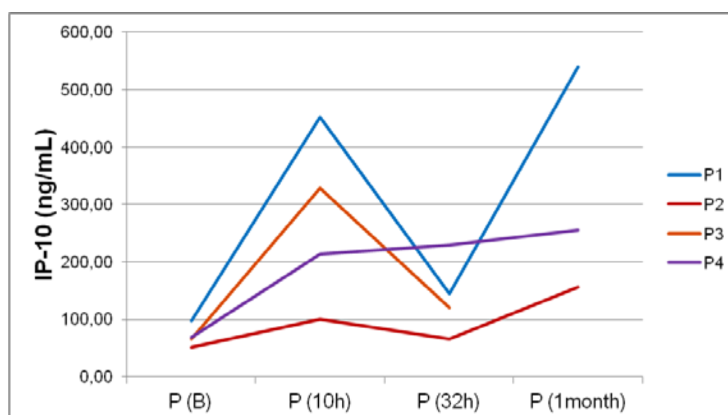

Figure 36 Plasma levels of IP10/CXCL10 were monitored in four patiens starting IFNβ therapy.

The increase of IP10 plasma levels were not as remarkable as those observed by our group in patients with hepatitis C virus (HCV) starting treatment with peg–IFN-α2/ribavirin therapy [1]. As IFNα and IFNβ share the same receptors on the surface of T cells (IFNAR1 and IFNAR2) we expected to find higher plasma levels of IP10 after IFNβ treatment. Therefore, we decided to continue with this line of investigation and studied the IP10 production in whole blood after stimuli with IFNα and IFNβ in two RR-MS male patients and two male PP-MS patients and 4 male healthy controls. We observed a similar intracellular production of IP10 after stimuli with IFNα (1,000 IU/mL and 250 IU/mL) and IFNβ (1,000 IU/mL and 250 IU/mL) in all the subjects studied (Figure 37) and ELISA IP10 results in the plasma was also very similar in all of them after 24 hours at both concentrations, reflecting a normal response to type 1 IFN in the MS patients studied.

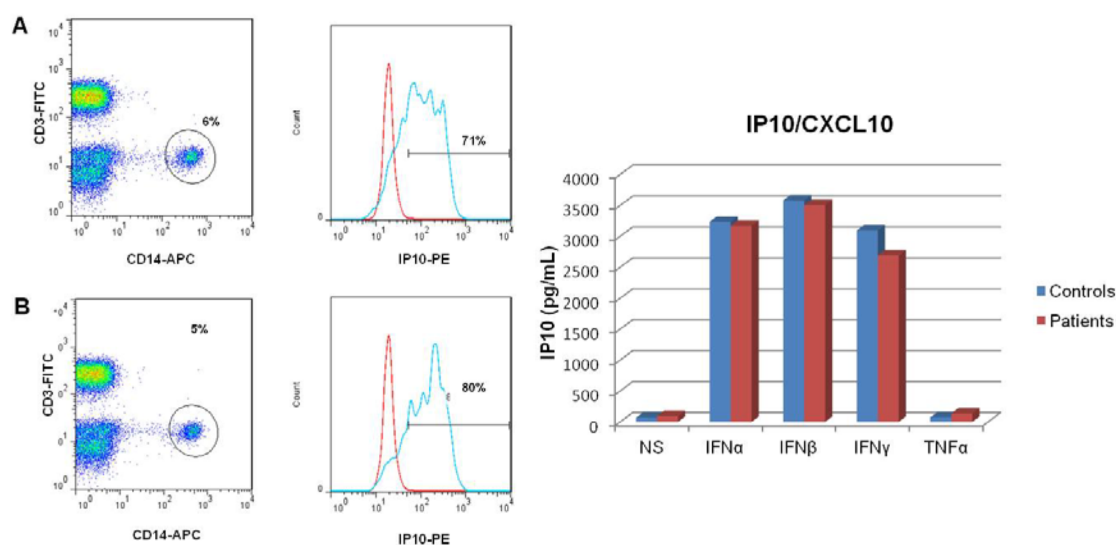

Figure 37 **(A)** Intracellular expression of the chemokine IP10 in the peripheral blood monocytes of a representative patient after IFNα stimulus (1,000 IU/mL) and **(B)** IFNβ-1a (1,000 IU/mL). The adjacent graph displays the mean protein expression of IP10 in the plasma of the blood stimulated.

4.2.2.7. CXCR3 cell surface expression on circulating T cell subsets

The analysis of the cell surface expression of CXCR3, receptor of the chemokine CXCL10/IP10, revealed that the different clinical groups had a different CXCR3 expression on the surface of their T cells subsets.

Higher circulating frequencies of $CD3^+CXCR3^+$ T cell subset were observed in the PP-MS patients with respect to SP-MS patients ($p=0.009$); RR-MS patients in remission ($p=0.02$); non responders to IFNβ ($p=0.02$) and to healthy controls. PP-MS patients presented the highest circulating frequencies of $CD3^+CD4^+$ $CXCR3^+$ T cells among clinical forms of MS. Interestingly, frequencies of $CD3^+CD8^+$ $CXCR3^+$ T cells were the lowest in SP-MS patients ($p=0.01$ vs PP-MS; $p=0.009$ vs Relapses; $p=0.02$ vs RR-MS Act; $p=0.03$ vs HC). The fluorescence at the single cell level, median fluorescence intensity (MFI), of the three cell subsets was higher in the groups at RR-MS patients with active disease ($p<0.05$ vs HC) and relapse ($p<0.05$ vs progressive forms) (Figure 38).

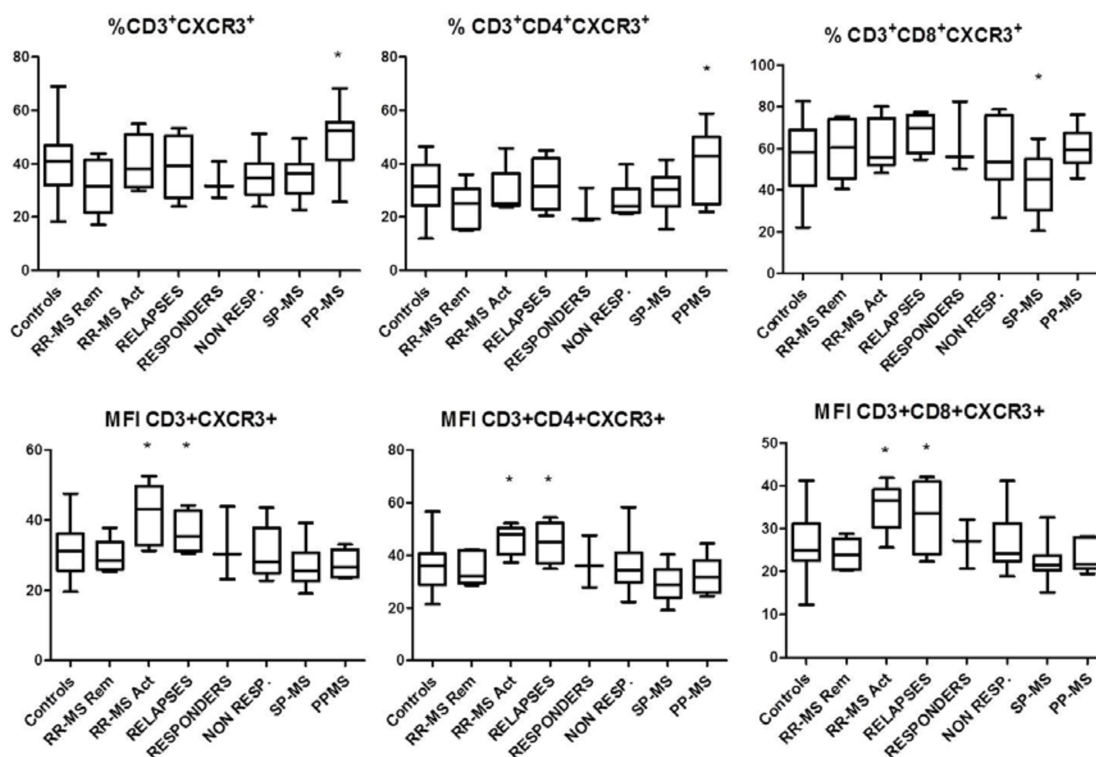

Figure 38 Circulating frequencies of $CXCR3^+$ T cells and mean fluorescence expression in the different clinical forms studied from the Validation cohort.

4.2.2.8. Gene markers in Responder MS patients to IFNβ

The analysis of a broad arrange of genes related with type I Interferon signature and with MS pathogenesis, enabled the search of selective genes stimulated by IFNβ in those patients who were under treatment with IFNβ and with a good clinical response. The comparison of the mRNA levels from PBMCs of the different clinical groups defined in this study enabled the identification of genes that were significantly overexpressed in the group of RESPONDERS to IFNβ than in the rest of clinical groups and than the cohort of healthy controls studied (Figure 39).

In an additional small cohort (n=4) of patients that were studied before starting treatment with IFNβ, after 10, 32 hours from the first injection of IFNβ and after 1 month (after 10 hours from the last injection); we observed an induction of most of the genes observed in the group of long-term responders to IFNβ; MCP1, MDA5, RIG-I, MX1, EIF2AK2, IL1RN, RSAD2 and TRIM25. This may reflect the maintained effect of IFNβ in gene induction (Figure 40).

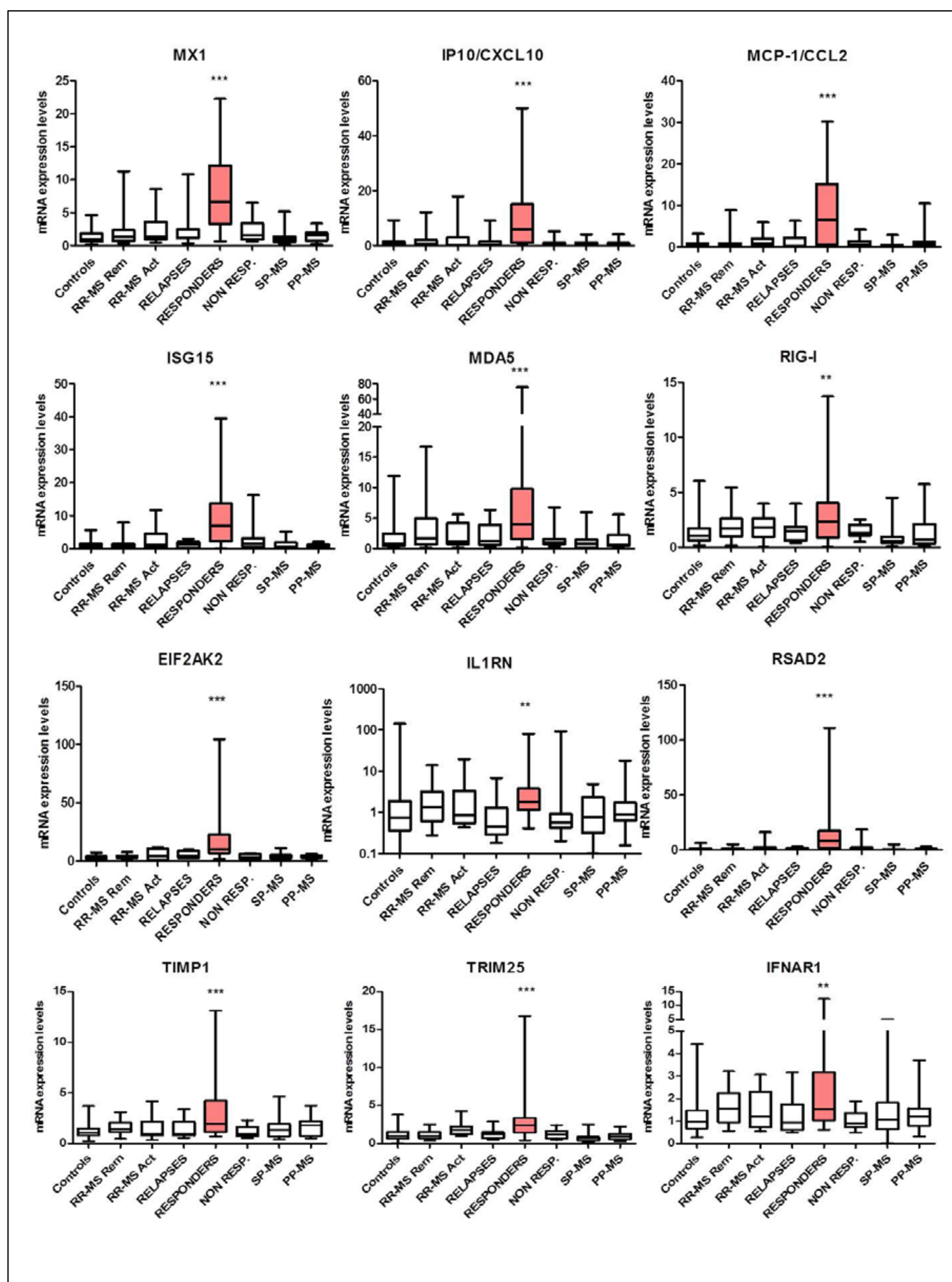

Figure 39 The genetic expression of different genes was enhanced in the peripheral blood mononuclear cells of patients under treatment with IFNβ (***p<0.0005, **p<0.005 Responders vs healthy controls).

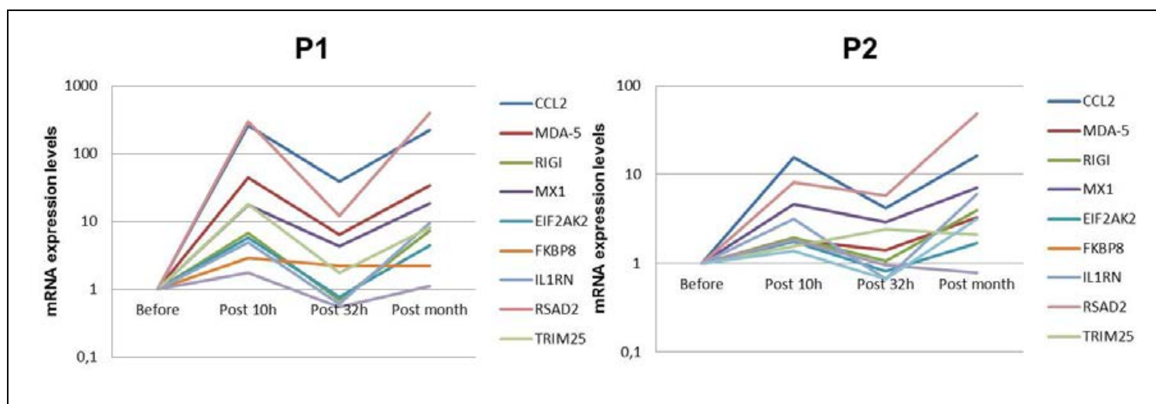

Figure 40 Changes in gene expression in patients starting IFNβ treatment. Time points: Before therapy, ten hours after the first injection with IFNβ, 32 hours after the first injection with IFNβ, and 1 month after the beginning of therapy but 10 hours after the last injection of IFNβ. In the figure are represented two of the patients as representative examples.

The gene expression of STAT (signal transducer and activator of transcription) molecules: STAT1, STAT2 and STAT 3; clue molecules in the downstream signaling of type I IFNs, was also increased in MS patients under long-term therapy with IFNβ (Figure 41). STAT6 gene expression was higher than in healthy controls (p=0.03) but not higher than untreated MS groups.

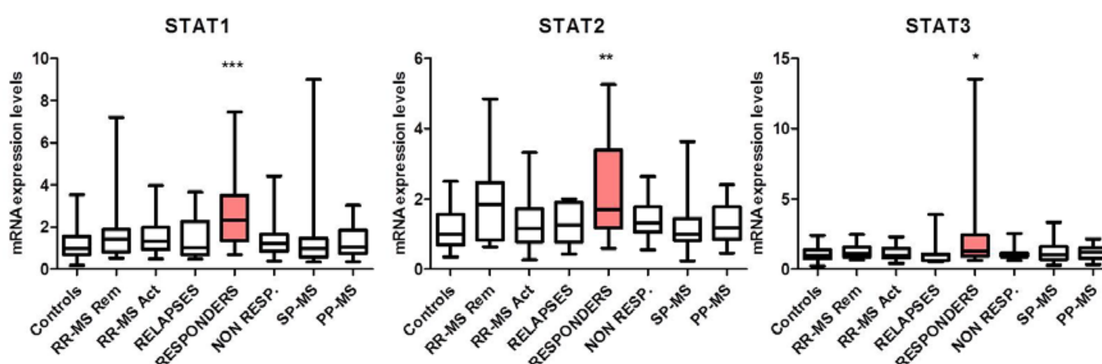

Figure 41 Genetic expression of STAT 1, 2 and 3 was enhanced in the peripheral blood mononuclear cells of patients under treatment with IFNβ (***p<0.0001, **p<0.005, *p<0.05 Responders vs healthy controls).

4.3. STUDY OF THE TYPE I INTERFERON SIGNATURE BY TLR3 STIMULATION IN MS

A series of functional assays with 13 MS patients and 11 HC were performed to test the hypothesis of whether the stimulation of TLR3, by different synthetic ligands (polyinosinic-polycytidylic acid, poly(I:C) and polyadenylic–polyuridylic acid poly(A:U)), in cultured PBMCs from MS patients induced an altered production of type I IFN measured by ELISA.

PBMCs were stimulated *in vitro* with different mitogens and antigens: soluble phytohemagglutinin (PHA) and soluble anti-CD3 and anti-CD28 (1μg/mL) in the presence or absence of the ligands of TLR3 (poly(I:C): 25 μg/mL and poly (A:U): 50μg/mL). As previously mentioned, we observed that in 61% of MS patients (8 out of 13), their PBMCs showed a marked proliferative defect to soluble anti-CD3 and anti-CD28 with normal proliferation to PHA (2 μg/mL) (Figure 43) with respect to the healthy controls included in the study. This defect in proliferation was only observed with soluble anti-CD3 and anti-CD28 at 1 μg/mL, in conditions with anti-CD3 bound to the plate at 5 μg/mL, the proliferation was restored.

The addition of the ligand of TLR3 poly(I:C) enhanced the proliferation of $CD4^+$ T cells only in the patients with defective proliferation to soluble anti-CD3 and anti-CD28 (Figure 42). The addition of exogenous IFNα (1,000 IU/mL) to soluble anti-CD3 and anti-CD28 TCR stimulus showed anti-proliferative effects in patients with normal proliferation.

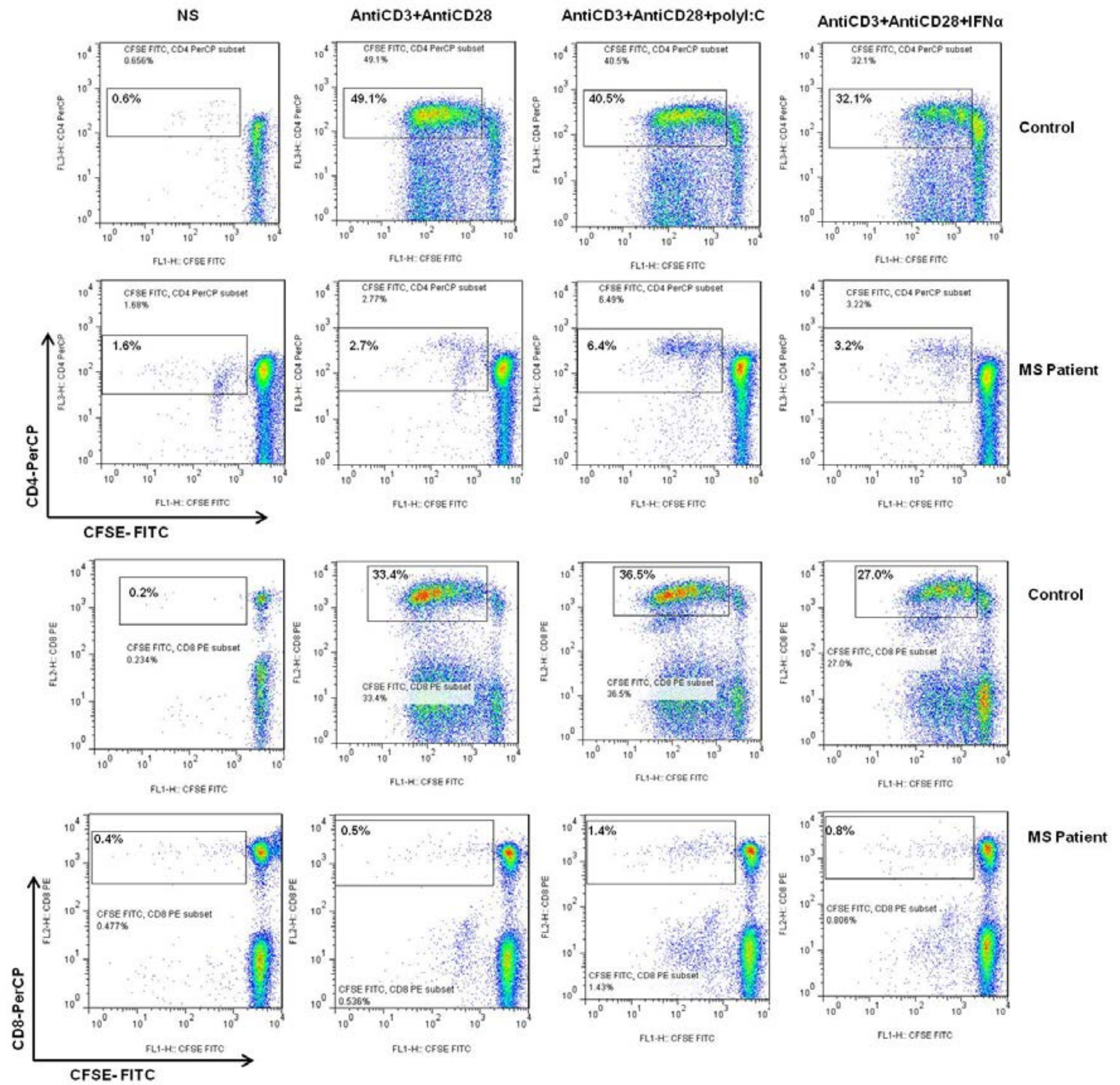

Figure 42 Representative examples of a healthy control with normal proliferation and an MS patient with defective proliferation to soluble anti-CD3 and anti-CD28. $CD4^+$ and $CD8^+$ T cells under the conditions: no stimulation, soluble anti-CD3 and anti-CD28, anti-CD3 plus anti-CD28 plus poly(I:C) (25 µg/mL) and anti-CD3 plus anti-CD28 plus IFNα (1,000 IU/mL). A modest increase in the proliferation of $CD4^+$ T cells was observed when the agonist of TLR3, poly(I:C) was added to soluble anti-CD3 and anti-CD28.

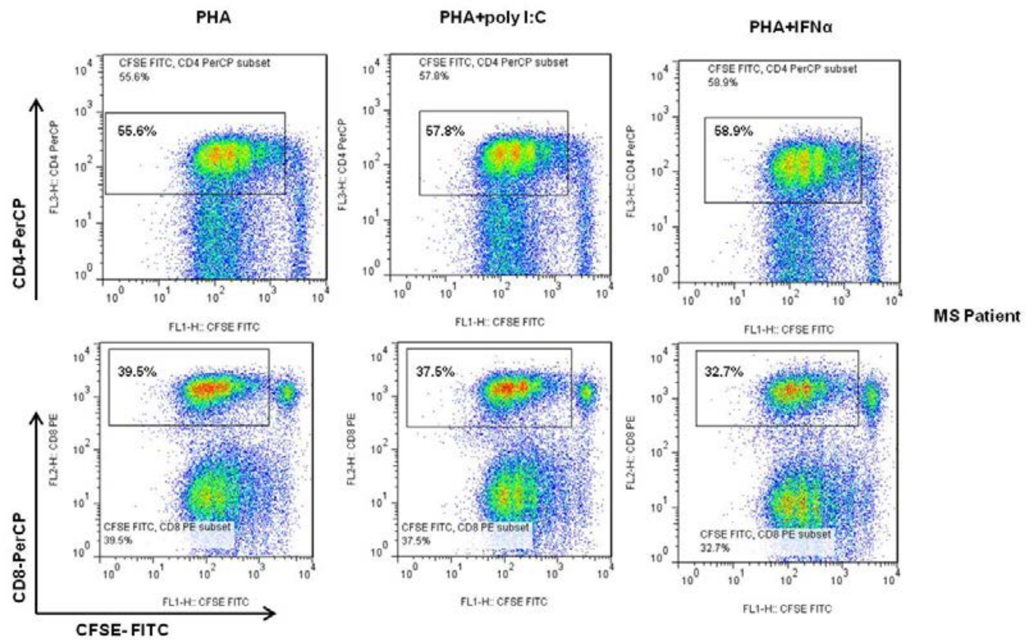

Figure 43 Representative example of the MS patient shown in Figure 39 with normal proliferation to PHA. $CD4^+$ and $CD8^+$ T cells under the conditions: no stimulation, PHA, PHA and poly(I:C) (25 µg/mL) and PHA plus IFNα (1,000 IU/mL).

The expression of the activation molecule CD69 on T cells was analyzed after stimulation of PBMCs with soluble anti-CD3 and anti-CD28, PHA and PMA/Ionomycin. CD69 expression on $CD4^+$ and $CD8^+$ T cells stimulated with PHA or PMA/Ionomycin was similar in MS patients and healthy controls (Figure 44), whereas CD69 expression on $CD4^+$ and $CD8^+$ T cells was diminished with respect to healthy controls in those patients with a defect in the proliferation to soluble anti-CD3 and anti-CD28 (Figure 44).

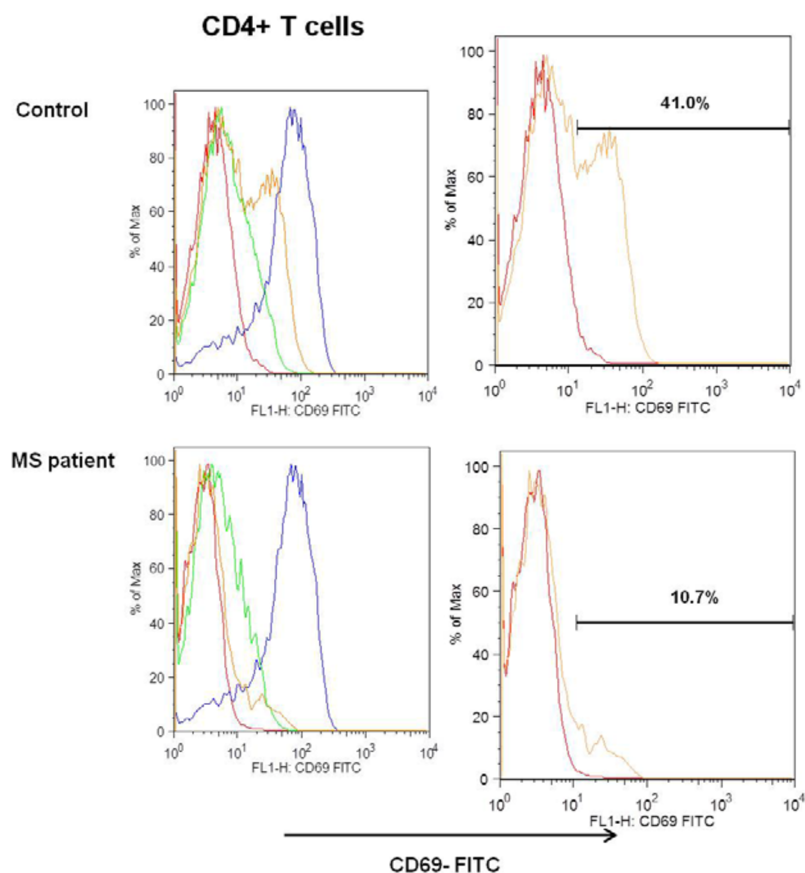

Figure 44 Activation of $CD4^+$ T cells from a healthy control and a patient with MS. CD69 expression in $CD4^+$ T cells after stimulation with PHA (blue line), PMA/Ionomycin (green line), soluble anti-CD3 and anti-CD28 (orange line) or unstimulated (red line). Numbers represent the percentage of $CD69^+$ cells. Cells were stimulated for 24 hours.

A single experiment was performed with stimulated PBMCs for 24 hours with poly(I:C) and poly(A:U) of an MS patient and a healthy control. The gene expression results by BioMark revealed that after 24 hours in the presence of the agonists of TLR3, poly(I:C) and poly(A:U), genes upregulated by type I IFN were differentially induced in the patient and the healthy control (ISGs) (Figure 45).

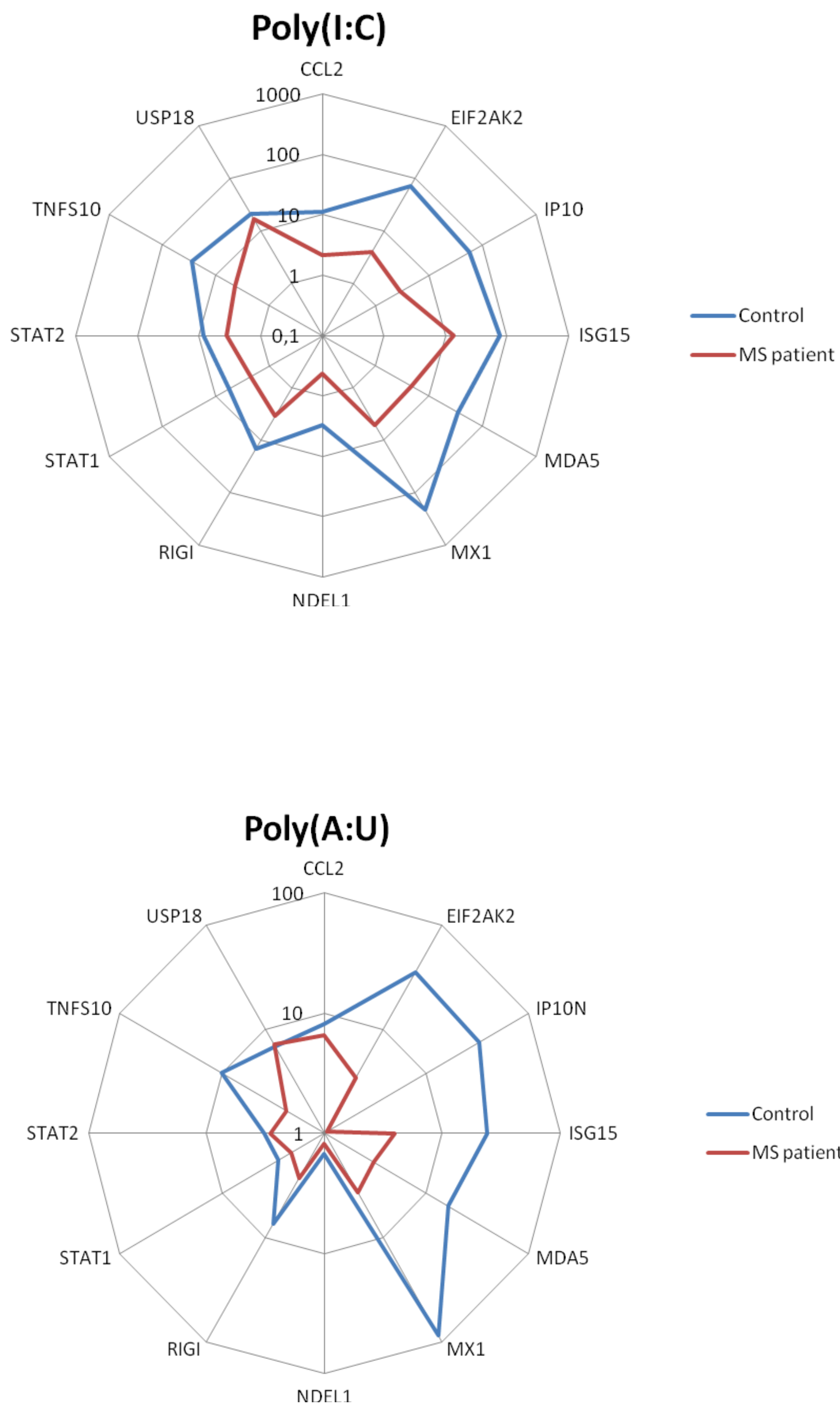

Figure 45 Induction of interferon stimulated genes after stimulation of PBMCs for 24 hours with the TLR agonists poly(I:C) and poly(A:U). The numbers represent the mRNA levels normalized to actin beta and expressed as fold increase.

The levels of IFNα after 24 hours in the cell culture from this patient supernatants were decreased with respect to the healthy control after stimulation with poly(I:C), the levels of I IFNα after stimulation with non redundant poly(A:U) were very low, both in the healthy control and the patient with MS (Figure 46).

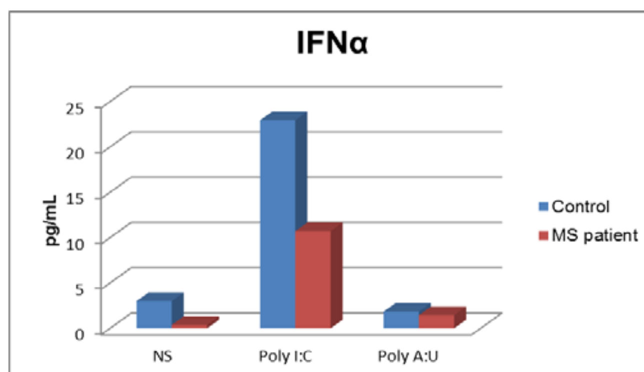

Figure 46 IFNα production, measured by ELISA, in PBMC ($2*10^6$) in response to poly(I:C) and poly(A:U).

The supernatants at 24 hours from different experiments performed were analyzed to test whether the production of type I IFN was impaired in the rest of MS patients. No differences in the production of IFNα by poly(I:C) stimulated PBMCs between the healthy controls and MS patients (Figure 47).

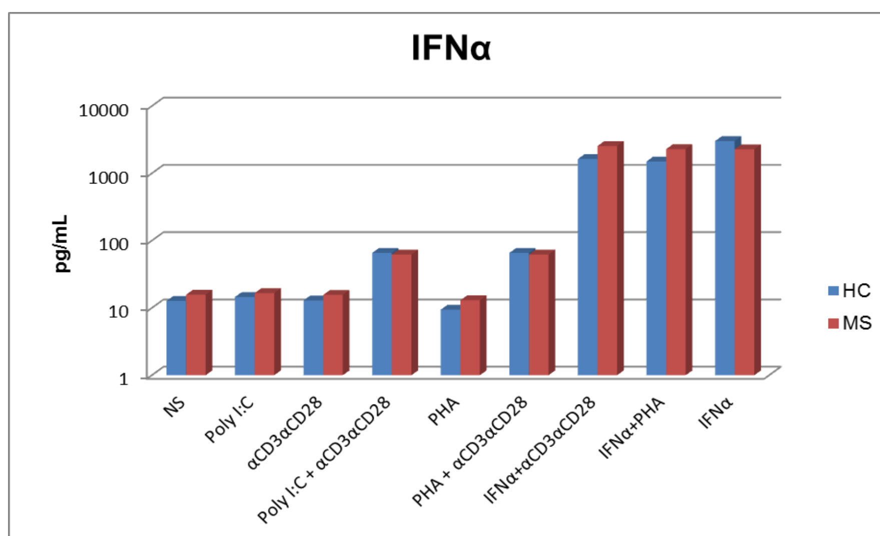

Figure 47 IFNα production, measured by ELISA, in PBMC (10^5) after different stimuli.

5. DISCUSSION

Multiple sclerosis (MS), the most common cause of neurological disability in young population after trauma, represents a significant personal, social and economic public health burden. MS is a chronic autoimmune inflammatory disorder of the CNS characterized by multiple demyelination lesions, axonal degeneration and oligodendrocyte and neuronal loss. Although CNS MRI and the presence of oligoclonal bands (OB) in the CSF have greatly helped in the diagnosis of MS, they are not capable of discriminating in a reliable and reproducible way between the inflammatory and progressive forms at MS onset or to predict evolution. The current challenges associated with MS stem from the lack of serum biomarkers that enable to distinguish MS patients from healthy controls and to discriminate among the different clinical forms of MS. Identification of such minimally invasive biomarkers of MS in the individual patient is an important issue. These biomarkers can also help to shed light on MS pathogenesis.

The present investigation shows a significantly decreased expression of DPP4 and DPP activity in the plasma of MS patients with respect to healthy controls, which suggests that DPP4 may have a role in the pathophysiology of MS. In addition, DPP activity correlated inversely with clinical disability score in MS. Among the most relevant analytes capable of differentiating between MS patients and healthy controls, the enzyme DPP4, IL-7, IL-1RA and Mig/CXCL9 were identified in this study. On the other hand, our data demonstrate that the divergent clinical and histology-based MS forms are associated with distinct profiles of circulating biomarkers, mostly chemokines and growth/angiogenic factors (HGF, Eotaxin, MCP-1, Rantes, EGF, MIP-1β, VEGF and FGFb); and different gene expression levels in their PBMCs (CLU, IRF-2 and LDLR). Finally, responder patients to type I IFN displayed high levels of plasma IP10 and MCP-1, and a specific expression pattern of IFN stimulated genes.

The molecule DPP4 is a multifunctional protein with important enzymatic and costimulatory acitivies. This is the first study to analyze simultaneously the plasma levels of sDPP4, DPP activity and DPP4 expression on T cells, in a large cohort of MS patients with different clinical forms. We report that MS patients show significantly diminished plasma levels of sDPP4 and lower DPP activity than healthy controls. These findings are in agreement with those

described in other autoimmune diseases such as systemic lupus erythematosus (SLE) [386] and rheumatoid arthritis (RA) [275], and may point out an underlying immunoregulatory and T cell activation defect in the pathophysiology of diverse autoimmune diseases.

MRI has dramatically transformed the horizon of MS, enabling an earlier diagnosis and treatment, and still only the presence of IgG OB within the CSF is used as routine biomarker. OB require a lumbar puncture, which is an invasive procedure with high sensitivity up to 95% but with less specificity as OB are present in other inflammatory CNS disorders [319]. OB are suggested to represent an altered B-cell activation within the CNS compartment. To this point, our results on plasma sDPP4 would represent an easy, non cruent and reproducible biomarker, as we have validated in two independent cohorts of MS patients and controls. Low levels of sDPP4 may suggest a T-cell impairment influencing the defects in B cell tolerance with subsequent intrathecal B-cell oligoclonal expansion. A preliminary cut-off level of 622 ng/mL of sDPP4 in our two independent cohorts showed a global PPV of 92.5% for MS. An effort should be made to further confirm our findings on the value of sDPP4 in prospective and larger series of MS patients and to compare with other non inflammatory neurological conditions.

The classification of MS patients according to their clinical forms has enabled us to observe differences in sDPP4 among the MS groups of patients. Interestingly, patients at active phase of the disease (RELAPSE and RR-MS Active) and progressive forms (SP-MS and PP-MS) had significantly lower levels of sDPP4 than non-active MS patients (RR-MS at remission). Moreover, DPP activity negatively correlated with EDSS. Accordingly, an inverse correlation of sDPP4 and disease activity was also observed in SLE patients [386]. The sDPP4 molecule may be modified post-translationally by glycosylation or sialation. Partial degradation may affect the detection of circulating sDPP4 by conventional ELISA. For instance, in RA the reduction in DPP4 activity was due to the glycosylation of DPP4 [387], whereas in SLE and Sjögren´s syndrome it was due to a reduced sDPP4 concentration [386, 387]. As circulating levels of sDPP4 in our MS patients' cohort correlated with the DPP activity, the reduced DPP activity is likely a reflection of lower levels of sDPP4.

Among MS patients, markedly low sDPP4 and the lowest DPP activity was found in the SP-MS group, a finding that was observed in both independent cohorts. Measuring DPP activity gives additional information to that of sDPP4 alone, as low DPP activity is associated with the progressive phase of the disease.

The current paradigm on MS pathophysiology states that circulating autoreactive CD4+ T cells migrate through the BBB to the CNS. CSF T cells express the chemokine receptor CXCR3 that possibly facilitates the entrance of T cells into the CNS [37]. Immunohistochemical studies of autopsy brain sections from MS patients have shown specific CXCR3 expression on most of the perivascular lymphocyte infiltrates, together with the expression of its ligand IP10/CXCL10. In the later stages of MS, the number of accumulated CXCR3^{+} T-cells in the intrathecal lesions was higher than in the early stages of the disease [265]. In this study, we found that PP-MS patients presented higher circulating frequencies of CD3^{+}CXCR3^{+} T and CD4^{+}CXCR3^{+} T cells than healthy controls and than the other groups of patients, while SP-MS patients had lower circulating frequencies of CD8^{+}CXCR3^{+} T cells. Balashov *et al.* [265] reported that peripheral CXCR3^{+} T cells with a skewed Th1 phenotype were increased in patients with chronic progressive MS. The high frequencies of circulating CD3^{+}CXCR3^{+} T cells in PP-MS may be reflecting a chronic activation of the immune system. Moreover, the expression at the single cell level (MFI) of CXCR3 in T cells was significantly higher in RR-MS patients with active disease and in RR-MS patients undergoing a clinical relapse, what would reinforce the role of these cells in immune activation. A direct correlation in the MS group was detected between sDPP4 expression and MIP-1α/CCL3 and MIP-1β/CCL4; and between DPP activity and IP10/CXCL10 in MS patients at relapse. According to previous findings of the group of Dr. Albert [1, 388], DPP activity would mainly affect chemokine gradients and might be driving the effector autoimmune response through the impaired regulatory loop. The data presented here compel new evidence of an underlying immunoregulatory defect in MS that could be playing a role in MS pathophysiology.

Previous studies have implicated the subset of CD4^{+}DPP4^{hi} T-cells in the pathogenesis of MS [281, 286, 289-291]. Hafler *et al.* were the first to describe CD4^{+} T-

cells coexpressing DPP4 in the CSF and peripheral blood of patients with progressive MS; naming this antigen T-cell–specific activation antigen Ta1+ [389]. Later on, several studies correlated $CD4^+DPP4^+$ and $CD4^+DPP4^{hi}$ T-cell subset with active MRI lesions and with clinical disease activity [281, 289-291]. In our MS population, $CD4^+DPP4^{hi}$ T-cell subset correlated with clinical disease activity in RR-MS patients. In addition, we found significantly higher percentages of circulating $CD8^+DPP4^{hi}$ T and $CD16^+CD56^+DPP4^+$ NK cells in MS patients than in healthy controls. An altered expression of DPP4 on the surface of NK cells has been reported in other diseases such as SLE [390] and chronic fatigue syndrome [391]. The overexpression of DDP4 on NK cells seems to regulate their proliferation and not to affect their cytotoxic function [392]. Thus, the increase in circulating frequencies of $CD16^+CD56^+DPP4^+$ NK cells may be reflecting activation of these cells in MS patients. Increased percentages of $CD8^+$ T cells coexpressing DPP4 have also been noted in other MS studies correlating with active MRI lesions [292]. This expanded subset of memory $CD8^+$ T-cells coexpressing DPP4 may show high migratory capacity, similarly to that described for $CD4^+DPP4^+$ T cells by Brezinschek *et al.* [393] given the role of membrane DPP4 on a relevant set of chemokines. Indeed, high numbers of $CD8^+$ T-cells of MS patients at acute phase of the disease that display more increased adhesion capabilities and migratory capacity than $CD4^+$ T-cells have been described, underlining a CD4/CD8 dichotomy in MS [394]. $CD8^+$ T-cells are the final effector cells inducing demyelination and axonal injury [395, 396], and the magnitude of the presence of CD8 T-cells at brain MS lesions is the best correlate with MS clinical severity [397, 398]. Jensen *et al.* observed that treatment with IFNβ decreased the expression of DPP4 on $CD8^+$ T cells. Our group has previously shown that, although MS patients disclose higher percentages of memory $CD8^+$ T-cells than healthy controls at baseline, IFNβ was able to significantly decrease $CD45RO^+$ memory $CD8^+$ T-cell subset while to expand $CD4^+$ and $CD8^+$ regulatory T-cells in a prospective 1-year follow-up study [399]. In our cohort of MS patients, the group under treatment with IFNβ (RESPONDERS) showed low levels of sDPP4. In contrast, this group showed the highest DPP activity among all groups of MS patients and the highest IP10/ CXCL10 concentration. Long-term treatment with IFNβ seems to restore DPP

activity to similar levels of those of healthy controls. Although this is a cross-sectional study and cannot analyze whether DPP activity could be an interesting biomarker of response to IFNβ, prospective studies should be performed to confirm our preliminary findings.

Apart from T cell activation marker, DPP4 is an alternative co-stimulatory molecule (reviewed by [400]). The crosslinking of DPP4 and CD3 with immobilized monoclonal antibodies can induce T-cell costimulation with the absence of antigen presenting cells [279]. In the clinical setting of MS, Fransson *et al.* described that patients with RR-MS in remission had poor proliferative capacity to anti-CD3 antibody and suggested that these cells were kept in anergy [401]. In some MS patients, we have observed a defect in PBMCs proliferation to anti-CD3 plus anti-CD28 antibodies that was restored after more potent T stimulation. In those patients with a T-cell proliferative defect, sDPP4 was undetectable in the supernatants of the cell cultures, while sDPP4 was detected in the cell cultures that proliferate and at similar concentrations as in healthy subjects, further pointing to an impaired T cell fitness in some patients with MS and to a secondary down-modulation of sDPP4 shedding. Moreover, the source of the sDPP4 present in the cell supernatants most probably came from the shedding of the PBMCs in proliferation. The origin of sDPP4 is still unclear, the hepatobiliary system and the shedding from peripheral blood T cells have been proposed as possible major sources [275].

sDPP4 concentration shows sex differences in healthy controls, with higher circulating levels of sDPP4 in men than in women, but these differences were lost in MS patients, which reinforces its use as a biomarker. Durinx *et al.* described that DPP4 activity in healthy men was slightly higher than in healthy women in a large population study [402]. In this work, we also observed a higher DPP activity in men than in women at any point of the menstrual cycle. We observed that circulating levels of 25(OH)-vitamin D showed an inverse correlation with the activity of DPP in MS patients and this negative correlation was skewed for MS women and not for MS men. Therefore, we observed that MS women with high vitamin 25(OH)-vitamin D levels had lower DPP activitiy, suggesting that vitamin D may affect differently women and men with MS in the enzymatic activity of DPP. The *in vitro* stimulation of PBMCs with the active

form 1,25(OH)$_2$ vitamin D$_3$ showed that DPP4 gene and protein expression was enhanced. Based on the clinical association between MS and vitamin D deficiency, the relationship with DPP activity warrants additional investigation.

In summary, our data suggest that the evaluation of sDPP4 and of DPP activity might provide additional information for clinical activity in MS patients. We can hypothesize that lower sDPP4 concentration and DPP activity might drive biased chemokine effects and highlight a novel role of this molecule in MS pathophysiology. Further larger and prospective studies should be accomplished to evaluate the clinical value of sDPP4 as an additional non cruent surrogate biomarker for MS.

In addition to the plasma analyte sDPP4, other proteins presented lower levels in MS patients compared to healthy controls, namely IL-7, IL1-RA and Mig/CXCL9.

The interleukin 7 (IL-7) is essential for T cell development and α-chain subunit of the receptor of IL-7 (IL-7Rα/CD127) is also present on the surface of B cell progenitors [403]. Genetic polymorphisms of IL-7Rα are associated with a higher risk of developing autoimmune diseases including rheumatoid arthritis [404], type 1 diabetes mellitus [405] and MS [86]. In the present study, we found that our cohort of MS patients disclosed lower plasma IL-7 compared to healthy controls. Our results are in agreement with those recently published by Kreft *et al.* [375] and in contrast with other authors that described increased levels of IL-7 in MS patients [406]. This may be due to the small sample size of published MS series and the heterogeneous distribution of patients. In our work, we studied a larger number of total patients with different clinical forms and we found that the group of non responder patients to IFNβ had the lowest IL-7 levels. In accordance, high serum IL-7 together with paired low IL-17F could be good biomarkers to identify those patients with Th1-driven MS that could respond to IFNβ treatment [376].

On the other hand, we observed that our MS patients uniformly expressed lower plasma sIL-1RA than healthy donors, which may be accounting for impaired antiinflamatory compensatory mechanism. Indeed, sIl-1RA is involved in the regulation of immunoinflammatory reactions as has been shown in IL-1RA

knockout mice that develop excessive inflammatory responses [378] and in sIL-1RA deficient children that present with autoinflammatory syndrome [407]. Long-term responders to IFNβ presented remarkably higher mRNA levels of IL1RN (gene than encodes IL-1RA). This is in agreement with an increase in the circulating IL-1RA detected ex vivo after therapy with IFNβ [380, 381, 408].

Finally, circulating Mig/CXCL9 was also found uniformly decreased in MS patients, with the exception of patients on IFNβ. By contrast, Mig and its related chemokine IP10, have been described to be increased in the CSF of MS patients, together with the overexpression of their receptor CXCR3 on T cells [263], suggesting that these cells might be recruited through a chemokine gradient into the CNS. Patients under treatment with IFNβ had higher Mig and IP10 levels, probably accounting for the effect of the drug in these chemokines.

The multivariate logistic regression model combining three biomarkers sDPP4, IL7 and Mig discriminated between the dichotomous variables MS/non- MS with an ameliorated sensitivity than individual proteins of 96.5% and classification accuracy of 79.7%. This study proposes the use of combinatorial biomarker analysis as an alternative tool with a better discriminating value. The results of the proposed model deserve further clinical validation in larger MS series.

An important goal of this study was to identify biomarkers enabling discrimination among clinical MS forms, in particular between RR-MS and progressive forms (SP-MS and PP-MS) which may be have relevant clinical implications.

To the best of our knowledge, this study is the first to describe significant differences in circulating levels of FGFb, HGF, VEGF, Eotaxin, MCP-1 and Rantes discriminating among MS clinical forms. The biomarker analysis described herein provides a revealing cross-section of the pathophysiological MS conditions. There is an urgent need to recognize and predict outcome in the individual MS patient that could enable more focused treatment strategies. Therefore, the identification and development of targeted therapies has moved to the forefront of the MS translational research.

This study shows that low expression of FGFb/FGF-2 is a selective marker of PP-MS, probably reflecting low remyelination related to progressive

neurodegeneration. FGFb is an emerging MS biomarker with a controversial role in promoting myelination by oligodendrocytes [315-317], since the knockout mice of FGFb in EAE showed that this growth factor has a neuroprotective/regenerative role avoiding nerve fiber degeneration and axonal loss and favoring remyelination in the CNS [318]. In MS, FGFb is increased in the CSF and serum of MS patients, with the highest levels in clinically active MS patients undergoing relapse and in SP-MS patients with disability progression [409]. FGFb is a main mitogen of oligodendrocyte precursor cells (OPCs), mainly expressed at MS periplaques where macrophagic and/or activated microglial and perivascular astrocyte-related remyelination, further supporting its role in neurorepair and neuroprotection, respectively, as well as in maintaining the integrity of the BBB [314]. We found that patients with PP-MS had significantly lower levels of FGFb than the other clinical groups, representing a distinctive PP-MS feature. In contrast, patients with SP-MS and patients at relapse showed similar levels to those of healthy controls. This finding is in agreement with the study by Sarchielli *et al.* who reported an elevation of FGFb in the CSF of MS patients, and the highest levels at relapse and in SP-MS patients with recent increase in disability [409]. FGFb might be playing a compensatory role during the inflammatory attack and during the axonal insult that occurs at these stages of the disease. We speculate that PP-MS patients with markedly low FGFb circulating levels may be pointing to an impaired production with respect to other MS clinical forms, suggesting an additional defect in PP-MS to restore myelination and explain their rapid disability progression compared to other patients. FGFb could be used as a future therapeutic target to induce effective migration of MS lesions by olygodendrocite precursor cells and to favor remyelination of lesions.

In addition to plasma FGFb, another distinctive biomarker for PP-MS was the higher gene expression in PBMCs of the low density lipoprotein receptor (LDLR) compared to that in untreated RR-MS patients, SP-MS patients and healthy controls. The protein coded by LDLR gene is a cell surface LDL receptor essential in cholesterol homeostasis [410] and also a receptor for HCV virus and other viruses [411]. We observed an increased mRNA expression in PP-

MS patients what may suggest a different pathogenic mechanism in these patients that deserves further investigation.

The SP-MS form was characterized by overexpression of the vascular endothelial growth factor (VEGF) with respect to RR-MS and even to PP-MS patients, a prominent player in the complex and highly regulated process of angiogenesis and a proinflammatory factor [412-415] suggesting its role in the RR-MS to the SP-MS transition. An increase of VEGF-A in the initial phases of relapse is compatible with its pro-inflammatory activity that attracts monocytes and lymphocytes, upregulates immunomodulatory adhesion molecules, stimulates secretion of proinflammatory cytokines, and increases BBB permeability [416, 417]. Evidence for the occurrence of neovascularization in MS has been observed by contrast-enhanced MRI in the appearance of “ring enhancement” at the periphery, but not at the center of chronic lesions [418]. Another MRI study showed a direct correlation between VEGF levels and the magnitude of spinal cord lesions, suggesting that VEGF might be involved in MS spinal cord lesions [419]. In agreement, increased VEGF and its receptor VEGFR-1 are found in astrocytes in MS plaques during the inflammatory phase [420, 421]. As a direct evidence of this, intrastriatal injection of VEGF aggravates plaque inflammation at the site of injection [421]. Moreover, as VEGF expression is highly influenced by inflammatory cytokines and ischemia, the accumulation of VEGF may be not only a mediator but the result of MS inflammation. While all these findings may suggest that VEGF aggravates MS but may act also as a neuroprotective factor, protecting against axonal damage in MS. Thus, the precise role of VEGF in MS remains enigmatic. Possibly, VEGF exerts a dual role in MS lesions: increased levels of VEGF can amplify vascular permeability and thus inflammation through glial cells during the acute phase of the disease, but can also stimulate the proliferation of neurons and their axons during the chronic phases of the disease [417]. Recent evidence described low RNA VEGF levels in CSF and blood PBMCs of SP-MS patients with respect to RR-MS [422].

Of note, here we propose a suitable method for classification of MS clinical forms. By using a multivariate logistic regression model combining four relevant biomarkers, there was significant overall model fit discriminating a dichotomous RR-MS versus progressive forms, with a specificity of 89.9% and classification

accuracy of 82.6%. In all the MS patients studied, having normal (similar to HC) plasma HGF and Eotaxin levels and low plasma EGF and MIP-1β levels were prognostic risk factors for being classified as a progressive patient with MS (SP-MS or PP-MS). At the RR-MS stage of the disease, HGF levels were diminished with respect to progressive clinical forms; and circulating levels of the β-chemokines MCP-1/CCL2, Eotaxin/CCL11 were diminished with respect to progressive clinical forms and to healthy controls, suggesting a potential role for these diminished molecules in the initial stages of MS pathogenesis. MCP-1/CCL2 levels have been reported to be decreased in MS patients [264, 384, 423-425] although its expression in MS lesions is increased [271]. MCP-1/CCL2 stimulates *in vitro* the production of HGF by a mouse macrophage cell line [426] and *in vivo* Müller *et al.* reported that low levels of MCP-1/CCL2 correlated strongly with low levels of HGF at the central compartment [308]. We have found that plasma levels of the β-chemokines: MCP-1/CCL2, Rantes/CCL5 and Eotaxin/CCL11 correlate strongly with plasma HGF in MS, suggesting a physiopathological link between HGF and these β-chemokines. The levels of Rantes/CCL5 were markedly elevated in the progressive forms with respect to healthy controls and to RR-MS patients. Thus, high circulating levels of Rantes/CCL5 might be reflecting a more progressive and disabling disease course. Rantes/CCL5 and its receptors CCR1, CCR3 and CCR5 have been detected in active demyelinating plaques on immune and microglia cells [263, 427]. Its expression in serum is higher in RR-MS patients with active lesions and clinical activity [258, 424, 428] and its concentration decreases in CSF after corticosteroid therapy [429].

On the other hand, we found that plasma EGF and the β-chemokine MIP-1β/CCL4 were diminished in SP-MS and PP-MS clinical forms with respect to RR-MS patients and to healthy controls. Scalabrino *et al.* found lower levels of CSF EGF in RR-MS and SP-MS patients with respect to other non-inflammatory neurological diseases, but they found no differences in serum EGF among MS clinical forms [313]. However, the number of progressive MS patients of the study was very limited (10 SP-MS and 9 PP-MS patients). In this study, we did find significantly diminished plasma EGF levels in progressive MS compared to RR-MS and to healthy controls. MIP-1β/CCL4 shares the same chemokine receptor, CCR5, with Rantes/CCL5 and with MIP-1α/CCL3. The target cells of

MIP-1β are CD8+ T lymphocytes and MIP-1β has been detected in actively demyelinating plaques on macrophages and microglia [257, 419].

Additionally, HGF, MCP-1/CCL2, Eotaxin/CCL11 and Rantes/CCL5 that were all at higher levels in progressive MS patients than in RR-MS correlated strongly with EDSS, the classical indicator of clinical disability. In contrast, EGF and MIP-1β that were diminished in progressive patients inversely correlated with EDSS.

Gene expression studies showed differences in the mRNA levels in PBMCs of the gene clusterin (CLU) and of the interferon regulatory factor 2 (IRF2) in RR-MS patients with respect to the progressive clinical forms (SP-MS and PP-MS). Clusterin is encoded by the CLU gene, and is an ubiquitous glycoprotein also known as apolipoprotein J and as complement lysis inhibitor (CLI) [430]. Proteomic analyses have found clusterin in the CSF and blood of MS patients and have suggested this protein as a putative biomarker for MS [431, 432]. Clusterin is overexpressed in neurodegenerative diseases such as Alzheimer's disease and Parkison's disease with a potential neuroprotective role [433]. In this study a higher expression of clusterin at the mRNA level was found in the progressive forms of MS (SP-MS and PP-MS) compared to both RR-MS patients and healthy controls. This high expression of clusterin in progressive patients may argue to a possible role of this protein in MS pathogenesis, especially in the progressive stage of the disease where neurodegeneration is more prominent than in the RR-MS phase. Indeed, there is evidence that clusterin can cross the BBB by diverse mechanisms [434] and the subcutaneous administration of recombinant clusterin promotes remyelination in experimental models [435]. Overall, these data indicate that clusterin may have a potential neuroprotective role and the results from this study suggest clusterin as a putative gene expression biomarker with higher expression in the progressive forms of MS. By contrast, the IRF2, an endogenous negative regulator of type I IFN signals [436] and IFN production, was markedly diminished in the progressive forms compared to RR-MS patients. IRF2 mRNA has been found in MS lesions [437]. This finding may be relevant in the pathogenesis of the progressive stages of MS, where IFNβ therapy is not effective.

The high degree of biological and clinical heterogeneity that characterizes MS, wherein inflammatory and neurodegenerative pathways vary has hampered the clinical effect of targeted therapies and emphasizes the need to identify those patients most likely to benefit from a particular treatment.

IP10/CXCL10 and MCP-1/CCL2 have previously been reported to be at higher levels in patients under treatment with IFN-β [327-329, 438] and have been proposed as biomarkers for IFNβ response [327, 329]. In this work, we observed that long term treatment with IFNβ translates into persistent IP10 plasma levels in these patients, with significantly higher values than those found in healthy controls and in the rest of groups with RR-MS, including non responders to IFNβ. In addition, we observed that MCP-1 discriminated between responder and non responder patients to IFNβ therapy. Moreover, we observed that SP-MS patients had significantly higher circulating IP10 than patients in a RR-MS stage and than healthy controls. IP10 is a chemokine expressed by astrocytes that is upregulated and highly expressed in active demyelinating lesions [257, 264]. This chemokine is increased in serum and in the CSF of MS patients during exacerbations (reviewed in [384] and [257]). However, in our cohort of patients we did not observe higher plasma levels of IP10 in MS patients at relapse. Interestingly, we remarked that progressive patients, especially SP-MS had increased circulating levels of IP10. Several studies have reported increased endogenous production of IFNγ by activated T lymphocytes expressing CCR5 from progressive MS patients [439, 440]. As IP10 is induced by IFN, a high endogenous production of the Th1 cytokine, IFNγ, by Th1 CCR5+ lymphocytes might be reflecting a positive endogenous loop of IP10 production.

Gene expression studies have been largely used for the identification of differential expression of genes in MS brain lesions and in PBMCs. As IFNβ treatment is a long-term therapy, we focused in comparing the genes induced at short term in the patients starting therapy with genes induced at long term. The PBMCs gene expression revealed that several genes induced by IFNβ treatment in a small group of patients starting IFNβ therapy were also overexpressed in the group of long-term responders to IFNβ. Although this is a cross-sectional study and the same patients were not followed over time, we observed that a set of genes: MCP-1, MDA5, RIG-I, MX1, EIF2AK2, IL1RN,

RSAD2 and TRIM25, were induced in the four patients starting therapy and also increased in long-term IFNβ responders.

The search for specific biomarkers of IFNβ bioactivity in patients with MS continues to be an interesting topic of research [441]. Some genes observed in this study as MX1, RSAD2, IP10, MCP-1, ISG15 and EIF2AK2 have been reported to be upregulated in MS patients under treatment with IFNβ [329, 441, 442]. Genes reported in this study, such as MDA5, RIGI or TRIM25, could be further evaluated as putative specific biomarkers of IFNβ bioactivity.

A suboptimal secretion of IFNα and β in response to different viruses and alterations in the type I IFN signaling with low expression of ISGs in active MS has been reported [443, 444]. However, in this study no differences in the production of IFNα after TLR3 stimulation of PBMCs were observed in a series of patients.

The present study illustrates the unique and informative role of plasma and gene expression profiling in improving our understanding of diverse pathophysiological pathways underlying the different MS clinical forms and response to treatments. The fact that among a combination of a wide set of human cytokines, chemokines, soluble receptors and growth factors, the most striking differences were noted for chemokines and growth factors, underlines the importance of these proteins in the clinical course and pathophysiology of a CNS-specific autoimmune disease, such as MS. Autoreactive immune cells have CNS tropism and migrate to their target organ, attracted by chemokines either through direct effect or by the activation of their leukocyte integrins [445, 446]. Growth factors have outstanding relevance peripherally and also in the central compartment, where they can participate in neovascularization and remyelination to counteract the inflammatory attacks. Our findings suggest that factors involved in the chemokine inflammatory response and growth/angiogenic factors may differentially account for divergent MS clinical courses. It is crucial that ongoing work in the field of non-invasive biomarkers will be aimed at unraveling the origins and function of the identified biomarkers, which could also favor more focused and personalized therapies.

6. CONCLUSIONS

1. Plasma dipeptidyl peptidase 4 (DPP4), a key point regulator of chemokines antagonism, was significantly lower in our cohort of multiple sclerosis (MS) patients than in healthy controls.

2. Dipeptidyl peptidase activity was significantly decreased in our MS patients than in healthy controls and correlated inversely with clinical disease activity score in MS (EDSS).

3. A multivariate logistic regression model with DPP4, interlekin 7 and monokine induced by gamma interferon (Mig) allowed the discrimination of MS patients with a classification accuracy of 79.7% in our cohort of 129 MS patients and 53 healthy controls.

4. A multivariate logistic regression model with hepatocyte growth factor (HGF), eotaxin, epidermal growth factor (EGF) and macrophage inflammatory protein-1β (MIP-1β) allowed the discrimination between relapsing-remitting and progressive (secondary and primary progressive) forms of MS with a classification accuracy of 82.6% in the present study. This model might have clinical implications for the individual patient.

5. Low plasma levels of basic fibroblast growth factor (FGFb) and high mRNA expression levels in peripheral blood mononuclear cells of low density lipoprotein receptor (LDLR) are distinctive features of primary progressive MS in our cohort of MS patients.

6. High plasma vascular endothelial growth factor (VEGF) appears to identify secondary progressive with respect to relapsing-remitting MS patients in this study.

7. The plasma levels and mRNA expression levels in peripheral blood mononuclear cells of interferon gamma-induced protein 10 (IP10) and monocyte chemotactic protein-1 (MCP-1) are increased in our MS patients with good response to interferon β treatment.

8. An increased mRNA expression levels in peripheral blood mononuclear cells of the clusterin (CLU) gene identifies progressive (secondary and primary progressive) MS forms in our cohort of patients.

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8. APPENDIX

8.1. GENES BIOMARK

ACTB	Actin Beta
ADAM17	A Disintegrin Metalloproteinase Domain 17 / Tumor Necrosis Factor-α Converting Enzyme
ATG5	Autophagy 5
ATG12	Autophagy 12
B2M	Beta-2-Microglobulin
CASP1	Caspase 1
CCL2	Chemokine, CC Motif, Ligand 2 / Monocyte Chemotactic Protein 1
CCL4	Chemokine, CC Motif, Ligand 4 / Macrophage Inflammatory Protein 1β
CCL11	Chemokine, CC Motif, Ligand 11 / Eotaxin
CD81	CD81 Antigen / Target of Antiproliferative Antibody 1
CD27	CD27 Antigen / Tumor Necrosis Factor Receptor Superfamily, Member 7
CLU	Clusterin
CXCL9	CXC Motif Chemokine Ligand 9 / Monokine induced by Gamma Interferon
CXCL10	CXC Motif Chemokine Ligand 10 / Interferon-Gamma-Inducible Protein 10
CXCR3	CXC Motif Chemokine Receptor 3
CYLD	CYLD Gene
CYP24A1	1,25-Dihydroxyvitamin D3 24-Hydroxilase
DPP4	Dipeptidyl Peptidase / CD26
EGF	Epidermal Growth Factor
EIF2A	Eukaryotic Translation Initiation Factor 2A
EIF2AK2	Eukaryotic Translation Initiation Factor 2- Alpha Kinase 2
FKBP8	FK506-Binding Protein 8
GADPH	Glyceraldehyde 3 Phosphate Dehydrogenase
GATA3	Gata-Binding Protein 3
GUS	Beta-Glucuronidase
HGF	Hepatocyte Growth Factor
HPRT	Hypoxantine Guanine Phosphoribosyltransferase
HSPA8	Heat Shock 70Kd Protein 8
IFNAR1	Interferon Alpha, Beta and Omega Receptor 1
IFNAR2	Interferon Alpha, Beta and Omega Receptor 2
IFNA2	Interferon Alpha 2
IFNA4	Interferon Alpha 4
IFNA5	Interferon Alpha 5
IFNA6	Interferon Alpha 6
IFNA7	Interferon Alpha 7
IFNA8	Interferon Alpha 8
IFNB1	Interferon Beta 1
IGFBP3	Insulin-Like Growth Factor Binding Protein 3
IL1B	Interleukin 1 Beta
IL1RN	Interleukin 1 Receptor Antagonist
IL2RA	Interelukin 2 Receptor Alpha / CD25
IL2RB	Interelukin 2 Receptor Beta / CD122
IL2RG	Interleukin 2 Receptor Gamma
IL6	Interleukin 6
IL6R	Interleukin 6 Receptor
IL7RA	Interelukin 7 Receptor Alpha
IL8	Interleukin 8
IL10	Interleukin 10
IL10RB	Interleukin 10 Receptor beta
IL12p35	Interleukin 12 subunit p35
IL12p40	Interleukin 12 subunit p40
IL18	Interleukin 18
IL18BP	Interleukin 18 Binding Protein
IL18RAP	Interleukin 18 Receptor Accessory Protein
IRF2	Interferon Regulatory Factor 2
IRF3	Interferon Regulatory Factor 3
IRF5	Interferon Regulatory Factor 5
IRF7	Interferon Regulatory Factor 7

IRF9	Interferon Regulatory Factor 9
ISG15	Interferon Stimulated Gene 15
JAK1	Janus Kinase 1
LDLR	Low Density Lipoprotein Receptor
MAVS	Mitochondrial Antiviral Signaling Protein
MDA5	Melanoma Differentiation-Associated Gene 5 / Interferon-Induced Helicase C Domain-Containing Protein 1
MX1	Myxovirus Resistance 1
NDEL1	Nude like protein 1
p65	NFKB, p65 subunit
PAFAH1B1	Platelet-Activating Factor Acetylhydrolase, Isoform 1B, Alpha subunit
PRF1	Perforin 1
PGR	Progesterone Receptor
RIGI	Retinoic Acid Inducible Gene I
RNF125	Ring Finger Protein 125
RSAD2	Radical S-Adenosyl methionine domain containing protein 2
SCARB1	Scavenger Receptor Class B, Member 1
SMAD7	SMA- and MAD-Related Protein
SOCS3	Suppressor of Cytokine Signaling 3
SOCS7	Suppressor of Cytokine Signaling 7
STAT1	Signal transducer and activator of transcription 1
STAT2	Signal transducer and activator of transcription 2
STAT6	Signal transducer and activator of transcription 6
TBP	TATA box binding protein
TCF7	Transcription Factor 7
TIMP1	Tissue inhibitor of metalloproteinase 1
TGFB1	Transforming growth factor beta 1
TGFB2	Transforming growth factor beta 2
TLR2	Toll like receptor 2
TLR3	Toll like receptor 3
TLR4	Toll like receptor 4
TLR7	Toll like receptor 4
TLR8	Toll like receptor 8
TLR9	Toll like receptor 9
TNF	Tumor Necrosis Factor
TNFRSF1A	Tumor Necrosis Factor Receptor Superfamily, Member 1A
TNFSF10	Tumor necrosis factor ligand superfamily, member 10
TRIM25	Tripartite Motif-Containing protein 25
TYK2	Tyrosine Kinase 2
USP18	Ubiquitin-specific protease 18
VDR	Vitamin D Receptor
ZNF148	Zinc Finger Protein 148

Resumen en Castellano del Trabajo de Tesis

Biomarcadores de las Formas Clínicas de Esclerosis Múltiple: Desarrollo de Estrategias Personalizadas basadas en Perfiles Inmunológicos de la Vía del Interferón de Tipo I.

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Madrid, 2013

INTRODUCCIÓN

La esclerosis múltiple (EM) es una enfermedad autoinmune crónica inflamatoria y desmielinizante del sistema nervioso central (SNC). La EM es la principal causa de discapacidad neurológica no traumática en adultos jóvenes [1]. Esta enfermedad compromete gravemente la calidad de vida de los pacientes y tiene un elevado coste e impacto socioeconómico para los pacientes, sus familias y la sociedad en su conjunto.

Según el Atlas mundial de la EM realizado en 2008 por la Organización Mundial de la Salud (OMS), la incidencia media global es de 2,5 casos por cada 100.000 habitantes (rango 1,1-4,0), mientras que la prevalencia global es de 30 casos por cada 100.000 habitantes (rango 5-200) [2]. La EM es más frecuente en Europa, Norteamérica, Australia y Nueva Zelanda con prevalencias mayores a 60 casos por 100.000 habitantes.

En la EM episodios repetidos de desmielinización inflamatoria resultan en la formación de placas escleróticas con distinto grado de pérdida de axonal [3]. Patológicamente se caracteriza por la pérdida o alteración de la vaina de mielina que cubre los nervios en el cerebro y la médula espinal, produciendo lesiones multifocales en el SNC [4] que puede llevar a un degeneración axonal y progresiva disfunción neurológica.

La EM es una enfermedad altamente heterogénea en cuanto a sus síntomas clínicos y curso de la enfermedad. Los síntomas más comunes incluyen alteraciones visuales, pérdida de equilibrio y coordinación, espasticidad, alteraciones sensoriales, incontinencia vesical e intestinal, dolor, debilidad, fatiga y parálisis [5]. El curso de la enfermedad comienza con en más del 80% de los pacientes con un ataque agudo, un síndrome clínico aislado (CIS). Si ocurren nuevos ataques, estos se dan con distintas frecuencias pero rara vez exceden 1,5 al año [5]. Esta forma clínica se conoce como esclerosis múltiple recurrente-remitente (RR-MS), y se caracteriza por brotes recurrentes con total o parcial recuperación y un curso inflamatorio de la enfermedad que se puede modificar con terapia. Después de unos 15 años desde el comienzo de la enfermedad [6], la progresión ocurre en aproximadamente un 65% de los pacientes que entran en una fase secundaria-progresiva (SP-MS).

Aproximadamente un 20% de los pacientes tienen un comienzo progresivo sin brotes, conocido como esclerosis múltiple primaria-progresiva (PP-MS) [5]. Para las formas progresivas de la enfermedad (SP-MS y PP-MS) los tratamientos existentes en la actualidad son incapaces de detener la progresión y el deterioro neurológico. Existen algunos pacientes con la forma menos agresiva de la enfermedad, llama EM benigna. Estos pacientes no tienen una elevada discapacidad después de diez o más años de enfermedad y tienen actividades laborales y domésticas normales. Sin embargo, el adjetivo benigno, no significa que estos pacientes carezcan de síntomas de la enfermedad, sino que la incapacidad es más lenta y no sucede en todos los pacientes [7]. El grado de discapacidad de los pacientes de EM suele medirse según la escala EDSS (Expanded Disability Status Scale), que se extiende numéricamente de 0 (examen neurológico normal) hasta 10 (muerte por EM). Esta escala se utiliza ampliamente como estándar para evaluar a los pacientes con EM pero proporciona mucha importancia a la capacidad para andar y no tiene en cuenta el deterioro cognitivo [8].

La etiología de la EM es aún desconocida. Se considera que la EM es un desorden complejo desencadenado en individuos susceptibles genéticamente por distintos factores ambientales y estocásticos [9]. Entre los factores ambientales propuestos destacan la influencia de las migraciones y la higiene en la infancia, el posible papel de los virus (familia de los herpes virus) y la asociación con la vitamina D. El principal factor genético asociado a EM es el HLA-DRB1*15:01 [10].

El diagnóstico de la EM se basa principalmente en la evidencia clínica. Los síntomas pueden ser provenir del sistema sensorial, motor y autónomo. Las lesiones deben estar diseminadas en el tiempo (al menos dos o más episodios de disfunción neurológica separados al menos 30 días) y en el espacio (al menos dos lesiones independientes en el SNC) [5]. Cuando las manifestaciones clínicas no son suficientes para un diagnóstico adecuado, los rasgos paraclínicos pueden ayudar a establecer el diagnóstico. Las pruebas paraclínicas más utilizadas son las imágenes de resonancia magnética (MRI: Magnetic Resonance Imaging) y el examen del líquido cefalorraquídeo (LCR). La MRI muestra anormalidades de la sustancia blanca en más del 95% de los

pacientes con EM y muestra la diseminación anatómica de las lesiones; al utilizarse la MRI de manera seriada puede mostrar nuevas placas en ausencia de episodios clínicos [5]. El gadolinio es un material de contraste que es captado por las lesiones y placas recientes, y permite detectar rupturas en la barrera hematoencefálica (BHE) [11]. El análisis del LCR, obtenido mediante una punción lumbar, permite detectar bandas oligoclonales (OB: oligoclonal bands) de inmunoglobulina G (IgG) a nivel del SNC y no en el suero de los pacientes. Las OB están presentes hasta en el 90% de los pacientes con EM [5]. Sin embargo, la detección de OB no es exclusiva de pacientes con EM y tiene valor diagnóstico una vez que otras causas de inflamación han sido descartadas [12]. Otras pruebas complementarias del LCR son el cociente de albúmina, el índice de IgG y el contaje celular [12]. Los criterios generalmente empleados para diagnosticar la EM son los criterios de Poser [13] o los criterios revisados de McDonald [14] [15] que tienen en cuenta síntomas clínicos y pruebas paraclínicas.

Aunque la etiología de la EM es desconocida, se considera que la EM es una enfermedad autoinmune Th1/Th17, donde los linfocitos T autorreactivos específicos de mielina alcanzan el SNC, atravesando la BHE y desencadenando los eventos patológicos que llevan a una desmielinización y daño axonal. Los linfocitos T activados expresan distintos receptores de quimiocinas e integrinas que facilitan la migración al SNC. El infiltrado inflamatorio de las placas escleróticas está compuesto por una infiltración perivascular de linfocitos T ($CD8^+$>$CD4^+$) α/β y ɣ/δ, monocitos, algunos linfocitos B y células plasmáticas [16]. El grado de daño axonal es proporcional al número de macrófagos y linfocitos T $CD8^+$ presentes en las lesiones [17]. Los linfocitos B son también importantes en la fisiopatología de la EM, ya que se activan anormalmente en células plasmáticas que producen intratecalmente inmunoglobulinas detectadas como OB [18]. La apoptosis es también un mecanismo importante de regulación de la inflamación en EM. Regula la eliminación de linfocitos T autorreactivos, el anormal funcionamiento de la apoptosis puede desembocar en daño y lesión en el SNC [19].

La EM es una enfermedad altamente heterogénea no sólo en sus formas y manifestaciones clínicas, sino también en el curso de la enfermedad y la respuesta a terapia. La búsqueda de biomarcadores que ayuden a

diagnosticar, estratificar y predecir la respuesta al tratamiento y la discapacidad clínica se ha incrementado en los últimos años y son especialmente necesarios. Los biomarcadores potenciales necesitan ser validados para uso clínico y el principal obstáculo en el descubrimiento de biomarcadores es que muy pocos han sido estandarizados para su uso clínico [20]. La definición de biomarcador puede definirse como "una característica medible y evaluada objetivamente como indicador de un proceso biológico normal, patogénico o una respuesta farmacológica a una intervención terapéutica [21]". En la EM, los biomarcadores se pueden clasificar según su uso propuesto como [22]: predictores del riesgo de presentar la enfermedad, diagnósticos, clasificadores del estadio y forma clínica de la enfermedad o predictores de la historia natural de la enfermedad o de respuesta al tratamiento. Las citoquinas son muy importantes en la regulación de la actividad y la función del sistema inmunológico; tienen un claro papel en la patogénesis de la EM y han sido ampliamente estudiadas como biomarcadores potenciales. Se han descrito biomarcadores candidatos en suero como Fas y MIF de empeoramiento neurológico en formas progresivas de EM [23]. En los pacientes en brote las citoquinas TNFα, IL-12, IL-17 and IFNɣ están aumentadas mientras que IL-10 está disminuida en el LCR y suero [20]. La producción sistémica de IL-12, que estimula la producción de otras citoquinas proinflamatorias, es anterior a los brotes clínicos y se correlaciona con la actividad clínica [20]. Todas estas citoquinas dan una idea de la naturaleza Th1 y Th17 del brote inmunológico. Las quimiocinas y sus receptores también juegan un importante papel en la fisiopatología de la EM. Actúan atrayendo el reclutamiento activo de linfocitos autorreactivos desde la periferia al SNC e inducen la secreción de nuevas citoquinas proinflamatorias, que promueven la desmielinización final y la pérdida neurológica [24,25]. Algunas de las quimiocinas más importantes descritas en EM son: CXCL8, Rantes/ CCL5 [26], CXCL13 [27, 28], IP10/CXCL10 [29-31] y su receptor CXCR3 [32,33]. La mayoría de las β quimiocinas o de los ligandos de quimiocinas CCL se encuentran agrupadas en el cromosoma 17q11.2-12, que se ha asociado con EM en distintos estudios [34-36]. Una de estas β quimiocinas, la eotaxina o CCL11 se ha estudiado en el modelo animal de EM, la encefalitis experimental autoinmune (EAE), y niveles elevados de eotaxina se asociaron con un fenotipo más leve de la enfermedad, BHE más conservada, una

especificidad antigénica más reducida y un fenotipo antiinflamatorio Th2 [37]. La molécula multifuncional dipeptidilpeptidasa 4 (DPP4, también conocida como CD26) está implicada en la fisiopatología autoinmune con un atribuible papel inmunoregulador. DPP4 es una serina proteasa con actividad X-prolil dipeptidilpeptidasa expresada en muchos tipos celulares, incluyendo a las células del sistema inmunológico [38,39]. También existe en forma soluble presente en el suero y otros fluidos biológicos como sDPP4 [39, 40]. En las células DPP4 tiene un papel de co-estimulación celular [41, 42], además está implicada en la regulación de las células T [43] y timocitos [44]. Las quimiocinas reguladas por DPP4 son muchas como: MIP-1α/ CCL3, MIP-1β/CCL4, Rantes/CCL5, Eotaxin/CCL11, Mig/CXCL9, IP10/CXCL10, I-TAC/CXCL11 y SDF-1α/CXCL12 [40, 45]. Los estudios en EM se han centrado en la caracterización de linfocitos T que expresan DPP4 en su superficie [46-50] y los han correlacionado con marcadores de actividad de la enfermedad clínica y MRI. Los factores de crecimiento tienen también relevancia en los desórdenes del SNC y podrían ser considerados como posibles biomarcadores de EM. El factor de crecimiento hepático (HGF) tiene multitud de funciones y es producido por la microglía, células precursores de oligodendrocitos, astrocitos y neuronas [51-54]. Además, el HGF periférico muestra efectos inmunomoduladores: promoviendo la adhesión de los linfocitos B [55], la migración de las células T [56] y el reclutamiento de células dendríticas (DCs) [57, 58]. El HGF se ha encontrado aumentado en enfermedades del SNC, como EM y la enfermedad de Alzheimer [59]. Aunque otros estudios encontraron distintos hallazgos [60, 61]. El factor de crecimiento epidérmico EGF tiene efectos positivos sobre la proliferación y diferenciación de neuronas, astrocitos y oligodendrocitos [62, 63] y su coadministración con la hormona del crecimiento en EAE mejora el fenotipo y la supervivencia [64]. En EM se ha encontrado en niveles bajos en el LCR comparado con otras enfermedades neurológicas no inflamatorias [65]. Por último el factor de crecimiento básico de los fibroblastos (FGFb), tiene un papel muy importante en el crecimiento, diferenciación, migración y crecimiento de los progenitores de la glia del SNC [62], y si expresión está aumentada en las places activas de EM [66]. Su papel en EM es controvertido [67-69] pero recientemente el ratón sin (knockout) FGFb ha revelado un papel neuroprotector en EAE [70]. El IFNβ sigue siendo la terapia más utilizada en EM. Sin embargo, todavía hay más de un

40% de pacientes que no responden a esta terapia [71]. Numerosos estudios han intentado buscar biomarcadores de expresión génica que permitan predecir la respuesta terapéutica al IFNβ [72-74]. Las quimiocinas IP10/CXCL10 y MCP-1/CCL2 se han propuesto como biomarcadores de respuesta al IFNβ [75, 76]. Finalmente los biomarcadores humorales más utilizados son las bandas oligoclonales de IgG [20], otros como los anticuerpos neutralizantes de IFNβ [77] que pueden ser indicativos de que el tratamiento con IFNβ no está siendo eficaz, las cadenas kappa ligeras en el LCR puede correlacionarse con pronóstico de discapacidad [78] y los anticuerpos IgG elevados al antígeno nuclear del virus Epstein-Barr (EBNA-1) se correlacionan con lesiones que captan gadolinio [79].

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OBJETIVOS

La esclerosis múltiple se caracteriza por un curso clínico y una respuesta al tratamiento heterogénea lo que puede representar distintas etiologías, dados los diferentes patrones patológicos que exhiben las distintas formas clínicas. En la actualidad, no existen biomarcadores séricos para la EM, lo que sería necesario para mejorar el diagnóstico así como la evaluación de la actividad de la enfermedad y la respuesta al tratamiento. Una identificación temprana de las formas clínicas de la EM, especialmente la primaria progresiva supone hoy día un reto para los clínicos. El hecho que las formas de la enfermedad todavía no disponen de terapias para frenar la neurodegeneración y la discapacidad hace todavía más urgente la necesidad de biomarcadores que puedan anticipar el comienzo de la progresión. La identificación de tales biomarcadores de EM en el paciente es un aspecto importante que permitiría el establecimiento de terapias más personalizadas para la enfermedad.

El propósito de esta tesis ha sido identificar biomarcadores en sangre periférica que pudieran distinguir entre pacientes con EM y controles sanos y reflejar diferencias fisiopatológicas en las formas clínicas de EM.

Objetivos concretos:

I. Identificar biomarcadores plasmáticos que discriminen entre pacientes con EM y controles sanos.

II. Caracterizar la expresión de DPP4 en EM y examinar la hipótesis de que DPP4 tiene un papel importante en la fisiopatología de la enfermedad, dado que esta enzima DPP4 tiene un papel muy importante en la inmunoregulación de quimiocinas clave para la EM.

III. Identificar biomarcadores plasmáticos que permitan estratificar a los pacientes con EM según su forma clínica.

IV. Identificar biomarcadores relacionados con las vías de señalización del interferón de tipo I que podrían ayudar a desarrollar estrategias personalizadas en EM y proporcionar información sobre los mecanismos fisiopatológicos de la enfermedad.

APORTACIONES FUNDAMENTALES DE LA TESIS DOCTORAL

El presente trabajo de tesis ha permitido identificar una serie de biomarcadores en plasma periférico y a nivel de expresión génica de ARNm (messenger RNA) en las células mononucleares de sangre periférica (PBMCs) en pacientes con EM.

Nuestro estudio ha encontrado una expresión disminuida de sDPP4 y una reducida actividad dipeptidil peptidasa (DPP) en el plasma de los pacientes con EM en comparación con los controles sanos. En concreto, la actividad DPP más baja hallada entre los grupos de pacientes con EM fue en el grupo de pacientes SP-MS. La actividad DPP se correlaciona inversamente con la escala del estado de discapacidad (EDSS).

En este trabajo hemos encontrado que las diferentes formas clínicas de EM se asocian con distintos perfiles de biomarcadores circulantes, principalmente quimiocinas y factores de crecimiento/angiogénicos (HGF, Eotaxina/CCL11, MCP-1/CCL2, Rantes/CCL5, EGF, MIP-1β/CCL4, VEGF y FGFb); y también con distintos niveles de expresión génica (CLU, IRF2 y LDLR). En concreto, los pacientes con RR-MS presentaban niveles circulantes disminuidos de HGF, Eotaxina/CCL11, MCP-1/CCL2, Rantes/CCL5, mientras que los pacientes en las fases progresivas de la enfermedad (SP-MS y PP-MS) presentaban niveles disminuidos de EGF y MIP-1β/CCL4. El grupo de pacientes con PP-MS presentaba específicamente niveles en plasma disminuidos de FGFb mientras que los pacientes con SP-MS presentaban niveles circulantes más elevados del factor de crecimiento vascular VEGF que los pacientes con RR-MS. A nivel de ARNm, los pacientes en las fases progresivas de la enfermedad presentaban una mayor expresión del gen de clusterina (CLU) que los pacientes RR-MS y que los controles sanos. La clusterina se encuentra aumentada en enfermedades neurodegenerativas como el Parkinson y el Alzheimer, en las que se le ha atribuido un posible papel neuroprotector. En nuestros pacientes con EM, aquellos con mayor expresión se encontraban en la fase progresiva y con mayor neurodegeneración de la enfermedad. Por otro lado, la expresión génica del factor regulador de interferón (IRF2) se

encontraba disminuida en los pacientes progresivos respecto a aquellos con RR-MS. Los pacientes con PP-MS presentaban específicamente niveles de expresión génica del receptor de lipoproteínas de baja densidad (LDLR) más altos que el resto de pacientes con EM y los controles sanos. Todos estos hallazgos diferenciales en las distintas formas clínicas de EM, apoyan y proporcionan nuevos indicios de la heterogeneidad de la enfermedad. En los pacientes tratados a largo plazo con IFNβ se han observado en este estudio niveles más altos en plasma de IP10/CXCL10 y MCP-1/CCL2 y una expresión génica incrementada de distintos genes como MCP1, IP10, MDA5, RIG-I, MX1, EIF2AK2, IL1RN, RSAD2 y TRIM25 respecto a controles sanos y a otros grupos clínicos de EM.

En este trabajo hemos realizado mediante análisis de regresión logística multivariante combinaciones de biomarcadores plasmáticos que nos han permitido aumentar la exactitud para identificar pacientes con EM o distinguir pacientes RR-MS de pacientes progresivos. Un modelo que incluye a DPP4, IL-7 y Mig como factores de protección de no tener la enfermedad permite la discriminación en nuestra cohorte entre pacientes con EM y controles sanos con un 79.7%. Por otro lado un modelo que incluye a HGF, Eotaxina como factores de riesgo y EGF y MIP-1β como factores de protección de tener una forma progresiva de EM; permite una discriminación entre formas RR-MS y progresivas (SP-MS y PP-MS) del 82.6%.

CONCLUSIONES

1. Los niveles plasmáticos de dipeptidil peptidasa 4 (DPP4), un regulador esencial en el antagonismo de las quimiocinas, estaban significativamente disminuidos en nuestra cohorte de pacientes con EM respecto a controles sanos.
2. La actividad dipeptidil peptidasa era significativamente más baja en nuestros pacientes con EM que en controles sanos y se correlacionaba inversamente con la escala del estado de discapacidad en EM (EDSS).
3. Un modelo de regresión logística multivariante con DPP4, interleucina 7 y la monoquina inducida por el interferón gamma (Mig) permitió la discriminación de pacientes con EM con una exactitud en la clasificación del 79.7% en nuestra cohorte con 129 pacientes con EM y 53 controles sanos.
4. Un modelo de regresión logística multivariante con el factor de crecimiento hepático (HGF), eotaxina, factor de crecimiento epidérmico (EGF) y la proteína inflamatoria de macrófagos 1β (MIP-1β) permitió la discriminación entre pacientes con EM recurrente-remitente y pacientes progresivos (EM secundaria y primaria progresiva) con una exactitud en el diagnóstico del 82.6% en el presente estudio. Este modelo puede tener implicaciones clínicas para el paciente individual.
5. En nuestra cohorte de pacientes, niveles plasmáticos bajos del factor básico de crecimiento de fibroblastos (FGFb) y altos de la expresión de ARNm en células periféricas mononucleares de sangre periférica del receptor de lipoproteínas de baja densidad (LDLR) diferencian a los pacientes con EM primaria progresiva del resto de formas clínicas.
6. Niveles plasmáticos elevados del factor de crecimiento vascular endotelial (VEGF) parecen identifican pacientes EM secundaria progresiva respecto a pacientes con EM recurrente-remitente en este estudio.
7. Los niveles plasmáticos y la expresión en células periféricas mononucleares de sangre periférica de ARNm de la proteína inducida por interferón-gamma (IP10) y la proteína quimiotáctica de monocitos 1 (MCP-1) están aumentados en nuestros pacientes respondedores al tratamiento con interferón β.

8. Una elevada expresión de ARNm en las células periféricas mononucleares de sangre periférica del gen de la clusterina (CLU) identifica a las formas progresivas (secundaria y primaria progresiva) en nuestra cohorte de pacientes.

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