# Search of prognostic biomarkers in patients with multiple sclerosis

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**Dedicated to my parents and teachers**

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# Abstract

The aim of the thesis was to identify biomarkers for multiple sclerosis (MS) prognosis, in particular disease activity biomarkers and interferon-β (IFNβ) treatment response biomarkers. The results presented in this thesis show an increase in the expression of sialic acid binding Ig-like lectins 1 (SIGLEC1) in progressive phases of MS (particularly during primary progressive MS - PPMS). Detailed analysis has demonstrated that both inflammatory and resident monocytes contributed to increased SIGLEC1 expression in PPMS. We also reported that SIGLEC7 expression was elevated in relapsing-remitting MS (RRMS) patients during relapses. Our results also indicate deficient expression of ubiquitin specific peptidase 18 (USP18) in RRMS patients as compared to controls. Further investigation revealed that haplotype *CG* carriers showed lower USP18 gene expression levels and higher clinical disease activity compared to *CG* non-carriers. Moreover, *AA* homozygosis for an intronic polymorphism of USP18 was associated with the responder phenotype. In conclusion, our results suggest the implication of SIGLEC1 in chronic progressive phases of MS and of SIGLEC7 in acute disease activity. We also demonstrated the implication of USP18 in MS pathogenesis and the therapeutic response to IFNβ. Based on these results, we propose SIGLEC1, SIGLEC7 and USP18 as potential disease activity biomarkers of MS, and USP18 as response biomarker to IFNβ.

## Resumen

El objetivo de esta tesis ha sido la identificación de biomarcadores pronósticos en la esclerosis múltiple (EM), principalmente biomarcadores de actividad de la enfermedad y biomarcadores de respuesta al tratamiento con interferón beta (IFNβ). Los resultados expuestos en esta tesis muestran un incremento en la expresión del gen "sialic acid binding Ig-like lectins 1" (SIGLEC1) en fases progresivas de la EM (principalmente en pacientes con EM primariamente progresiva - EMPP). Un análisis más detallado demostró que tanto los monocitos residentes como los inflamatorios contribuyeron al incremento de la expresión de SIGLEC1 en pacientes con EMPP. También observamos que la expresión de SIGLEC7 se encuentró aumentada en la EM recurrente-remitente (EMRR) durante el brote. Nuestros resultados también mostraron una expresión deficiente del gen "ubiquitin specific peptidase 18" (USP18) en pacientes con EMRR comparado con controles. Experimentos adicionales mostraron que los pacientes portadores del haplotipo *CG* presentaron niveles de expresión del gen USP18 disminuidos y un incremento en la actividad clínica de la enfermedad en comparación con los no portadores del haplotipo *CG*. Además, los pacientes homocigotos *AA* para un polimorfismo intrónico del gen USP18 presentaron una buena respuesta al tratamiento con IFNβ. En conclusión, nuestros resultados sugieren la implicación de SIGLEC1 en la fase crónica progresiva de la EM, y un papel de SIGLEC7 en la actividad aguda de la enfermedad. También sugieren la implicación de USP18 en la patogenia de la

EM y la respuesta terapéutica al IFNβ. En base a estos resultados, proponemos SIGLEC1, SIGLEC7 y USP18 como biomarcadores de actividad de la enfermedad, y USP18 como biomarcador de respuesta al IFNβ.

# Preface

The present dissertation is divided into 3 main parts. The first part provides an introduction to MS, with a special focus on what is known regarding its pathogenesis, complexity, and the need of biomarkers in the disease. The second part represents the main core of the dissertation and describes the 4 scientific publications associated with this work. A third and final part is dedicated to a brief discussion on main findings and the conclusions derived from the 4 studies.

## Abbreviations

| | |
|---|---|
| AUC | Area under curve |
| cDNA | complementary DNA |
| CIS | Clinically isolated syndrome |
| EDSS | Expanded Disability Status Scale |
| FACS | Flow cytometry |
| GEO | Gene expression omnibus |
| IFN | Interferon |
| IFNβ | Interferon beta |
| IFNγ | Interferon gamma |
| IFNAR | Interferon receptor |
| IL | Interleukin |
| ISRE | IFN-stimulated response element |
| LPS | Lipopolysaccharide |
| MFI | Mean fluorescence intensity |
| MRI | Magnetic resonance imaging |
| MS | Multiple sclerosis |
| MxA | Myxovirus resistance protein A |
| NABs | Neutralising antibodies |
| NK | Natural killer |
| PBMCs | Peripheral blood mononuclear cells |
| PHA | Phytohaemagglutinin |
| PPMS | Primary progressive multiple sclerosis |
| RRMS | Relapsing-remitting multiple sclerosis |
| RT-PCR | Real-time polymerase chain reaction |
| SD | Standard deviation |

| | |
|---|---|
| SEM | Standard error of mean |
| SIGLEC | Sialic acid binding Ig-like lectins |
| SNP | Single nucleotide polymorphism |
| SOCS3 | Suppressor of cytokine signalling 3 |
| SPMS | Secondary progressive multiple sclerosis |
| USP18 | Ubiquitin specific peptidase 18 |

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# PART I

# BACKGROUND

# Chapter 1

# Review of Literature

# Chapter 1

# Review of Literature

## 1.1 Introduction to Multiple sclerosis

Multiple sclerosis (MS) is a chronic neurological disease that affects the central nervous system (CNS). It mainly affects young adults between 20-40 years of age and women are affected about twice as often as men. The etiology of MS is unknown; however, it is assumed that both a complex genetic background and environmental triggers contribute to disease manifestation. MS is one of the most frequent causes of severe neurological disability among young adults, and hence the socioeconomic costs due to MS are high.

Studies consider that MS has a prevalence ranging between 2 and 150 cases per 100,000 populations. It has been estimated that approximately 2.5 million people in the world are affected by MS, 1 million of whom are Europeans, and around 45,000 are in Spain (according to the Multiple Sclerosis International Federation - http://www.msif.org/).

## 1.2 Clinical forms of MS

MS is a heterogeneous disease in its clinical course. As shown in figure 1A, MS is classified into four major clinical forms: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), and progressive-relapsing MS (PRMS) (Lublin and Reingold 1996). Approximately 80-85% of MS patients present a RR course, the majority of whom will develop a SP course over time. Around 15-20% of MS patients have PP or PR courses of the disease.

Patients who have had only a single bout of disease but are not yet diagnosed with MS, are referred to as clinically isolated syndromes (CIS), and is considered the first manifestation of MS.

**Fig.1.A**

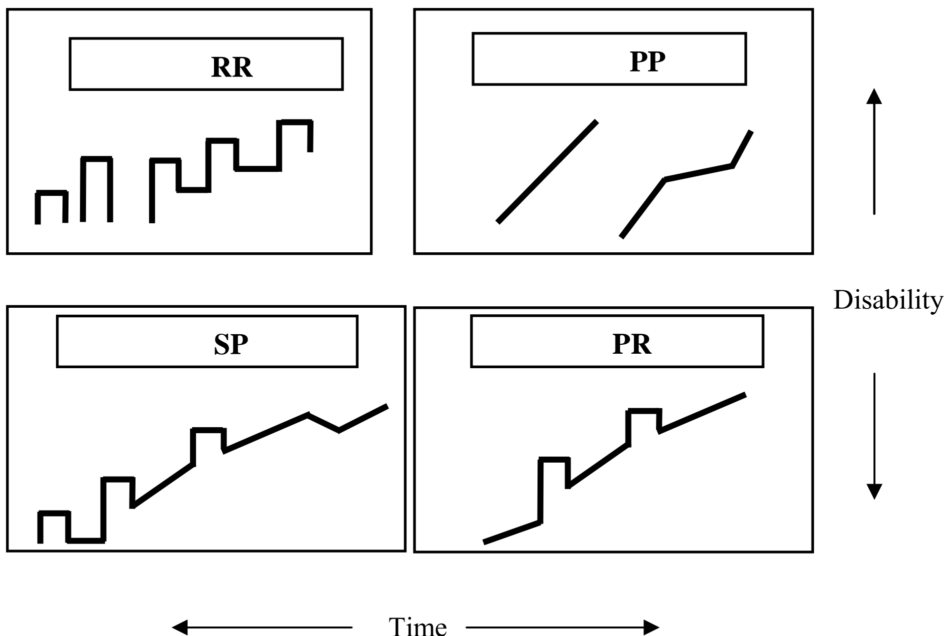

*Figure 1A. Clinical forms of MS. RRMS is defined by relapsing and full recovery from the relapse or by relapsing and less recovery from the relapses. Primary progressive is defined as progression of MS from the beginning and sometimes with some remissions. Secondary progressive in its initial phase is defined as RR, followed by irregular progression and minor remission. Progressive-relapsing showed progression from beginning with relapses.*

## 1.3 Multiple sclerosis: A complex disease

MS is a complex disease caused by aggregation of genetic, environment, epigenetic and life style factors. In MS, genetic factors represent a small component of the risk associated with the disease. Another component of the development of disease phenotype is triggered by environment and lifestyle. It is thus important to understand the gene-environment interactions which can help in a better comprehension of this complex disease.

During the last two decades, many investigative groups dedicated important efforts to identifying the individual genes that conferred susceptibility to MS. The main conclusion that evolved from these efforts was that the HLA-class II region on chromosome 6p21, specifically the HLA-DRB1*15 haplotype, contributed by far the most to genetic susceptibility in MS, and results from many MS genetic studies supported this association (MS Genetic Group 1996; Ebers et al. 1996). Despite the evidence that MS was a complex genetic trait with multiple genes contributing to disease susceptibility, genetic studies aiming to identify additional risk genes for MS were rather disappointing, as many of the candidate genes identified in one study were not confirmed in others. It has

not been until recently that, thanks to the development of new genotyping technologies, for instance the single nucleotide polymorphisms (SNP) arrays, additional genes located outside the HLA region have been proposed as solid candidates for MS genetic risk. In this scenario, it is important to highlight the results from a recent Genome-Wide Association study (GWAS) conducted by the International Multiple Sclerosis Genetics Consortium (IMSGC) in close to 10,000 DNA samples from MS patients and over 17,000 samples from healthy controls, which led to the confirmation of previously known susceptibility genes and identified 29 novel susceptible loci (IMSGC et al. 2011). Results from this powerful GWAS suggest a primary role of genes related with the immune system in the risk for MS.

## 1.4 Environmental factors

### *1.4.1 Vitamin D*

Vitamin D is considered an important environmental factor responsible for initiation of MS. This can be reflected directly by looking on the world map where the countries with more sunlight have fewer cases of MS and vice-versa. There are many studies which support a role of vitamin D in MS. To name a few: a strong genetic association of vitamin D binding sites has been observed with MS disease (Ramagopalan et al. 2010); serum levels of 25-hydroxyvitamin D have a prognostic role in patients with clinically isolated syndromes (Martinelli et al. 2013); levels of 25-hydroxyvitamin D have also been found to correlate with MS

severity (Mowry et al. 2012; Shahbeigi et al. 2013); and vitamin D intake has been associated with a decrease in the number of brain active lesion and a reduction of clinical relapses (Wingerchuk et al. 2005). Altogether, these findings reflect the importance of vitamin D in MS.

### *1.4.2 Smoking*

Smoking has recently emerged as an important risk factor for MS susceptibility (Handel et al. 2011), and may also play additional roles in the disease. In this context, smoking has been proposed as a triggering agent responsible for the transition from RRMS to SPMS (Healy et al. 2009; Roudbari et al. 2013), and the risk of developing neutralising antibodies (NABs) to IFNβ is higher in smokers than in non-smokers (Hedström et al. 2013).

### *1.4.3 Viruses*

Viruses are considered as potential factors responsible for triggering MS disease. One of the leading viruses that is being discussed as etiological factor in MS is the Epstein-Barr virus (EBV). Evidence linking EBV infection and MS comes from epidemiological studies: (i) Virtually all MS patients are seropositive for EBV; (ii) MS risk is increased among individuals with history of infectious mononucleosis compared to those who acquired EBV earlier in life (Thacker et al. 2006); (iii) Years before MS onset, individuals who will develop MS present heightened humoral responses against

EBV nuclear antigens (EBNA), which are primarily expressed during latent infection (Sundström et al. 2004).

Other viruses such as cytomegalovirus and the human herpesvirus 6 are probably also playing important roles as etiological factors in MS.

## 1.5 Biology of MS

### *1.5.1 CD4 cells*

MS research was mainly focused on the role of CD4+ T cells in disease pathogenesis. The idea that MS is primarily a CD4+ T cell-mediated disease arises from studies in the animal model, experimental autoimmune encephalomyelitis (EAE), in which the disease is driven by myelin-specific CD4+ T cells and can be adoptively transferred to unaffected animals by the injection of these T cells reactive against myelin.
Over the last years, new research has focused on the roles played by different subsets of CD4+ T cells in the disease. After activation, naïve T cells will differentiate into various T cell populations with different effectors functions. Th1 cells release IFNγ which activates macrophages that in turn kill intracellular pathogens. Th2 cells release anti-inflammatory cytokines like IL4 which are known for killing extracellular pathogens. A disruption in the balance between Th1 and Th2 cytokines has been reported to be involved in MS pathogenesis (Steinman and Conlon 2001).

Th17 cells represent another important lineage of T cells. The ratio of Th17 /Th1 is an important factor which is associated with infiltration of T cells and brain inflammation (Stromnes et al. 2008). Th17 T cell clones show increased expression of activation markers and adhesion molecules as compared to Th1 T cell clones (Brucklacher-Waldert et al. 2009). The frequency of Th17 cells in CSF from MS patients has been significantly increased at the time of relapses as compared to the remission phases of the disease (Brucklacher-Waldert et al. 2009). These findings clearly point towards an important role of Th17 cells in the immunopathogenesis of the disease.

Naturally occurring regulatory T cells (CD4+CD25+ Treg) represent a very small subset of the CD4+ T cell populations that has also been involved in MS pathogenesis. Several studies have proposed defects in the capacity of Tregs from MS patients to suppress the activation of myelin-specific T cells in the periphery, particularly in patients with RRMS (Venken K. 2006). It is important to mention that these studies focused on the suppressive properties of Tregs from peripheral blood mononuclear cells (PBMCs), but their role in preventing inflammation in the CNS remains controversial (Venken, K. 2006).

### *156.2 CD8 cells*

Numerous studies point to an important role of CD8+ T cells in MS pathogenesis: CD8+ T cells are predominant in the inflammatory infiltrate in CNS lesions (Babbe et al. 2000); MS shows association

with MHC class I alleles (Link et al. 2012). Adoptive transfer of activated myelin-specific CD8+ T cell clones has shown to induce severe EAE (Huseby et al. 2001); amyloid precursor protein (APP) expression in damaged axons has been correlated with the number of CD8+ T lymphocytes migrating to the lesion (Bitsch A et al. 2000); CD8 T cells expressing granzyme B have been observed in demyelineated axons (Sauer et al. 2013).

### *1.5.3 B cells*

Humoral immunity also plays an important role in MS pathogenesis: (i) A steady production of oligoclonal immunoglobulins is observed in the CSF of MS patients (Skulina et al. 2004); (ii) B cells isolated from CSF and brain lesions of MS patients are clonally expanded (Obermeier et al. 2008); (iii) B cells may directly participate in the demyelination process by secreting antibodies which target oligodendrocytes (O´Connor et al. 2005); and (iv) the presence of follicle-like aggregates in the meninges of some MS patients suggests that B-cell responses can be maintained locally within the CNS and contribute to pathogenic process (Magliozzi et al. 2007).

## 1.6 Interferon beta: First MS treatment

Interferons (IFNs) are a diverse family of pleiotropic cytokines with both anti-viral and anti-proliferative functions. There are two types of IFNs: type I IFNs, which include IFNα, IFNβ and IFNϖ, and play important roles in antiviral responses. Type II IFNs, which

include IFNγ, are mainly secreted by T lymphocytes and natural killer (NK) cells. IFNβ, a type I IFN, binds to a specific cell surface receptor and initiates a signaling pathway that will eventually lead to the secretion of IFN-stimulated gene products.

There are 4 IFNβ formulations available, two IFNβ-1a administered intramuscularly once per week (Avonex®) and subcutaneously three times weekly (Rebif®); and two IFNβ-1b administered subcutaneously every other day (Betaferon® and Extavia®). Many biological effects have been proposed for IFNβ in MS, for instance, IFNβ has been shown to decrease the expression of molecules that are needed for antigen presentation, such as the MHC and costimulatory molecules, and it has been shown to decrease the expression of adhesion molecules and metalloproteinases, thus reducing the trafficking of peripheral blood cells into the CNS (Dhib-Jalbut and Marks 2010).

IFNβ was the first disease modifying therapy approved by the FDA for the treatment of MS. In patients with CIS, IFNβ is effective in delaying conversion to clinically definite MS (Comi et al. 2001; Jacobs et al. 2000; Kappos et al. 2006). In patients with RRMS, IFNβ has demonstrated beneficial effects on reducing the relapse rate, delaying the time to sustained disability progression, and decreasing brain disease activity as assessed by magnetic resonance imaging (MRI) (The Interferon β Multiple Sclerosis Study Group et al. 1993; Jacobs et al. 1996; PRISMS et al. 1998). In patients with SPMS, IFNβ demonstrated positive effects on relapses and brain

MRI activity (European Study Group et al. 1998; Panitch et al. 2004; Cohen et al. 2002).

## 1.7 Responders and non-responders to IFNβ

Despite the beneficial effects of IFNβ in MS treatment, the drug is partially effective, and its long-term impact on disease progression remains unclear. Moreover, there is a relatively large proportion of patients who will show a lack of response to IFNβ, which is estimated to vary between 20-55% of treated patients depending on the clinical and radiological criteria used to assess treatment failure (Río et al. 2002). It should be mentioned that the response criteria that are used to classify patients into responders and non-responders to IFNβ are not applied until one or two years of follow-up, period of time during which many patients will be treated with no benefit and at the expense of a high socioeconomic cost. In this context, the search of biomarkers related with the response to IFNβ becomes an important issue to identify patients at high risk for treatment failure (see next section on biomarkers).

One of the reasons for a lack or intermittent response to IFNβ is the development of NABs associated with this treatment (Goodin et al. 2007). IFNβ is a recombinant formulation which is immunogenic and may thus result in antibody responses against the protein. The prevalence of NABs varies considerably among the different IFNβ studies and is influenced by factors such as treatment duration, drug doses, and administration route (Ross et al. 2000). It has been reported that patients positive for NABs may revert to NAB

negativity during continued therapy with IFNβ, and patients who remain NAB negative during the first two years of treatment rarely develop NABs (Sorensen et al. 2005). The effect of NABs on the clinical efficacy of IFNβ has been the subject of considerable debate (Sorensen et al. 2005; Goodin et al. 2007; Polman C et al. 2010). However, evidence exists from different studies about a detrimental role of persistent high-titre NAb positivity on the clinical efficacy of IFNβ, especially with regards to relapses and MRI outcomes (Sorensen et al. 2005).

## 1.8 Biomarkers in MS

A biomarker can be defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention” (Biomarkers Definitions Working Group et al. 2001). In MS, molecular biomarkers can be grouped into the following four categories: predictive, diagnostic, disease activity, and treatment response biomarkers.

- Predictive biomarkers are those which can identify individuals at risk for developing MS.
- Diagnostic biomarkers have the potential to differentiate between MS patients and patients with other autoimmune/neurological disorders or healthy individuals.
- Disease activity biomarkers can be measured in patients with relapsing-remitting and progressive disease courses and may help to differentiate between benign and aggressive disease courses.

- Treatment response biomarkers can be measured before or during treatment and will help in the identification of patients who will or will not respond to treatment, or patients at high risk for adverse drug effects.

It is relevant to mention that all these biomarkers have clear prognostic implications. As an example, biomarkers of lack of response to a particular treatment have associated a worse prognosis compared to biomarkers identified in patients who have good responses to that treatment.

## 1.9. Importance of biomarkers in MS

As it was mentioned before, MS is quite a heterogeneous disease in many aspects. In this context, there is a strong need for biomarkers that capture these different aspects of disease heterogeneity and helps, for instance, (i) in MS diagnosis and disease stratification; (ii) in the prediction of disease course; (iii) in the identification of new therapies that may be beneficial for the disease; and (iv) in the development of a personalized therapy based on the prediction of treatment response and identification of patients at high risk for side effects.

Despite that many biomarkers have been proposed as candidates in MS, very few of them have been properly validated or are currently used in clinical practice. In addition, there are inconsistent results for some of the biomarkers, and there is limited overlap in the results between studies. In this scenario, more efforts are needed to

move forward in the field of biomarkers in MS and identify meaningful biomarkers that can be used in MS clinical practice.

## 1.10 Technologies for biomarker discovery in MS

Over the past few decades, new and massive technologies have come into existence and provided high-throughput and unbiased identification of biomarkers. These technologies have been termed collectively "omics" and mainly include genomics (large-scale study of the whole DNA sequence), transcriptomics (genome-wide determination of the expression levels of coding and/or non-coding RNAs, proteomics (large-scale study of protein profiling), metabolomics (large-scale identification of disease-specific metabolite signatures), and epigenomics (large-scale study of epigenetic modifications).

It is important to highlight that, at present; one of the most optimized and reliable omic technologies is transcriptomics. Within transcriptomics, gene expression profiling has been mainly performed with microarrays, and this technology has been widely used in MS (reviewed in: Comabella and Martin 2007; Sanchez-Pla et al. 2012). In this regard, gene expression microarrays have helped to:

- Identify transcriptional differences between different clinical forms of MS, or between MS patients and healthy controls or patients with other autoimmune disorders (Bomprezzi et al. 2003; Satoh et al. 2005; Tian et al. 2011).

- Identify differentially expressed genes at the time of the CIS event, which may influence the risk of later conversion to MS (Corvol et al. 2008).
- Investigate the transcriptional changes associated with therapies used to treat MS patients (Weinstock-Guttman et al. 2003; Hong et al. 2004; Goertsches et al. 2010).

# HYPOTHESIS

# Hypothesis

1. Gene expression microarrays will allow the identification of highly specific IFNβ bioactivity markers with potential roles in MS pathogenesis.

2. USP18 is involved in MS prognosis as biomarker of disease activity and biomarker of response to IFNβ.

3. Members of the Siglec family play roles in MS prognosis as biomakers of disease activity.

4. Polymorphisms of the IL28B gene are associated with the response to IFNβ in MS patients and thus have a role as response biomakers in the disease.

# OBJECTIVES

# Objectives

1. Search of prognostic biomarkers in MS by (i) identifying genes that follow similar expression behaviour to the IFNβ bioactivity marker myxovirus resistance protein A (MxA); and (ii) evaluating their potential involvement in MS pathogenesis.

2. To investigate the role of USP18 in MS as (i) disease activity biomarker; and (ii) response biomarker to IFNβ.

3. To explore the potential implication of Siglecs in MS as disease activity biomarkers.

4. To investigate the role of IL28B in MS as IFNβ treatment response biomarker.

# PART II
# RESULTS

**Article 1.**

Malhotra S, Bustamante MF, Pérez-Miralles F, Rio J, Ruiz de Villa MC, Vegas E, Nonell L, Deisenhammer F, Fissolo N, Nurtdinov RN, Montalban X, Comabella M. Search for specific biomarkers of IFNβ bioactivity in patients with multiple sclerosis. PLoS One. 2011;6(8):e23634. doi: 10.1371/journal.pone.0023634. Epub 2011 Aug 23.

# Chapter 2

# Search of prognostic biomarkers in MS

# Search for specific biomarkers of IFNβ bioactivity in patients with multiple sclerosis

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## Abstract

Myxovirus A (MxA), a protein encoded by the *MX1* gene with antiviral activity, has proven to be a sensitive measure of IFNβ bioactivity in multiple sclerosis (MS). However, the use of MxA as a biomarker of IFNβ bioactivity has been criticized for the lack of evidence of its role on disease pathogenesis and the clinical response to IFNβ. Here, we aimed to identify specific biomarkers of IFNβ bioactivity in order to compare their gene expression induction by type I IFNs with the MxA, and to investigate their potential role in MS pathogenesis.

Gene expression microarrays were performed in PBMC from MS patients who developed neutralizing antibodies (NAB) to IFNβ at 12 and/or 24 months of treatment and patients who remained NAB negative. Nine genes followed patterns in gene expression over time similar to the *MX1*, which was considered the gold standard gene, and were selected for further experiments: *IFI6*, *IFI27*, *IFI44L*, *IFIT1*, *HERC5*, *LY6E*, *RSAD2*, *SIGLEC1*, and *USP18*. In vitro experiments in PBMC from healthy controls revealed specific induction of selected biomarkers by IFNβ but not IFNγ, and several markers, in particular *USP18* and *HERC5*, were shown to be significantly induced at lower IFNβ concentrations and more selective than the *MX1* as biomarkers of IFNβ bioactivity. In addition, *USP18* expression was deficient in MS patients compared with healthy controls (p=0.0004).

We propose specific biomarkers that may be considered in addition to the MxA to evaluate IFNβ bioactivity, and to further explore their implication in MS pathogenesis.

# Chapter 2

# Search of prognostic biomarkers in MS

## 2.1 Introduction

In 1993, IFNβ became the first FDA-approved drug for the treatment of relapsing-remitting MS (RRMS), and since then it has widely been used in clinical practice. IFNβ has demonstrated beneficial effects on decreasing the number of clinical relapses and disease activity measured by magnetic resonance imaging (The interferon beta group 1993). The mechanisms of action by which IFNβ produces its therapeutic effects in MS are not yet fully understood, however, IFNβ beneficial effects are most likely associated with its immunomodulatory properties.

IFNβ is a type I IFN that binds a heterodimeric cell surface receptor composed of the IFN receptor 1 (IFNAR1) and 2 (IFNAR2) subunits and activates the JAK-STAT signaling pathway. As a result, IFN-stimulated gene factor 3 (ISGF3) complexes are formed and translocated to the nucleus where they bind to IFN-stimulated response elements (ISREs) and initiate the transcription of type I IFN-responsive genes (Platanias et al. 2005). Among the different type I IFN-responsive genes, MxA, a GTPase protein encoded by the *MX1* gene with potent antiviral activity (Haller et al. 2002), has proven to be one of the most sensitive and specific biomarkers of IFNβ bioactivity (Pachner et al. 2003; Gilli et al. 2006). MxA

expression is significantly reduced during the development of neutralizing antibodies (NABs) (Deisenhammer et al. 1999; Pachner A et al. 2003; Hesse D. et al. 2009), and its measurement has provided the basis for in vitro and in vivo assays to determine the presence of NABs (Bertolotto A et al. 2003; Gneiss C. et al. 2006). However, MxA has no clear roles as prognostic biomarker on disease pathogenesis or in the therapeutic response to IFNβ.

In the present study, we aimed to identify new biomarkers of IFNβ bioactivity in order to compare their specificities as genes induced by type I IFNs with the MxA, and evaluate their potential implication in MS pathogenesis.

## 2.2 Materials and Methods

### *2.2.1 Ethics Statement*

The study was approved by the Hospital Universitari Vall d'Hebron Ethics Committee and all patients gave written informed consent to be included in the study.

### *2.2.2 Gene expression microarrays*

PBMC from RRMS patients were collected before and during IFNβ treatment and stored in liquid nitrogen until used. Gene expression microarrays (Affymetrix Human Genome U133 Plus 2.0) were performed in PBMC from 8 RRMS patients at baseline and after 3, 12 and 24 months of IFNβ treatment. All patients were females and the mean age (SD) was 43.1 years (8.8). Four patients were treated

with subcutaneous IFNβ -1b (Betaferon), and the remaining received subcutaneous IFNβ-1a (Rebif). Four patients were negative for NABs at 12 and 24 months and 4 patients developed NABs at 12 and/or 24 months (one patient was NAB positive at 12 and 24 months, another patient was negative at 12 and positive at 24 months, and 2 patients were positive at 12 and negative at 24 months).

Quality control, preprocessing and analysis of microarray data MX1, which was chosen as our 'gold standard' gene. To achieve this purpose, graphics of MX1 gene expression (202086_at affy ID) over time were generated for the 8 patients included in the study, and searched for genes that followed the same pattern in gene expression. First, for each patient, behaviour of MX1 was analyzed at each time point and determined whether MX1 gene expression decreased or increased in each time interval: 0–3 months, 3–12 months, and 12–24 months. Next, genes that followed the same increase-decrease pattern in gene expression to the MX1 were selected. The final list of genes was generated with all common genes in the 8 study patients. The absolute value of change in gene expression was set at 0.8, because 0.83 was the minimum increase in gene expression observed for MX1 in one of the patients from baseline to the 3 months time point. Pathway analysis was performed with Ingenuity Pathway Analysis (Ingenuity Systems, version 9.0 www.ingenuity.com) using two separate lists of genes; on one side the 816 unique transcripts of up-regulated genes and, on the other side, the 329 unique transcripts list of down-regulated genes. Search of potential binding sites for transcription factors in

promoter regions of selected genes was performed using the TRANSFAC database.

We searched potential binding sites for STAT transcription factors in promoter regions of selected IFNβ -induced genes. For this, we downloaded corresponding matrixes from the TRANSFAC database (Matys V et al. 2006) (public release 7.0 2005). Unfortunately, matrixes were of poor quality and therefore we decided to use Transcription Factor ChIP-seq from ENCODE track in UCSC genome browser as an additional control. We downloaded five matrixes for STAT factors from TRANSFAC database (public release 7.0 2005). M00223 matrix was designed to recognize all STATs binding sites, M00224 matrix was designed to recognize only STAT1 factor, M00225 matrix was designed to recognize STAT3. Finally, M00459 and M00460 matrixes were designed to recognize STAT5B homodimer and STAT5A homotetramer respectively. We searched candidate sites in 5kb upstream sequences for selected genes.

Microarray data are stored in the Gene Expression Omnibus (GEO) repository and are available at http://www.ncbi.nlm.nih.gov/geo/ with the following entry number: GSE26104. NABs were determined in serum samples at baseline and after 12 and 24 months of treatment by means of the MxA induction bioassay, as described elsewhere (Gneiss, C., et al., 2006), and titers equal or higher than 20 neutralizing units were deemed positive results.

### *2.2.3 Validation of selected IFNβ bioactivity markers by real time PCR (RT-PCR)*

In 4 patients, expression levels of selected genes were also determined by real time RT-PCR in order to validate microarray findings. Total RNA was taken from the same samples that had been used for the microarrays. Complementary DNA (cDNA) was synthesized from total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, U.S.A). Amplifications were performed in duplicate using Taqman probes specific for the genes selected from microarray studies (Applied Biosystems). The housekeeping gene GAPDH was used as an endogenous control. The threshold cycle ($C_T$) value for each reaction, and the relative level of gene expression for each sample were calculated using the $2^{-\Delta\Delta CT}$ method (Li et al. 2010). In brief, GAPDH was employed for the normalization of the quantity of RNA used. Its $C_T$ value was subtracted from that of the specific genes to obtain a ΔCT value. The differences (ΔΔCT) between the ΔCT values obtained for the untreated baseline samples (calibrators) and the ΔCT values for the IFNβ-treated samples (at 3, 12 and 24 months) were determined. The relative quantitative value for the treated samples was then expressed as $2^{-\Delta\Delta CT}$, representing the fold change in gene expression normalized to the endogenous control and relative to the calibrators.

### *2.2.4 Dose- and time-dependent induction of selected IFNβ bioactivity markers*

### *2.2.4a For dose-dependent experiments*

Fresh PBMC from 6 healthy controls [3 females / 3 males; mean age: 27.5 years (7.1)] were isolated by Ficoll-Isopaque density gradient centrifugation (Gibco BRL, Life Technologies LTD, UK), washed twice and resuspended in culture medium (RPMI medium 1640 supplemented with 10% fetal bovine serum, 4mM L-glutamine, 25mM Hepes buffer, 50 units/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL). PBMC ($2x10^6$cells/ml) were cultured for 24 hours with intramuscular IFNβ-1a (Avonex), Rebif, Betaferon, and human recombinant IFNγ at different concentrations: 0.1, 10, 100, and 1000 IU/ml. After cell culture, mRNA expression levels of selected IFNβ bioactivity markers were determined by real time RT-PCR, as previously described. Changes in gene expression were always compared with cells cultured in the absence of IFNβ (referred to as 0 IU/ml; calibrators).

### *2.2.4b For time-dependent experiments*

Freshly isolated PBMC from 7 healthy controls [3 females / 4 males; mean age: 27.5 years (5.7)] were cultured as previously described in the presence or absence of 100 IU/ml of Avonex, Rebif, Betaferon, and human recombinant IFNγ for 2, 4, 6, 8, and 24 hours. After cell culture, gene expression levels for selected markers were determined by real time RT-PCR, as described above. Changes in gene expression were always referred to a baseline uncultured condition (0 h; calibrators). Previously, gene expression levels obtained for the different biomarkers in untreated cells

cultured for the same time points were subtracted from the values obtained after treatment with IFNβ and IFNγ.

### *2.2.5 NAB-induced gene expression inhibition*

Undiluted and serially diluted serum (1:3, 1:9, 1:27, 1:81, 1:243, 1:729) collected from a patient treated with Betaferon who developed NABs at high titres (>1280) was preincubated for 1 hour in the presence or absence of 100 IU/ml of Betaferon. Subsequently, freshly isolated PBMC from 3 healthy controls [2 females / 1 male; mean age: 24.7 years (2.1)] were cultured for 8 hours with preincubated medium. After cell culture, mRNA expression levels of selected IFNβ bioactivity markers were determined by real time RT-PCR, as described above. IFNβ-induced expression levels were compared with those obtained from cells cultured without IFNβ in the presence of serum from a NAB negative patient (calibrators). PBMC cultured with 100 IU/ml of Betaferon was used as positive control.

### *2.2.6 Abrogation of gene expression induced by non-specific cell activation*

Freshly isolated PBMC from 3 healthy controls [2 females / 1 male; mean age: 29.3 years (4.9)] were cultured for 8 hours in preincubated medium with Phytohaemagglutinin (PHA) (5ug/ml) plus Lipopolysaccharide (LPS;1ug/ml) in the presence or absence of undiluted high-titre NAB positive serum (>1280) with and without anti-IFNγ antibodies (100ng/ml) at 37º C for 1 hour. After cell

culture, gene expression of selected biomarkers was determined by real time RT-PCR, as previously described. Cell activation-induced expression levels were compared with those obtained from unstimulated cells cultured in the presence of serum from a NAB negative patient (calibrators). PBMC cultured with 100 IU/ml of Betaferon in the presence or absence of high-titre NAB positive serum were used as positive controls of NAB-induced inhibition.

### *2.2.7 Gene expression levels for selected bioactivity markers in MS and controls*

Fresh PBMC were isolated from 14 untreated RRMS patients [64.3% females; mean age (standard deviation (SD)): 42.1 years (9.6); mean number of relapses in the previous 2 years: 0.9 (0.9); mean disease duration: 12.4 years (7.1); median Expanded Disability Status Score (EDSS) at the time of blood collection (interquartile range): 2.0 (1.5-3.0)]. A group of 15 healthy controls [53.3% females; mean age: 30.5 years (6.2)] was also included in the study. After RNA extraction, mRNA expression levels for selected biomarkers were determined by real time RT-PCR, as described above. Gene expression values obtained for MS patients were referred to the expression levels observed in controls (calibrators).

### *2.2.8 Statistical analysis*

#### *2.2.8a For dose-dependent experiments*

The following parameters were considered: (i) Sensitivity was evaluated by the LLOQ and defined as the minimum IFNβ concentration that induced a statistically significant increase in gene expression when compared with the untreated condition, and it was calculated by paired t-tests adjusting for multiple testing using the Bonferroni approach. (ii) Selectivity was defined as the total induction in gene expression observed in the presence of different concentrations of type I but not type II IFNs, and it was calculated by comparing the Area under curve (AUC) obtained for IFNβ and IFNγ. The p-value associated with the AUC of the difference was calculated by means of a t-type statistic that uses the critical value from a t-distribution with Satterthwaite's approximation (Matys V et al. 2006) to the degrees of freedom for calculation of confidence intervals.

*2.2.8b For time-dependent experiments*

Selectivity was defined as the total induction in gene expression obtained at the different time points of in vitro cell culture in the presence of type I but not type II IFNs, and it was analyzed by computing the AUC of the difference, as described above.

*2.2.8c NAB-induced gene expression inhibition*

It was evaluated by comparing the NAB-positive serum dilutions that were associated with reductions in gene expression of selected biomarkers greater than 25% and 50% of the expression levels obtained for the positive control condition.

*2.2.8d Gene expression levels in MS patients and controls*

A Mann-Whitney´s test was used to test for significant differences in gene expression levels between MS patients and controls. Insomuch as 10 different genes were evaluated, Bonferroni correction was used to correct the alpha level for multiple comparisons (alpha=0.005). Statistical calculations were performed with R language and the SPSS 11.5 package (SPSS Inc, Chicago, IL) for MS-Windows.

## 2.3 Results

The present study is designed to find out the genes which show differential expression in response to treatment of IFNβ and to study differential expression in gene expression at basal level between patients and controls

*2.3.1 Microarray studies identify biomarkers of IFNβ bioactivity with similar gene expression patterns to the MX1*

**Table.2.1**

| Affymetrix probe set | Symbol | Description | Other aliases and designations |
|---|---|---|---|
| 202086_at | *MX1** | myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) | MxA |
| 204415_at | *IFI6* | interferon, alpha-inducible protein 6 | IFI-6-16, G1P3 |
| 202411_at | *IFI27* | interferon, alpha-inducible protein 27 | ISG12 |
| 204439_at | *IFI44L* | interferon-induced protein 44-like | C1orf29 |
| 203153_at | *IFIT1* | interferon-induced protein with tetratricopeptide repeats 1 | IFI56, ISG56 |
| 219863_at | *HERC5* | hect domain and RLD 5 | CEB1, CEBP1 |
| 202145_at | *LY6E* | lymphocyte antigen 6 complex, locus E | RIGE |
| 213797_at / 242625_at | *RSAD2* | radical S-adenosyl methionine domain containing 2 | viperin |
| 219519_s_at | *SIGLEC1* | sialic acid binding Ig-like lectin 1, sialoadhesin | CD169 |
| 219211_at | *USP18* | ubiquitin specific peptidase 18 | ISG43 |

***Table 2.1*** Selected markers of IFNβ bioactivity from gene expression microarrays

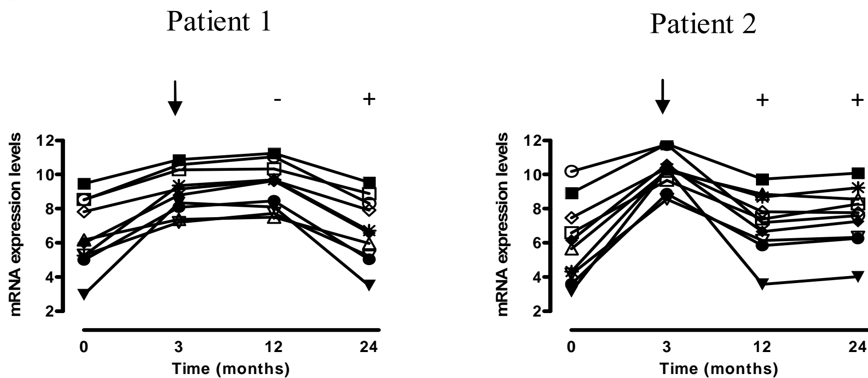
*Appendix table 1 and 2* show the top canonical pathways that were identified in down- and up-regulated genes respectively during IFNβ treatment compared to the baseline condition. As expected, the type I IFN signaling pathway was one of the most significant pathways identified among up-regulated genes.

In order to identify new markers of IFNβ bioactivity, we first performed gene expression microarrays in PBMC collected at different time points from IFNβ-treated patients who developed NABs at 12 and/or 24 months and patients who remained NAB negative during the follow-up.

Nine genes fulfilled the conditions described in the Methods section and followed patterns of gene expression over time similar to the MX1, the gold standard gene, and were chosen for further experiments (Table 2.1)

As shown in Figure 2.A, selected genes were significantly induced by IFNβ treatment after 3 months of treatment and their expression levels were reduced by the presence of NABs and reversed in NAB negative conditions.

**Fig.2.A**



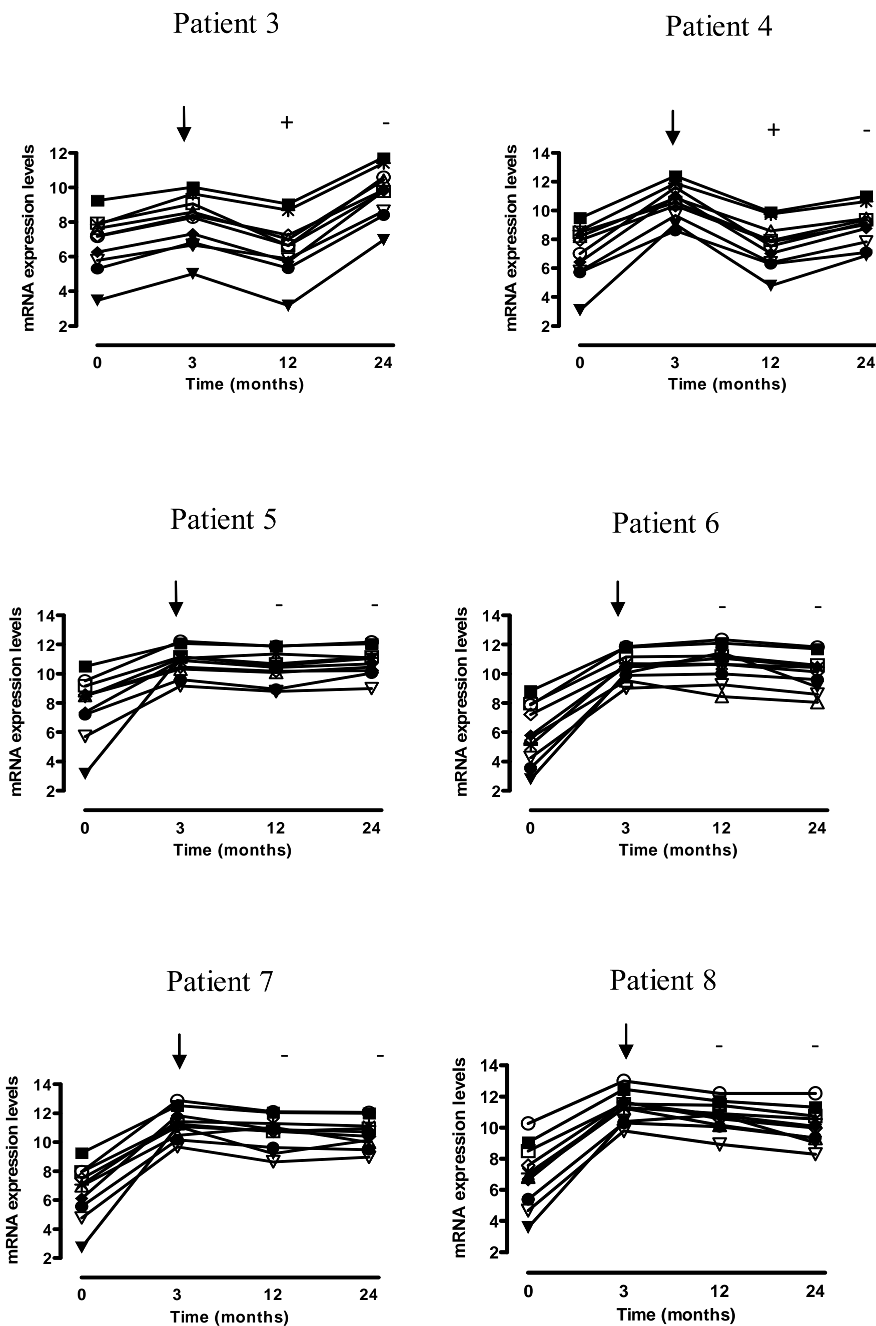



*Figure 2. (A) Changes in gene expression observed with microarrays for selected IFNβ bioactivity markers at baseline (T=0), and after 3, 12, and 24 months of*

*treatment. Four patients developed NABs at 12 and/or 24 months (Patients 1-4) and 4 patients remained NAB negative at these time points (Patients 5-8). (B) Validation of microarray findings by real time RT-PCR in representative patients belonging to the different categories (Patients 1, 2, 3, and 5). Given the much stronger induction in gene expression observed for IFI27, graphs corresponding to its expression were depicted separately for the sake of clarity. Open squares: Ly6E; open circles: IFIT1; open triangles: IFI6; open inverted triangles: USP18; open diamonds: HERC5; asterisks: IFI44L; solid squares: MX1; solid circles: SIGLEC1; solid inverted triangles: IFI27; solid diamonds: RSAD2. ↓: refers to induction in gene expression after 3 months of treatment. +: NAB positive determination. -: NAB negative determination*

### *2.3.2 Real time RT-PCR of selected genes in order to validate microarray findings*

As depicted in Figure 2.B, mRNA expression levels measured by PCR over time in NAB positive and negative patients mirrored those obtained with gene expression.

**Fig.2.B**

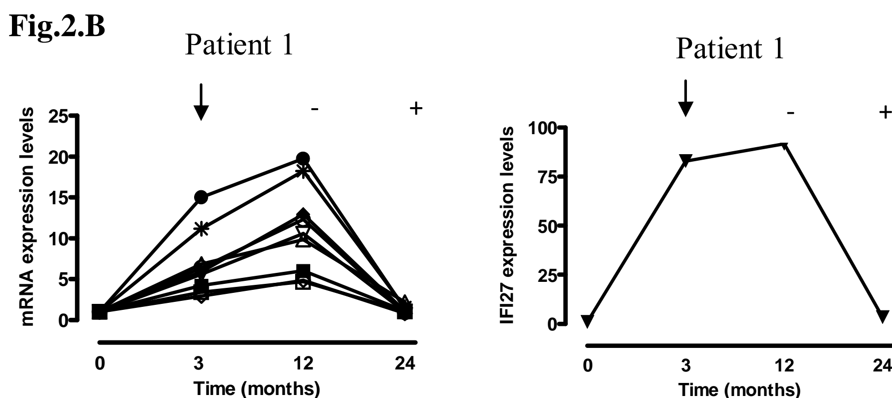


**Fig.2.C**

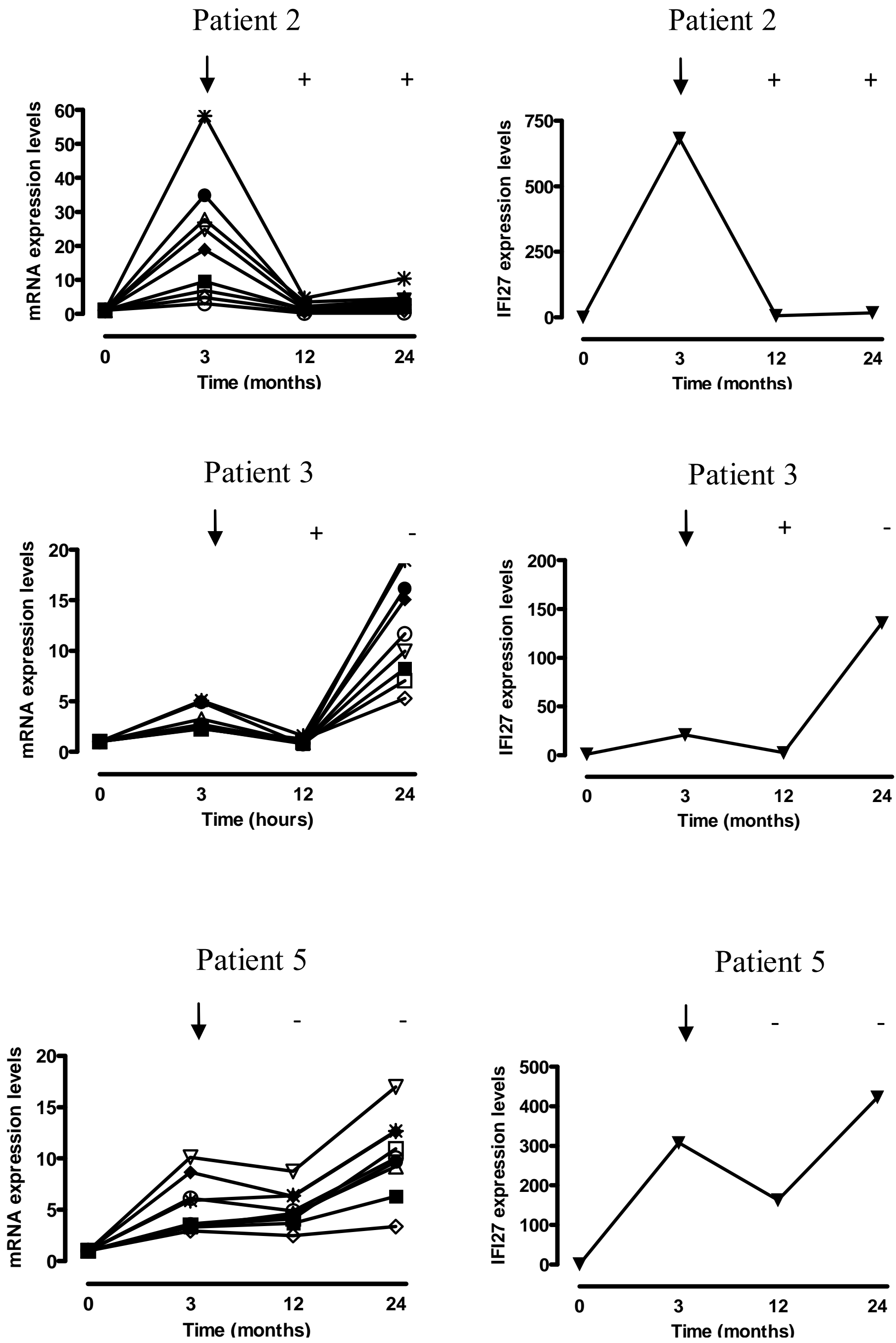
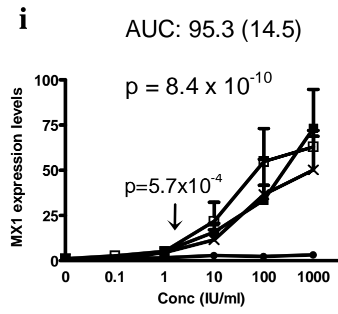


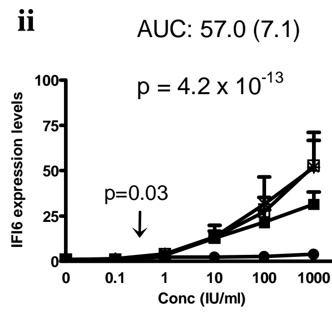
*Figure 2.B Validation of microarray findings by real time RT-PCR in representative patients belonging to the different categories (Patients 1, 2, 3, and 5). Given the much stronger induction in gene expression observed for IFI27, graphs corresponding to its expression were depicted separately for the sake of clarity.*

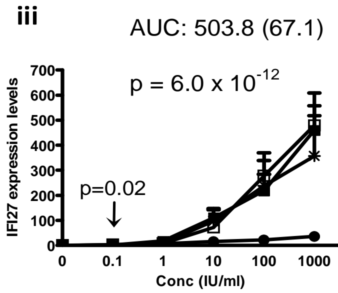
### *2.3.3 Selected IFNβ bioactivity markers are specifically induced by type I IFNs*

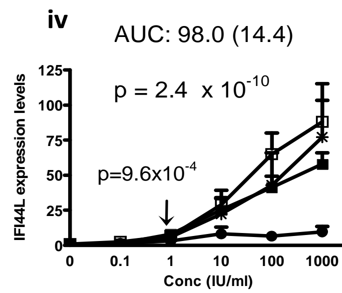
As a next step, we performed in vitro experiments to characterize the specific induction of selected biomarkers by type I (IFNβ) but not type II (IFNγ) IFNs. First, we cultured PBMC from healthy controls for 8 hours in the presence or absence of different concentrations of Avonex, Rebif, Betaferon, and IFNγ.

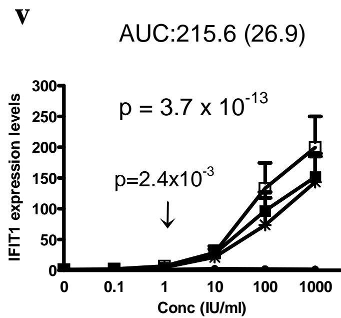
After cell culture, mRNA expression levels were determined by real time RT-PCR, as described in Methods. Results are expressed as fold change in gene expression relative to the untreated condition (0 IU/ml). Bars represent AUC: Standard error of mean (SEM) of the difference between IFNβ and IFNγ inductions, together with the associated p-value (selectivity). Arrows indicate the p-values resulting from the comparisons in gene expression between the different IFNβ concentrations and the untreated conditions (lower limit of quantification).

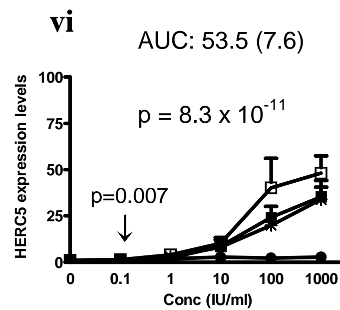

i
AUC: 95.3 (14.5)
p = 8.4 x 10-10
p=5.7x10-4
MX1 expression levels
Conc (IU/ml)

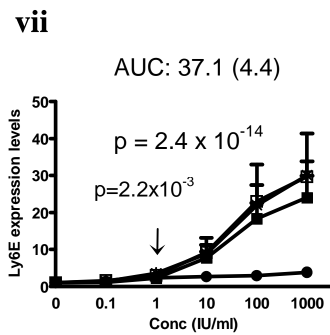

ii
AUC: 57.0 (7.1)
p = 4.2 x 10-13
p=0.03
IFI6 expression levels
Conc (IU/ml)

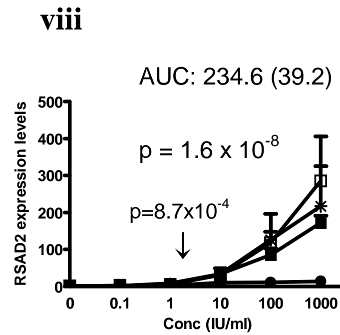

iii
AUC: 503.8 (67.1)
p = 6.0 x 10-12
p=0.02
IFI27 expression levels
Conc (IU/ml)


iv
AUC: 98.0 (14.4)
p = 2.4 x 10-10
p=9.6x10-4
IFI44L expression levels
Conc (IU/ml)


v
AUC:215.6 (26.9)
p = 3.7 x 10-13
p=2.4x10-3
IFIT1 expression levels
Conc (IU/ml)


vi
AUC: 53.5 (7.6)
p = 8.3 x 10-11
p=0.007
HERC5 expression levels
Conc (IU/ml)


vii
AUC: 37.1 (4.4)
p = 2.4 x 10-14
p=2.2x10-3
Ly6E expression levels
Conc (IU/ml)


viii
AUC: 234.6 (39.2)
p = 1.6 x 10-8
p=8.7x10-4
RSAD2 expression levels
Conc (IU/ml)

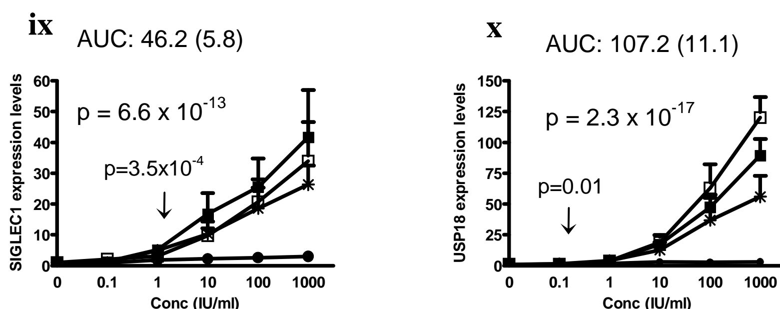


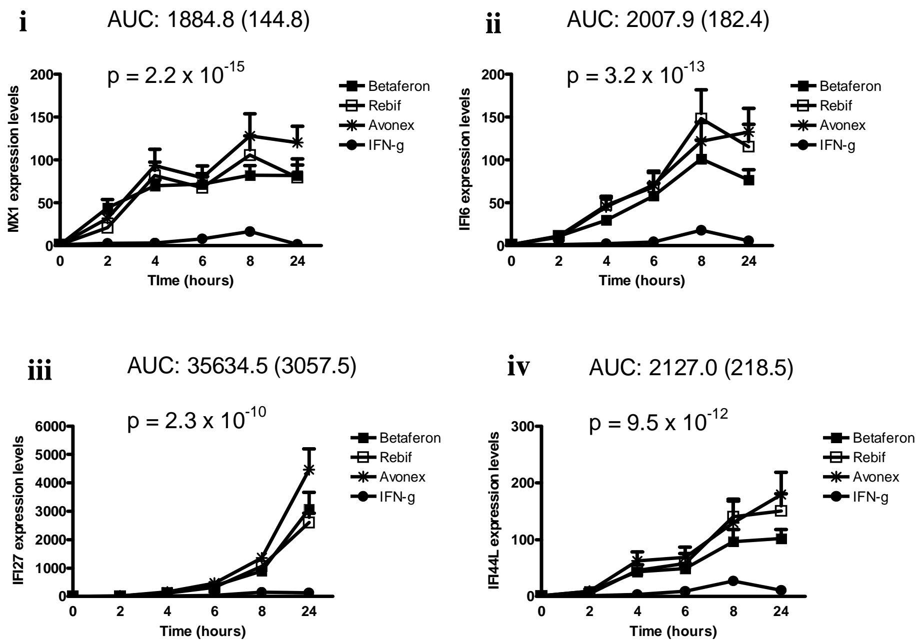

*Figure 2.C Dose-dependent induction in gene expression of selected IFNβ bioactivity biomarkers. PBMC from 6 healthy controls were cultured for 24 hours with Avonex, Rebif, Betaferon, and recombinant IFNγ at different concentrations (Conc; x-axis).*

As shown in Figure 2.C, all genes were selectively induced by IFNβ, as indicated by the differences in gene expression observed for IFNβ and IFNγ. The different types of IFNβ resulted in similar levels of gene expression and were considered together for calculations. Four genes had a lower limit of quantification (LLOQ) of 0.1 IU/ml: *HERC5* (p=0.007), *USP18* (p=0.01), *IFI27* (p=0.02), and *IFI6* (p=0.03) (Figure 2.C, arrows). The remaining genes included *MX1*, reached statistical significance in their gene expression inductions at higher IFNβ concentrations (LLOQ: 1 IU/ml). Except for *RSAD2*, all the selected biomarkers were shown to be more selective than the *MX1* gene, as indicated by the p-values associated with the AUC of the difference between IFNβ and IFNγ. *USP18* had the lowest p-value ($p=2.3 \times 10^{-17}$) and was considered to be the most selective IFNβ biomarker. Four genes (*IFI27*, *IFIT1*, *RSAD2*, and *USP18*) had stronger inductions in gene expression by IFNβ compared with the *MX1*, whereas *IFI6*, *IFI44L*, *HERC5* and *SIGLEC1* showed gene expression levels comparable

to the *MX1*. Finally, *Ly6E* was up-regulated at lower levels (Figure 2.C).

From these dose-dependent experiments, a concentration of 100 IU/ml was considered optimal for gene expression induction and selected for further experiments. Next, we cultured PBMC from healthy controls at different time points with 100 IU/ml of IFNβ and IFNγ.

**Fig.2.D**



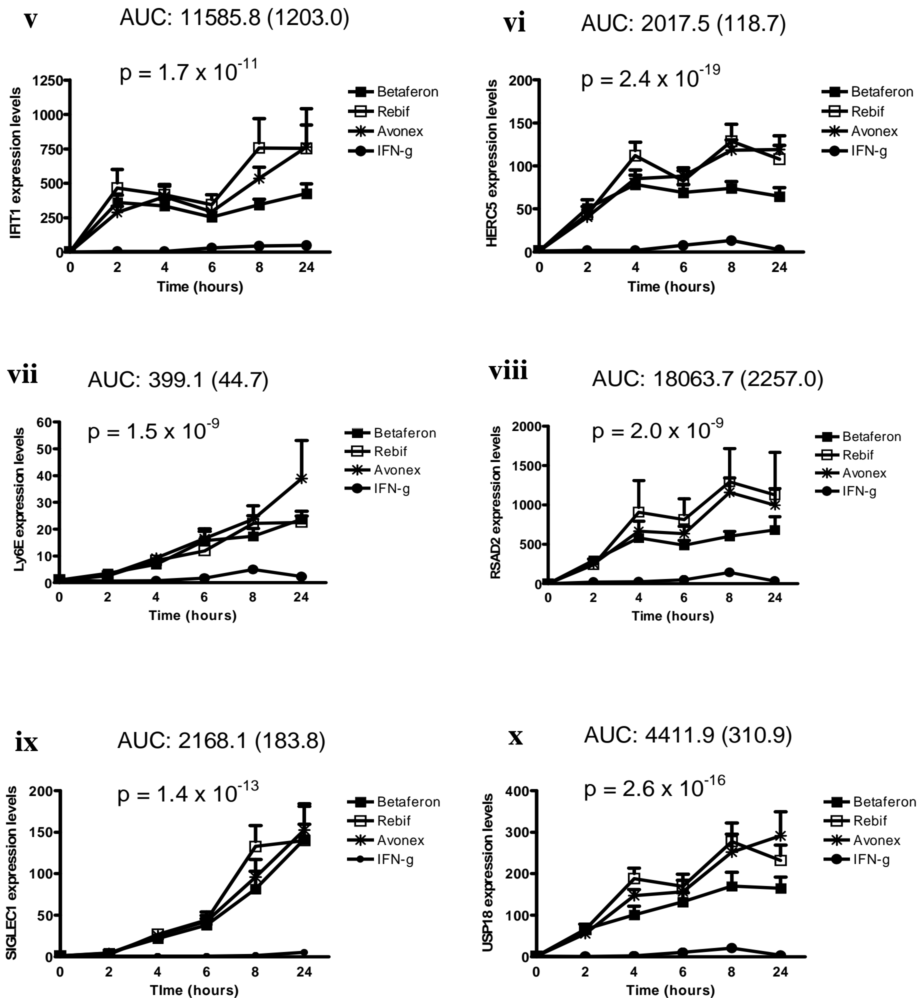



*Figure 2.DTime-dependent induction in gene expression of selected IFNβ bioactivity markers. PBMC from 7 healthy controls were cultured at different time points in the presence or absence of 100 IU/ml of Avonex, Rebif, Betaferon, and recombinant IFNγ*

At each time point, mRNA expression levels for each gene were determined by real time RT-PCR, as described under Methods. Results are expressed as fold change in gene expression relative to the uncultured condition (0 h) after subtraction of the expression

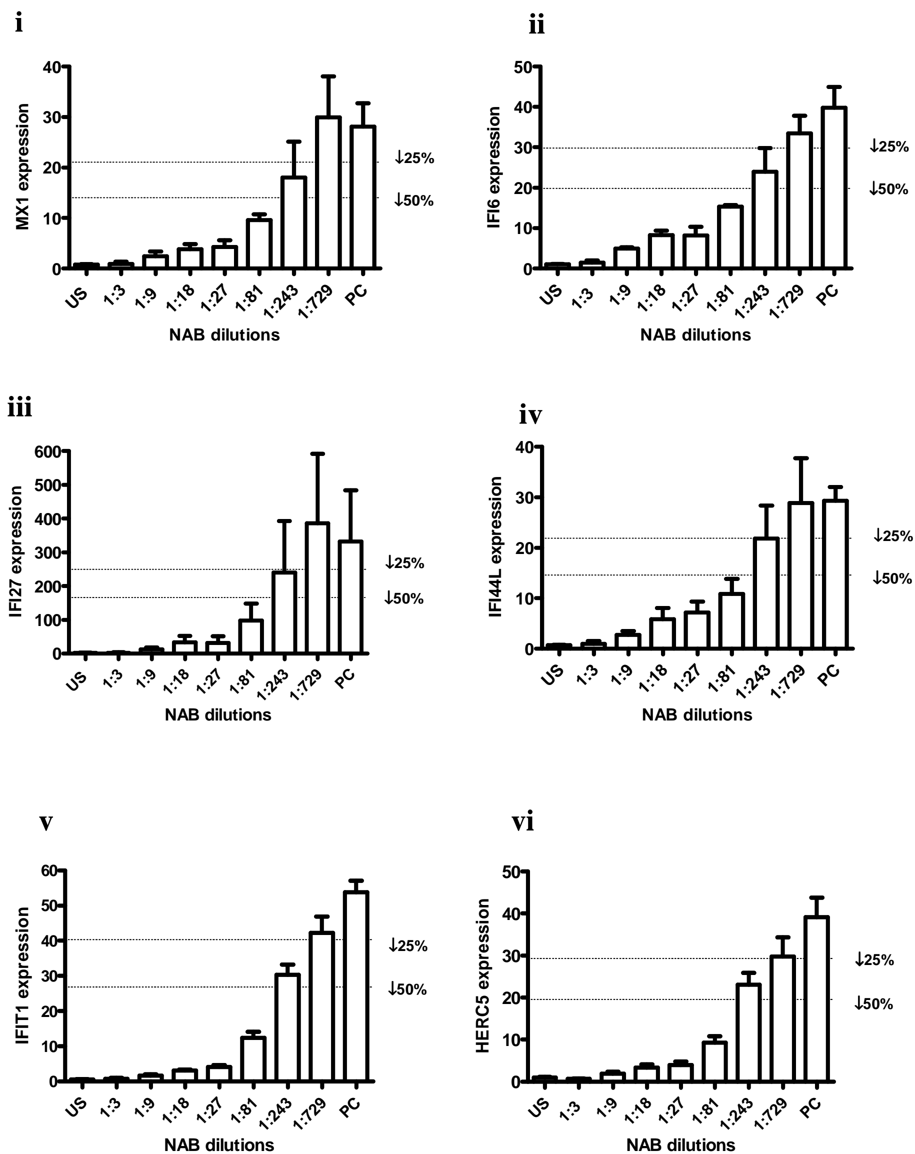
levels obtained for untreated cells. Bars represent SEM. AUC (SEM) of the difference between IFNβ and IFNγ inductions, together with the associated p-value (selectivity). As shown in Figure 2.D, comparisons of the AUC obtained for gene expression at the different time points revealed *HERC5* ($p=2.4 \times 10^{-19}$) and *USP18* ($p=2.6 \times 10^{-16}$) as the genes that were most selectively induced by IFNβ but not IFNγ. The remaining genes showed lower selectivity values compared with the *MX1* ($p=2.2 \times 10^{-15}$). Similar to the dose-dependent induction, *IFI27*, *IFIT1*, *RSAD2*, and *USP18* were more up-regulated at the different time points by IFNβ than the *MX1*. On the other hand, *IFI6*, *IFI44L*, *HERC5*, and *SIGLEC1* showed comparable levels of gene expression induction to the *MX1*, whereas *Ly6E* was the least induced gene at all time points For most of the biomarkers, peak levels of gene expression occurred after 8 hours of cell culture and this time point was chosen for further experiments.

These data indicate that, although all the selected genes are specifically induced by type I but not type II IFNs; several biomarkers appear to be induced at lower IFNβ concentrations and more selective than the *MX1*.

### *2.3.4 Gene expression of selected biomarkers is gradually inhibited by increasing NAB titres*

We next evaluated the capacity of high and low NAB titres to inhibit the expression of selected IFNβ bioactivity biomarkers.

**Fig.2.E**



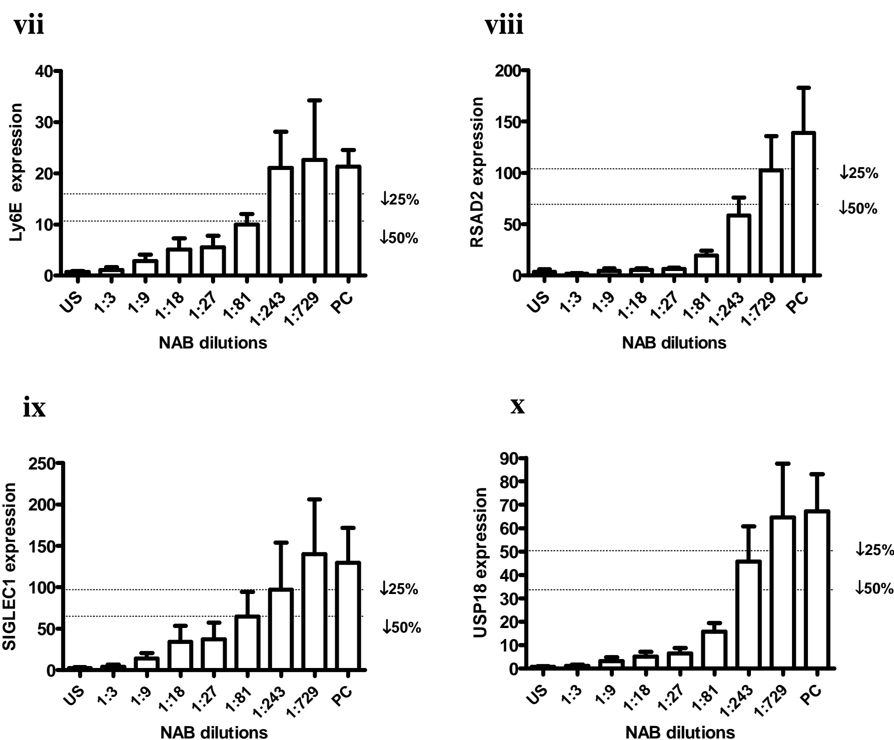



*Figure 2.E NAB-induced gene expression inhibition of selected biomarkers. Undiluted and increasingly diluted serum from an IFNβ-treated patient who developed high NAB titres were preincubated for 1 hour in the presence or absence of 100 IU/ml of Betaferon, and then added to PBMC from 3 healthy controls for 8 hours*

Results are expressed as fold change in gene expression relative to a condition of cells cultured without IFNβ and with a value of 1 after normalization (not shown in the graphs for the sake of clarity). Bars represent SEM. Dotted lines indicate the expression levels that correspond to 25% and 50% reductions in gene expression of the positive control condition. US: undiluted serum. PC: positive control. NAB: neutralizing antibodies to IFNβ.

As depicted in Figure 2.E, all biomarkers showed similar profiles of gene expression inhibition by different NAB dilutions, and gene

expression was greatly reduced by high NAB titres (undiluted serum and serum dilutions ranging from 1:3 to 1:27). At lower NAB titres (1:81 serum dilutions), except for *SIGLEC1* gene expression of selected biomarkers was reduced by more than 50% of the expression levels obtained for the positive control. At 1:243 serum dilutions, except for *SIGLEC1*, *IFI44L*, and *Ly6E* gene expression of the remaining biomarkers was reduced by greater than 25% of the positive control expression levels. Interestingly, *RSAD2* showed the highest degree of inhibition in gene expression by low NAB titres, and was the only IFNβ bioactivity biomarker whose expression was reduced by more than 25% of the positive control condition at the highest serum dilutions (1:729), and greater than 50% after 1:243 dilutions (Figure 2.E).

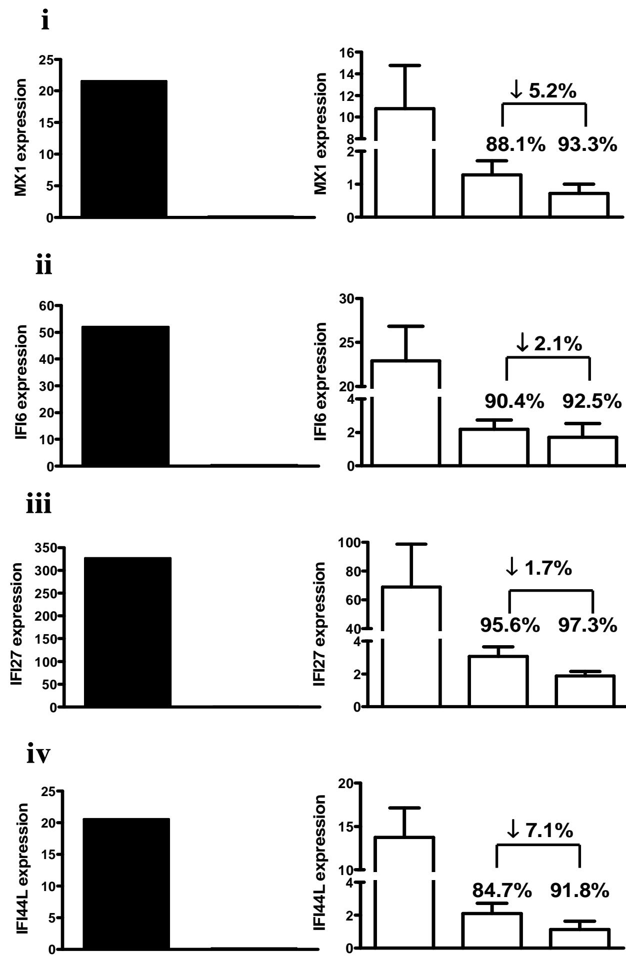
Despite similar levels of NAB-induced gene expression inhibition observed for selected biomarkers, these results point to *RSAD2* as the most sensitive biomarker to capture the blocking effect of low NAB titres.

### *2.3.5 Abrogation of gene expression of selected biomarkers following cell activation*

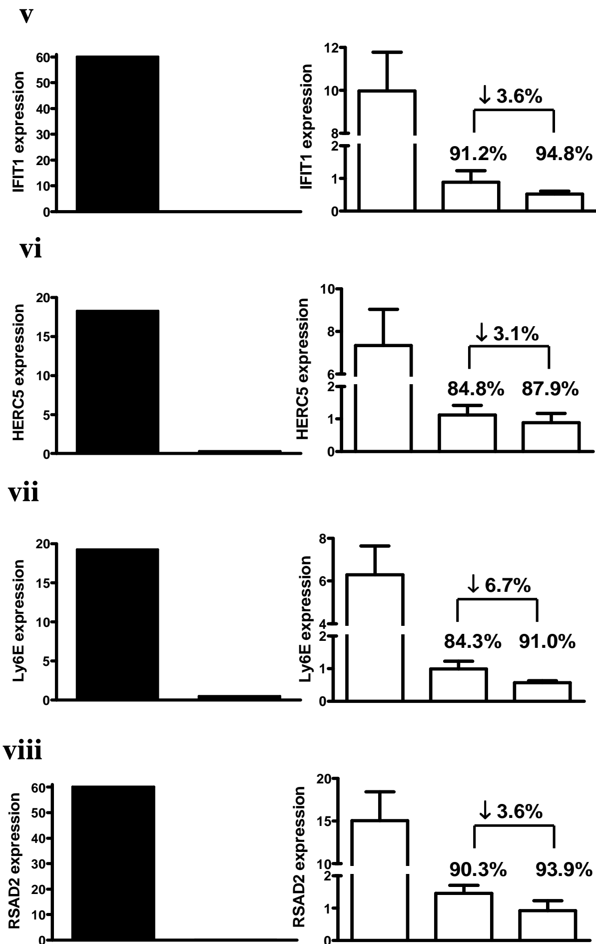
To evaluate whether selected biomarkers could be indirectly induced via the production of cytokines other than IFNβ, PBMC from healthy controls were non-specifically activated with LPS plus PHA in the presence or absence of a high-titre NAB positive serum.

As shown in Figure 2.F, IFNβ accounted for the majority of gene expression induced by non-specific cell activation, as IFNβ blocking was associated with a more than 80% reduction in the expression levels for *MX1*, *IFI44L*, *HERC5*, and *Ly6E*, and greater than 90% reduction for *IFI6*, *IFI27*, *IFIT1*, *RSAD2*, *SIGLEC1*, and *USP18*. As expected from dose- and time-dependent experiments, IFNγ contributed little to cell activation-induced gene expression, and IFNγ blocking only resulted in a small additional decrease in gene expression that ranged from 1.5% for *SIGLEC1* to 7.1% for *IFI44L* (Figure 2.F). These findings indicate that cell activation-induced up-regulation of selected biomarkers is mostly mediated by the effects of IFNβ, and other cytokines included IFNγ appear to contribute little (As shown on the next page).

# Fig.2.F




| | | | | | |
|---|---|---|---|---|---|
| IFNβ: | + | + | - | - | - |
| LPS + PHA: | - | - | + | + | + |
| NABs: | - | + | - | + | + |
| anti-IFNγ: | - | - | - | - | + |




| | | | | | |
|---|---|---|---|---|---|
| IFNβ: | + | + | - | - | - |
| LPS + PHA: | - | - | + | + | + |
| NABs: | - | + | - | + | + |
| anti-IFNγ: | - | - | - | - | + |

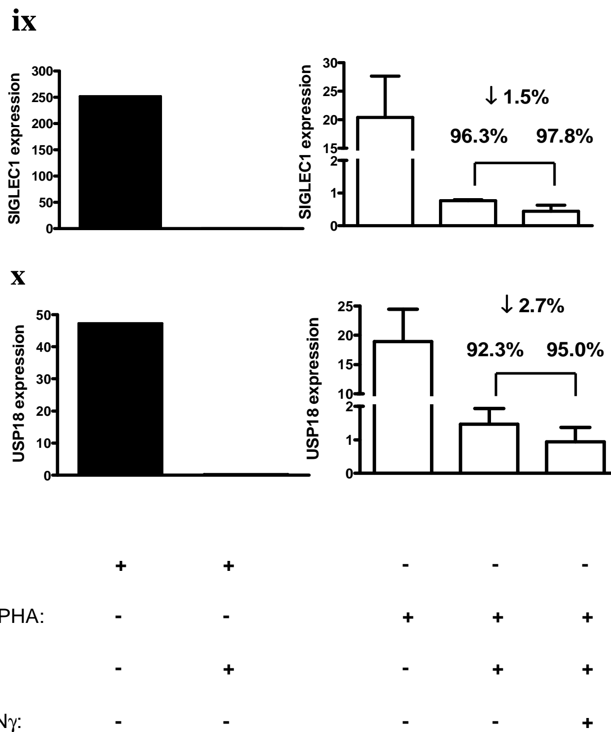


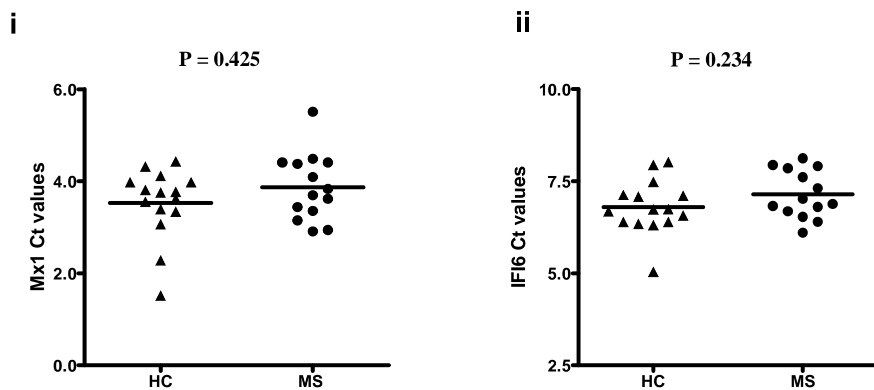

*Figure 2.F Abrogation of gene expression of selected biomarkers following non-specific cell activation (i-x). PBMC from 3 healthy controls were cultured for 8 hours in preincubated medium with PHA plus LPS in the presence or absence of undiluted high-titre NAB positive serum with and without anti-IFNγ antibodies, as described in Methods. Results are expressed as fold change in gene expression relative to a condition of unstimulated cells and with a value of 1 after normalization (not shown in the graphs for the sake of clarity). PBMC cultured with 100 IU/ml of Betaferon in the presence or absence of high-titre NAB positive serum were used as positive controls (graphs on the left). Bars represent SEM. Arrows indicate the difference in gene expression observed after the addition of anti-IFNγ antibodies. NAB: neutralizing antibodies to IFNβ.*

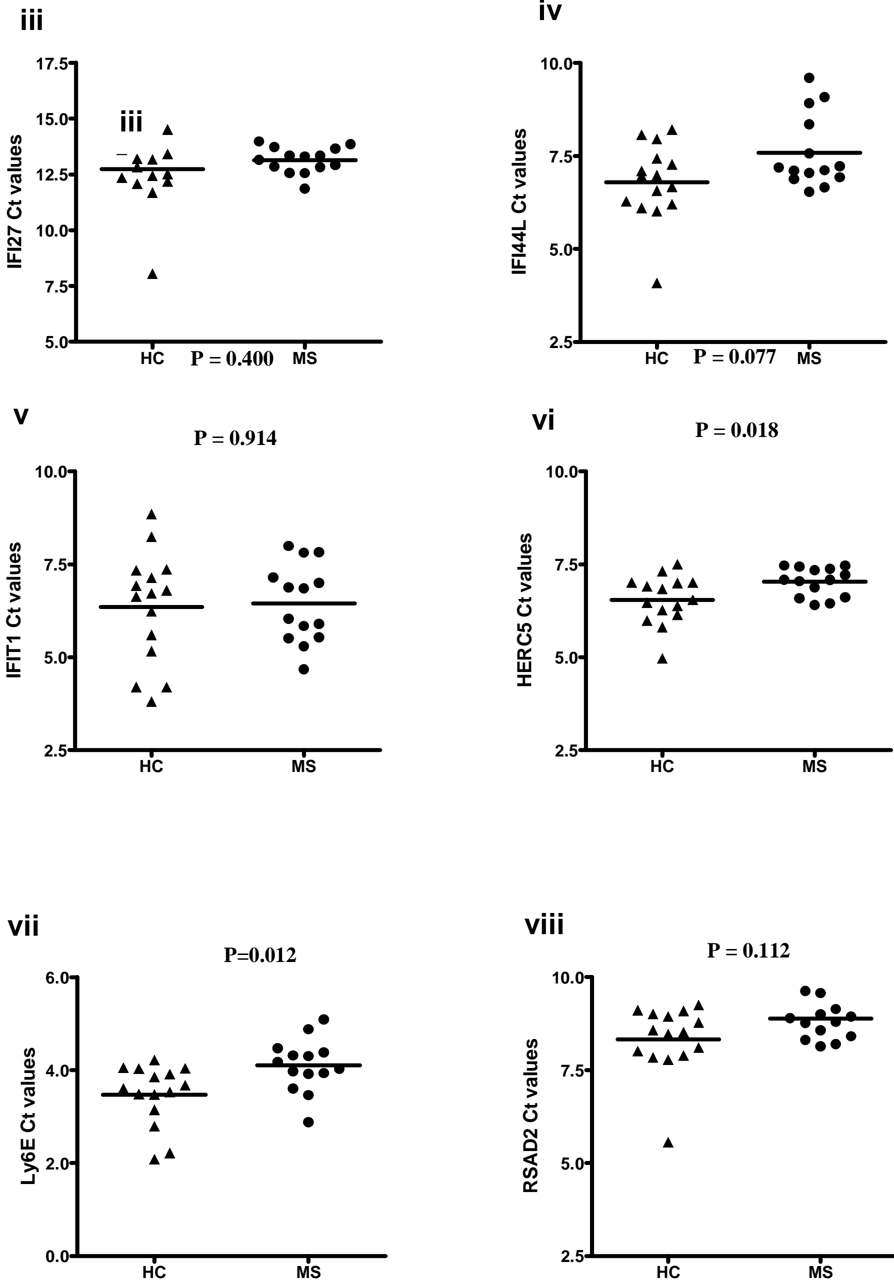
### *2.3.6 USP18 expression is deficient in MS patients*

We finally aimed to evaluate the potential implication of selected biomarkers in MS pathogenesis. To achieve this, expression levels for these biomarkers were compared between untreated RRMS

patients and healthy controls. Interestingly, only *USP18* survived correction for multiple testing, and expression levels for this gene were significantly lower in MS patients compared with controls (p=0.0004) (Figure 2.G). Trends towards lower expression in MS patients were also observed for *HERC5* (p=0.018) and *Ly6E* (p=0.012), although differences did not reach the threshold for statistical significance after Bonferroni correction (alpha=0.005). Expression levels for the remaining genes were similar between MS patients and healthy controls (Figure 2.G).

**Fig.2.G**




iii
iii
IFI27 Ct values
17.5
15.0
12.5
10.0
7.5
5.0
HC
P = 0.400
MS
iv
IFI44L Ct values
10.0
7.5
5.0
2.5
HC
P = 0.077
MS
v
P = 0.914
IFIT1 Ct values
10.0
7.5
5.0
2.5
HC
MS
vi
P = 0.018
HERC5 Ct values
10.0
7.5
5.0
2.5
HC
MS
vii
P=0.012
Ly6E Ct values
6.0
4.0
2.0
0.0
HC
MS
viii
P = 0.112
RSAD2 Ct values
10.0
7.5
5.0
2.5
HC
MS

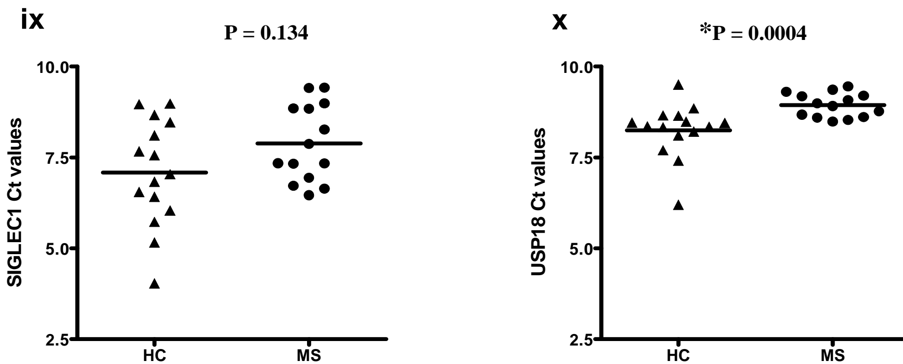



*Figure 2.G Comparison of gene expression levels of selected biomarkers in MS patients and controls. PBMC were collected from untreated RRMS patients (N=14) and healthy controls (N=15) and the mRNA expression levels for each gene were determined by real time RT-PCR. The y-axis represents the threshold cycle (CT) values obtained for each individual. CT is inversely related to quantity, and higher CT values are indicative of lower mRNA expression levels. MS: untreated RRMS patients. HC: healthy controls*

No significant correlations were observed between USP18 expression levels and variables such as gender, age at onset, EDSS scores at the time of blood collection, number of relapses in the 2 previous years, and disease duration (p>0.05).

## 2.4 Discussion

MxA is specifically induced by type I IFNs and has demonstrated to be a reliable and sensitive measure of the biological response to IFNβ (Pachner et al. 2003; Gilli F et al. 2006) However, it has no confirmed roles in MS pathogenesis or in the clinical response to IFNβ. By applying gene expression microarrays to PBMC from patients who developed NABs to IFNβ and patients who remained NAB negative, we identified 9 biomarkers that followed changes in

gene expression over time similar to the *MX1*, the gold standard gene. While some of these biomarkers have been used in previous studies to evaluate the biological response to IFNβ (Sellebjerg et al. 2009; Pachner et al. 2009) others have not been tested yet. In the present study, we compared the potential for selected biomarkers to evaluate IFNβ bioactivity. Interestingly, although *MX1* induction was highly selective for type I IFNs, dose- and time-dependent induction experiments revealed several biomarkers of IFNβ bioactivity that were more selective, and significantly induced by lower IFNβ concentrations and at higher levels than the *MX1*. The finding of similar profiles of gene expression inhibition by different NAB dilutions for all selected biomarkers supports their use to measure the in vivo effects of NABs on IFNβ bioactivity. Finally, the gene expression abrogation experiments following non-specific stimulation indicate that cytokines other than IFNβ contribute little to the expression of selected biomarkers and reinforce their specificity by type I IFNs. Although not proven in the present study, the low gene expression levels that remained after inhibiting the effects of both IFNβ and IFNγ were most likely due to the action of IFNα, another type I IFN.

*USP18* was one of the most selective biomarkers of IFNβ bioactivity, and was significantly induced at the lowest IFNβ concentration and up-regulated to a greater degree by type I IFNs compared to the *MX1* gene. Furthermore, it was the only biomarker found to be differentially expressed between MS patients and controls, which suggest that *USP18* may play a prognostic role in

the pathogenesis of MS. *USP18* codes for a type I IFN-inducible cysteine protease that deconjugates ISG15, a ubiquitin-like protein, from target proteins (Malakhov et al. 2002). Interestingly, USP18 has been shown to negatively regulate the type I IFN signalling pathway, and its deficiency results in enhanced and prolonged STAT1 phosphorylation (Malakhov et al. 2002; Malakhov et al. 2003; Malakhova et al. 2003). This action appears to be independent of its protease activity and mediated by the specific binding of USP18 to IFNAR2, which then blocks the interaction between JAK1 and the IFN receptor and results in inhibition of the downstream phosphorylation cascade (Malakhova et al. 2006). Although further studies are needed, it is tempting to speculate that a deficient expression of *USP18* in MS patients may lead to overactivation of the type I IFN pathway and have implications in the therapeutic response to IFNβ. In fact, overexpression of type I IFN-responsive genes has been associated with a decrease biological and clinical response to IFNβ in MS patients (Comabella et al. 2009a; Van Baarsen et al. 2008). Whether or not responders and non-responders to IFNβ differ in their allelic frequencies for *USP18* is an open question.

Together with *USP18*, *HERC5* was highly selective as IFNβ bioactivity biomarker, significantly induced at the lowest IFNβ concentration, and showed induction levels comparable to the *MX1*. *HERC5* codes for a protein ligase that is involved in the ISG15 conjugation process (ISGylation) upon stimulation with type I IFNs (Takeuchi et al. 2006).

Similar to the *MX1*, *RSAD2* was significantly induced at a concentration of IFNβ of 1 IU/ml, but showed stronger induction in gene expression by type I IFNs although with lower selectivity. *RSAD2* (also known as viperin) encodes an antiviral protein that is involved in innate immunity against the infection of many DNA and RNA viruses. Of note, *RSAD2* showed the highest degree of inhibition in gene expression by high dilutions of serum from a NAB positive patient. The inhibiting effect of high and low NAB titres on *RSAD2* expression was also evaluated in a recent study (Pachner et al. 2009). These findings suggest that RSAD2 measurement may be considered in the design of new and more sensitive assays to determine NABs.

Similar to the *MX1, SIGLEC1* and *Ly6E* had a LLOQ of 1 IU/ml. In dose-dependent induction experiments, they were shown to be more selective than the *MX1* as IFNβ bioactivity biomarkers. However, *Ly6E* induction levels were the lowest following stimulation with IFNβ, and *SIGLEC1* was the least sensitive biomarker to capture the blocking effect of low NAB titres. *SIGLEC1* (also known as CD169) codes for a macrophage-restricted sialic acid receptor, which mediates adhesive interactions with lymphoid and myeloid cells (Van den Berg et al. 2001). Although little is known on the function of Ly6E, Ly6 proteins may be playing roles in cell signalling and cell adhesion processes (Kosugi et al. 1998; Pflugh et al. 2002). Interestingly, *SIGLEC1* and *Ly6E* were found to be up-regulated in peripheral blood mononuclear cells (PBMC), mainly monocytes, from patients with other autoimmune disorders such as

systemic sclerosis and systemic lupus erythematosus compared with healthy controls (Biesen et al. 2008; Feng et al. 2006; Tang et al. 2008; York et al. 2007) and mRNA and protein levels were shown to correlate with disease activity in lupus patients (Biesen et al. 2008; Feng et al. 2006; Tang et al. 2008). Studies correlating *SIGLEC1* and *Ly6E* levels with disease activity or the response to IFNβ have not been performed in MS.

In dose-dependent induction experiments, *IFI6* and *IFI27* were significantly induced at lower IFNβ concentrations and more selective than the *MX1*. While *IFI6* showed comparable induction levels to the *MX1*, *IFI27* was by far the most up-regulated gene following stimulation with type I IFNs. Of note, *IFI27* was proposed as a sensitive marker of IFNβ bioactivity in a recent study (Sellebjerg et al. 2009), and in a one-year time course transcriptomic study *IFI6* was found among the genes consistently up-regulated by IFNβ (Serrano-Fernández et al. 2010). IFI6 and IFI27 belong to the FAM14 family of proteins and have roles in the regulation of apoptosis. *IFI6* encodes an anti-apoptotic protein that inhibits depolarization of mitochondrial membrane potential, cytochrome c release, and caspase-3 activity (Tahara et al. 2005). Interestingly, IFI6 has also been shown to antagonize the effects of TRAIL (TNF-related apoptosis-inducing ligand) by inhibiting the intrinsic apoptotic pathway through mitochondrial stabilization (Cheriyath  et al. 2007). The protein encoded by *IFI27* associates with or inserts into the mitochondrial membrane, and it's up-regulation has been reported to lead to decreased viable cell

numbers and enhanced sensitivity to DNA-damage induced apoptosis (Rosebeck et al. 2008). Given the important role that apoptosis plays in the pathogenesis of MS, further studies to explore the implication of IFI6 and IFI27 in disease pathogenesis are warranted.

Finally, LLOQ of *IFIT1* and *IFI44L* was the same as the *MX1* (1 IU/ml). Whereas in the dose-dependent experiments *IFI44L* showed similar selectivity and induction levels to the *MX1*, *IFIT1* appeared to be more selective and induced to a higher degree compared to the *MX1*. *IFIT1* encodes a protein that is rapidly induced in response not only to viral infections but also non-viral stimuli such as LPS, IL-1 and TNFα (Wathelet et al. 1987; Smith et al. 1996), and may be involved in cell apoptosis via interaction with the eukaryotic elongation factor-1A (eEF1A) (Li et al. 2010). Little evidence exists in the literature regarding the function of the protein encoded by *IFI44L*. However, it is important to mention that a related gene, *IFI44*, and *IFIT1* were found to be among the genes that best predicted the response to IFNβ treatment in MS patients (Comabella et al. 2009a).

**Article 2.**

Malhotra S, Morcillo-Suárez C, Brassat D, Goertsches R, Lechner-Scott J, Urcelay E, Fernández O, Drulovic J, García-Merino A, Martinelli Boneschi F, Chan A, Vandenbroeck K, Navarro A, Bustamante MF, Río J, Akkad DA, Giacalone G, Sánchez AJ, Leyva L, Alvarez-Lafuente R, Zettl UK, Oksenberg J, Montalban X, Comabella M. IL28B polymorphisms are not associated with the response to interferon-β in multiple sclerosis. J Neuroimmunol. 2011 Oct 28;239(1-2):101-4. doi: 10.1016/j.jneuroim.2011.08.004. Epub 2011 Sep 1.

# Chapter 3
# Role of USP18 as biomarker of MS disease activity and response to IFNβ

# Roles of the ubiquitin peptidase USP18 in multiple sclerosis and the response to interferon-beta treatment

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Word count: 2651

Running title: USP18 in MS and the response to intereferon-beta

Keywords: multiple sclerosis / USP18 polymorphisms / genetic susceptibility / interferon-beta / response to treatment

## Abstract

**Background and purpose:** Ubiquitin specific peptidase 18 (USP18) is a deubiquitinating enzyme that functions as a negative regulator of the type I interferon (IFN) signalling pathway and is specifically induced by type I IFNs. In the present study, we expanded previous observations by our group suggesting an implication of USP18 in multiple sclerosis (MS) based on the finding of a deficient expression of the gene in peripheral blood mononuclear cells from MS patients compared with healthy controls.

**Methods:** Two polymorphisms, rs2542109 (intronic) and rs9618216 (promoter), were genotyped in a cohort of 691 relapse-onset MS patients and 1028 healthy controls, and in 225 MS patients treated with IFNβ and classified into responders and non-responders after two years of treatment according to clinical criteria. Correlations between genotypes and expression levels for USP18 and its target ISG15 were performed by real-time polymerase chain reaction (PCR).

**Results:** Two USP18 haplotypes were significantly associated with MS, *TG* and *CG*. Additional experiments revealed that *CG* carriers were characterized by lower USP18 gene expression levels in peripheral blood mononuclear cells and higher clinical disease activity. Finally, *AA* homozygosis for the intronic polymorphism rs2542109 was associated with the responder phenotype; however

USP18 expression levels induced by IFNβ did not differ among MS patients carrying different rs2542109 genotypes.

**Conclusions:** Altogether, these results point to a role of USP18 in MS pathogenesis and the therapeutic response to IFNβ.

# Chapter 3

# Role of USP18 as biomarker of MS disease activity and response to IFNβ

## 3.1 Introduction

In the previous study conducted by our group and fully described in Chapter 2 (Malhotra et al. 2011), USP18 was identified as a highly sensitive and specific biomarker of IFNβ bioactivity, and compared with the "gold standard", MX1 gene, USP18 was induced at lower concentrations and up-regulated to a greater degree by type I IFNs. Among all the IFNβ bioactivity biomarkers investigated, USP18 was the only gene whose expression was found to be differentially expressed between healthy controls and multiple sclerosis (MS) patients, with lower expression levels in the latter group (Malhotra et al. 2011). Based on these previous findings, in the present study we aimed to further investigate the prognostic role of USP18 in MS as both disease activity biomarker and IFNβ treatment response biomarker.

## 3.2 Materials and Methods

### *3.2.1 Patient*

Patients To evaluate the role of USP18 polymorphisms in the genetic risk for MS, a total of 691 unrelated relapse-onset MS

patients were included in the study [65.5% females; mean age (SD) at examination: 42.4 years (11.0); mean disease duration: 13.7 years (9.7); median EDSS (interquartile range): 3.0 (1.5-6.0)]. All cases were of Spanish origin and satisfied Poser's and McDonald's criteria for clinically definite MS (Poser et al. 1983; McDonald et al. 2001). The healthy control population comprised of 1028 unrelated individuals [(52.8% females; mean age: 37.7 years (13.5)] recruited at our hospital blood bank.

To investigate the role of USP18 polymorphisms in response to IFNβ, a total of 225 RRMS patients treated with IFNβ were included in the study. Of these, 130 patients were labeled as responders [79.2% females; mean age at treatment onset: 33.5 years (8.7); mean disease duration: 13.7 years (7.1); median EDSS (interquartile range): 2.0 (1.5-2.5)] based on the absence of relapses and lack of progression on the EDSS during the first 2 years of treatment. On the other hand, 95 patients were labeled as non-responders [72.6% females; mean age at treatment onset: 34.3 years (10.1); mean disease duration: 13.3 years (7.3); median EDSS (interquartile range): 2.5 (1.5-3.5)] based on the presence of one or more relapses and confirmed increased of at least one point in the EDSS during the two-year follow-up period (Río et al. 2006). The study was approved by the local ethics committees and all patient samples were collected with written informed consent.

### *3.2.2 Genotyping of USP18 polymorphisms*

Genomic DNA was obtained from peripheral blood samples of MS patients and healthy controls using standard methods. Two single nucleotide polymorphisms (SNPs), rs2542109 and rs9618216, with minor allele frequencies >0.10 were selected from the Celera Discovery System SNP database and available from the Applied Biosystems Web store. SNP rs9618216 is a C/T transition substitution located in the 5' near USP18 gene region. SNP rs2542109 is an intronic A/G transition substitution. Genotyping was carried out by means of the 5′nuclease assay technology for allelic discrimination using TaqMan probes commercially available from the Assay on-Demand Service (Applied Biosystems, Foster City, CA). Genotyping was performed on a 7900 real- time PCR machine (Applied Biosystems).

### *3.2.3 Gene expression levels associated with USP18 haplotypes*

To assess whether USP18 haplotypes H2 (TG) and H3 (CG) correlated with differences in gene expression, the expression levels for USP18 and ISG15 were determined by real-time PCR in peripheral blood mononuclear cells (PBMC) from 37 untreated RRMS patients. Ten patients were homozygotes for haplotype H1 (CA), which was taken as a reference (CACA), 12 patients were heterozygotes for haplotype H2 (CATG) and 15 patients were heterozygotes for haplotype H3 (CACG). Briefly, total RNA was extracted from PBMC using an RNeasy Mini Kit (Quiagen, Santa Clarita, USA) and converted into cDNA with the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Amplifications were performed using Taqman probes specific for

USP18 and ISG15 (Applied Biosystems) on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. To determine USP18 and ISG15gene expression levels associated with USP18 haplotypes, the threshold cycle (CT) value for each reaction and the relative level of gene expression for each sample were calculated using the 2-ΔΔCT method (Livak et al. 2001) and results were expressed as fold change in gene expression in CATG and CACG heterozygotes relative to CACA homozygotes (calibrators).Gene and protein expression levels associated with SNP rs2542109 genotypes after induction with IFNβ

PBMC from 15 untreated RRMS patients, 7 AA homozygotes and 8 AG heterozygotes for SNP rs2542109, were cultured in complete media for 20 hours in the presence or absence of 100 IU/ml of IFNβ-1b (Betaferon®). After incubation, total RNA was extracted and the expression levels for USP18 and ISG15 were determined by real-time PCR as previously described, using GAPDH as endogenous control. The MX1 gene was used as positive control of IFNβ induction. Results were expressed as fold change in gene expression in IFNβ-treated samples relative to untreated samples (calibrators), as described above.

In a small subgroup of patients (4 AA homozygotes and 2 AG heterozygotes), IFNβ-induced protein expression levels for USP18 and ISG15 were also determined by western blot analysis. Briefly, PBMC from untreated and IFNβ-treated samples were lysed in lysis

buffer (RIPA with protein inhibitor) and the total amount of protein was measured using the Bradford method. Western blot was performed with anti-USP18, and anti-ISG15 monoclonal antibodies (Cell Signaling Technology, USA). β-actin was used as loading control. SDS protein gel were run, transferred onto membranes and incubated with primary antibodies. After incubation, immunoblots were washed and incubated with anti-rabbit secondary antibodies for 1 hour. Following washing to remove unbound secondary antibodies, detection of bounded antibodies was performed by western HRP substrate LuminateTM Forte (Millipore Corporation, Billerica, MA 01821) as per manufacturer's protocol.

### *3.2.4 Search of potential binding sites in the upstream promoter region of the USP18 gene*

A total of 213 matrixes were downloaded from public release of TRANSFAC database (Matys et al. 2006). A search for candidate sites was performed within a 13kb region covering the upstream region of the USP18 gene from the end of the TUBA8 gene and approximately the first 1kb of the USP18 gene. We considered the value of 0.95 as normalized score threshold for reporting potential binding sites.

### *3.2.5 Statistical analyses*

USP18 association analysis was performed with SNPator (Morcillo-Suarez, et al. 2008). For the analysis, the following comparisons between groups were considered: MS case group versus healthy controls, and IFNβ-responders versus non-responders. For each

comparison, allelic, genotypic, and haplotypic tests were performed. For genotype association analysis, each genotype was tested against the combination of the other two. For haplotype association analysis, each estimated haplotype was tested against the grouping of the rest. Haplotype estimation was performed using the software PHASE (Stephens et al. 2001) with default settings.

## 3.3 Results

### *3.3.1 Hardy–Weinberg equilibrium tests and linkage disequilibrium strength between SNPs*

SNPs rs2542109 and rs9618216 were in Hardy-Weinberg equilibrium ($p = 0.732$ and $p = 0.909$ respectively) and in strong linkage disequilibrium ($p < 10^{-100}$; D′= 0.886; R2 = 0.160).

### *3.3.2 USP18 polymorphisms and susceptibility to MS*

We first investigated the role of USP18 polymorphisms in the genetic risk for MS. As shown in Table 3.1,

For SNP rs2542109 allele frequencies were similar between MS patients and controls. At the genotype level, frequency of GG homozygotes was significantly lower in the MS group compared with the control group (OR=0.7; p=0.014).

For SNP rs9618216, allele T was associated with the MS group (OR=1.3; corrected p=0.017). Comparison of genotype frequencies for rs9618216 between MS patients and controls revealed a

negative association of CC homozygosity with the disease when compared with the control group (OR=0.8; p=0.033) (Table 3.1).

**Table. 3.1**

| SNP | Analysis | MS, n (%) | HC, n (%) | OR (95% CI) | p-value |
|---|---|---|---|---|---|
| rs2542109 | Allele | | | | |
| | **A** | 899 (67.4) | 1287 (64.9) | 1.1 (1.0 – 1.3) | 0.143 |
| | G | 435 (32.6) | 695 (35.1) | | |
| | Genotype | | | | |
| | GG | 63 (9.4) | 133 (13.4) | 0.7 (0.5 – 0.9) | **0.014** |
| | AG | 309 (46.3) | 429 (43.3) | 1.1 (0.9 – 1.4) | 0.222 |
| | AA | 295 (44.2) | 429 (43.3) | 1.0 (0.8 – 1.3) | 0.705 |
| rs9618216 | Allele | | | | |
| | C | 1218 (89.2) | 1843 (91.6) | 1.3 (1.0 – 1.7) | **0.017** |
| | T | 148 (10.8) | 169 (8.4) | | |
| | Genotype | | | | |
| | TT | 9 (1.3) | 5 (0.5) | 2.7 (0.9 – 8.0) | **0.068** |
| | CT | 130 (19.0) | 159 (15.8) | 1.2 (1.0 – 1.6) | **0.083** |
| | CC | 544 (79.7) | 842 (83.7) | 0.8 (0.6 – 1.0) | **0.033** |

*Table 3.1. Genotype frequency distribution in MS and HC*

OR: odds ratio. 95% CI: 95% confidence interval. Bold alleles denote risk alleles. Significant associations are shown in bold.

Table 3.2 shows the distribution of the resulting 4 USP18 haplotypes [H1 (CA) / H2 (TG) / H3 (CG) / H4 (TA)] in MS patients and healthy controls. Two haplotypes, H2 (TG) and H3 (CG), were found to be significantly associated with MS when compared with the control group (for H2: OR=1.4; p=0.009 / for H3: OR=0.8; p=0.002).

**Table 3.2**

| Haplotypes | MS, n (%) | HC, n (%) | OR (95% CI) | p-value |
|---|---|---|---|---|
| H1 (*CA*) | 925 (67.2) | 1328 (65.0) | 1.1 (0.9 – 1.3) | 0.185 |
| H2 (*TG*) | 139 (10.1) | 154 (7.5) | 1.4 (1.1 – 1.7) | **0.009** |
| H3 (*CG*) | 303 (22.0) | 545 (26.7) | 0.8 (0.7 – 0.9) | **0.002** |
| H4 (*TA*) | 9 (0.7) | 15 (0.7) | 0.9 (0.4 – 2.0) | 0.782 |

***Table 3.2.*** *Distribution of USP18 haplotypes in MS patients and controls*

### *3.3.3 Correlation between USP18 haplotypes and gene expression levels*

As a next step, we determined in PBMC from untreated RRMS patients the gene expression levels for *USP18* and its target *ISG15* associated with H2 (*TG*) and H3 (*CG*) haplotypes. Haplotype H1

(*CA*), which was the most prevalent and similarly distributed between MS patients and controls, was taken as a reference. As shown in Figure 3.A (i), *USP18* expression in *CACG* heterozygotes was significantly lower when compared with homozygotes for the reference haplotype (*CACA*) (p=0.022). Although *ISG15* expression was also lower in *CACG* heterozygotes, differences did not reach statistical significance when compared with the reference haplotype (p=0.146; Figure 3.A (ii). Conversely, *USP18* and *ISG15* expression levels were similar between *CATG* heterozygotes and *CACA* homozygotes (Figures 3.A) Finally, *USP18* and *ISG15* expression levels significantly differed between *CACG* and *CATG* heterozygotes (p=0.0006 for *USP18* and p=0.008 for *ISG15*; Figure 3.A).

**Fig 3 A**

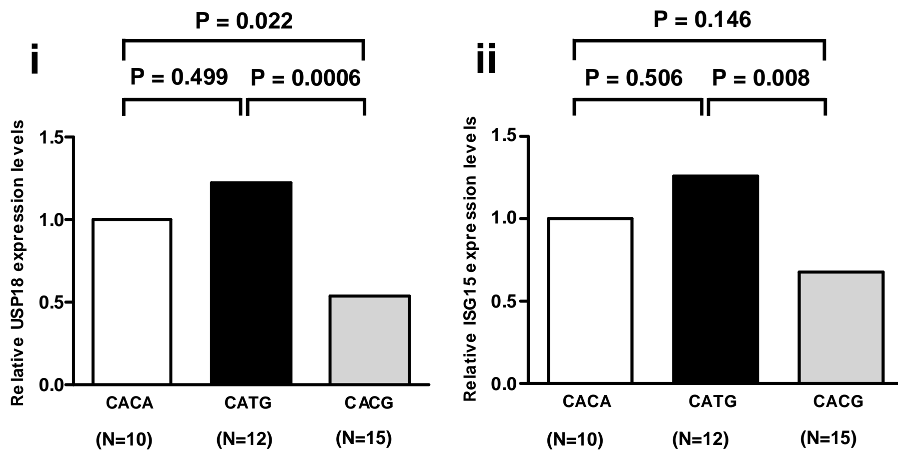


*Figure 3.A. USP18 (Left) and ISG15 (right) gene expression levels associated with the different USP18 haplotypes. Total RNA was extracted from PBMC of untreated RRMS, as described in Materials and Methods. USP18 and ISG15 gene expression was then determined by real-time PCR using GAPDH as endogenous control. CACA refers to patients homozygotes for the reference haplotype (H1: CA). CATG refers to patients heterozygotes for haplotype H2 (TG). CACG refers to patients heterozygotes for haplotype H3 (CG). Results are expressed as fold*

*change in gene expression in CATG and CACG heterozygotes relative to CACA homozygotes (reference haplotype).*

### *3.3.4 Potential binding sites in the USP18 promoter region*

Based on the finding of a decreased expression of *USP18* in MS patients carrying H3 (*CG*) haplotypes, we searched for potential binding sites of transcription factors in the promoter region of the gene that may account for the differences observed in gene expression.

As shown in Table 3.3, we found many potential binding sites for the signal transducers and activators of transcription (STAT) family of proteins. The consensus sequence for STAT TRANSFAC matrixes is very short and contains from three to five important positions; thus, it cannot be ruled out that some of our binding sites for STAT proteins may be predicted by chance. Other potential binding sites that were identified across the different upstream regions of the *USP18* gene were the binding sites for (i) the activator protein 2 (AP-2) gamma and AP-2 alpha; (ii) the nuclear factor of activated T-cells (NF-AT); (iii) the heat shock proteins HSF1 and HSF2; (iv) the nuclear factor (erythroid-derived 2)-like 1 (NFE2L1); and (v) the general transcription factor TBP (TATA box binding protein) with TATAAATA sequence that completely match to the consensus.

Table 3.3. List of potential binding sites identified in USP18 promoter region (on next page)

# Table 3.3

| ENCODE[&] | Position | Factor(s) | Matrices | Scores |
|---|---|---|---|---|
| -10kb | -10637 | STAT5A | M00499 | 0.953 |
| | -10620 | STAT(5A,4)* | M00499 M00498 | 0.986 0.934 |
| | -10606 | STAT(6,1) | M00500 M00496 | 0.950 0.906 |
| | -10576 | STAT(4,3) | M00498 M00497 | 0.966 0.946 |
| | -10431 | STAT(5A,4) | %* | 0.965 0.932 |
| | -10419 | NFE2L1 | M00285 | 0.969 |
| | -10223 | STAT(1,4,6,5A,3) | %* | 0.971 - 0.912 |
| | -10203 | STAT(6,1,5A,4) | %* | 0.958 - 0.900 |
| | -10065 | STAT(5A,4) | %* | 0.988 0.900 |
| | -10034 | STAT(6,3,5A,4) | %* | 0.965 - 0.900 |
| | -9673 | STAT(1,5A,3) | %* | 0.973 - 0.927 |
| | -9664 | TBP | M00471 | 1 |
| | -9650 | STAT(5A,1) | %* | 0.961 0.902 |
| | -9623 | STAT5A | M00499 | 0.955 |
| -1kb | -1099 | HSF(2,1) | M00147 M00146 | 0.959 0.925 |
| | -1069 | STAT(6,4,1,5A) | %* | 0.985 - 0.903 |
| | -956 | STAT(1,3) | %* | 0.952 0.916 |
| 0kb | -175 | AP-2 (alpha, gamma) | M00469 M00470 | 0.964 0.958 |
| | +18 | STAT (3,1,6,4) | %* | 0.966 - 0.902 |

ENCODE[&] refers to three regions of potential transcription binding sites according to ChipSeq data. STAT(5A,4)* means STAT5A and STAT4 factors. %* means that corresponding matrices were already listed above.

### *3.3.5 USP18 haplotypes and clinical variables*

We next aimed to correlate the haplotype-associated differences observed in USP18 expression with clinical parameters such as the number of relapses in the two years before blood collection and EDSS score at extraction. As depicted in Figure 3.B, MS patients carrying haplotype H3 (*CG*), which was associated with lower *USP18* expression, were characterized by significantly higher mean relapse rate (p=0.023) and EDSS scores (p=0.023) when compared with patients homozygotes for the reference haplotype (*CACA*). A trend towards higher mean relapse rate was also observed in the comparison between *CACG* and *CATG* heterozygotes (p=0.067). Mean number of relapses and EDSS scores were similar between *CATG* heterozygotes and patients carrying the reference haplotype.

**Fig. 3.B**

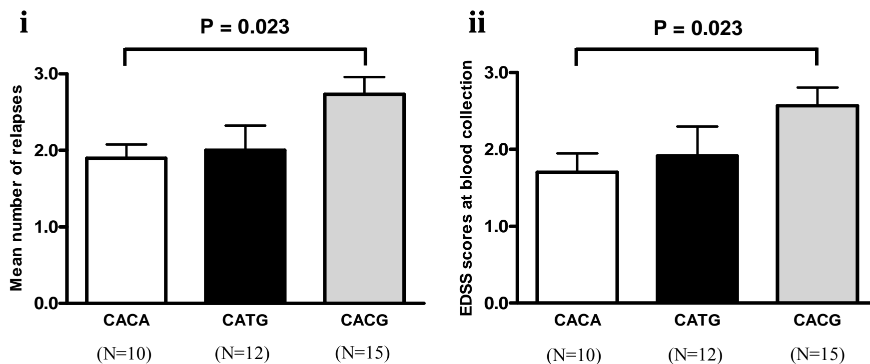

*Figure 3.B (i) Number of relapses in the two years before blood extraction and EDSS scores at blood collection (ii) in MS patients carrying the different USP18 haplotypes. CACA refers to patients homozygotes for the reference haplotype (H1: CA). CATG refers to patients heterozygotes for haplotype H2 (TG). CACG refers to patients heterozygotes for haplotype H3 (CG). Results are expressed as mean values (standard error of the mean (SEM)).*

### *3.3.6 USP18 polymorphisms and response to IFNβ*

Given the close interrelationship between USP18 and the type I IFN signaling pathway, we also investigated the potential role of *USP18* polymorphisms in the response to IFNβ. As shown in Table 3.4, for SNP rs2542109 the frequency of allele *A* was significantly higher in MS patients than in controls (OR=1.6; p=0.015). At the genotype level, *AA* homozygosis was positively associated with the disease (OR=1.8; p=0.041). For SNP rs9618216, allele and genotype frequencies did not significantly differ between IFNβ responders and non-responders (Table 3.4).

### *3.3.7 USP18 expression induced by IFNβ in AA for SNP rs2542109*

We finally investigated USP18 gene and protein expression induced by IFNβ in MS patients carrying the risk genotype (*AA*) for SNP rs2542109. As shown in Figure 3.C in vitro culture of PBMC with IFNβ resulted in a strong and statistically significant induction of the gene expression of *USP18*, its target *ISG15* and the positive control *MX1*, and no differences in the magnitude of induction were observed between *AA* homozygotes and *AG* heterozygotes.

**Table 3.4**

| SNP | Analysis | R, n (%) | NR, n (%) | OR (95% CI) | p-value |
|---|---|---|---|---|---|
| rs2542109 | Allele | | | | |
| | **A** | 181 (72.4) | 113 (61.4) | 1.6 (1.1 – 2.5) | **0.015** |
| | G | 69 (27.6) | 71 (38.6) | | |
| | Genotype | | | | |
| | GG | 9 (7.2) | 14 (15.2) | 0.4 (0.2 – 1.1) | 0.058 |
| | AG | 51 (40.8) | 43 (46.7) | 0.8 (0.5 – 1.4) | 0.382 |
| | AA | 65 (52.0) | 35 (38.0) | 1.8 (1.0 – 3.1) | 0.041 |
| rs9618216 | Allele | | | | |
| | C | 236 (90.8) | 165 (89.7) | 1.1 (0.6 – 2.1) | 0.700 |
| | T | 24 (9.2) | 19 (10.3) | | |
| | Genotype | | | | |
| | TT | 3 (2.3) | 1 (1.1) | 2.1 (0.2 – 2.1) | 0.500 |
| | CT | 18 (13.8) | 17 (18.5) | 0.7 (0.3 – 1.5) | 0.350 |
| | CC | 109 (83.8) | 74 (80.4) | 1.3 (0.6 – 2.5) | 0.510 |

***Table 3.4.*** *Allele and genotype frequency distribution in responders and non-responders to IFNβ.*

OR: odds ratio. 95% CI: 95% confidence interval. Bold alleles denote risk alleles. R: responders to IFNβ. NR: non-responders to IFNβ. Significant associations are shown in bold.

**Fig. 3.C**

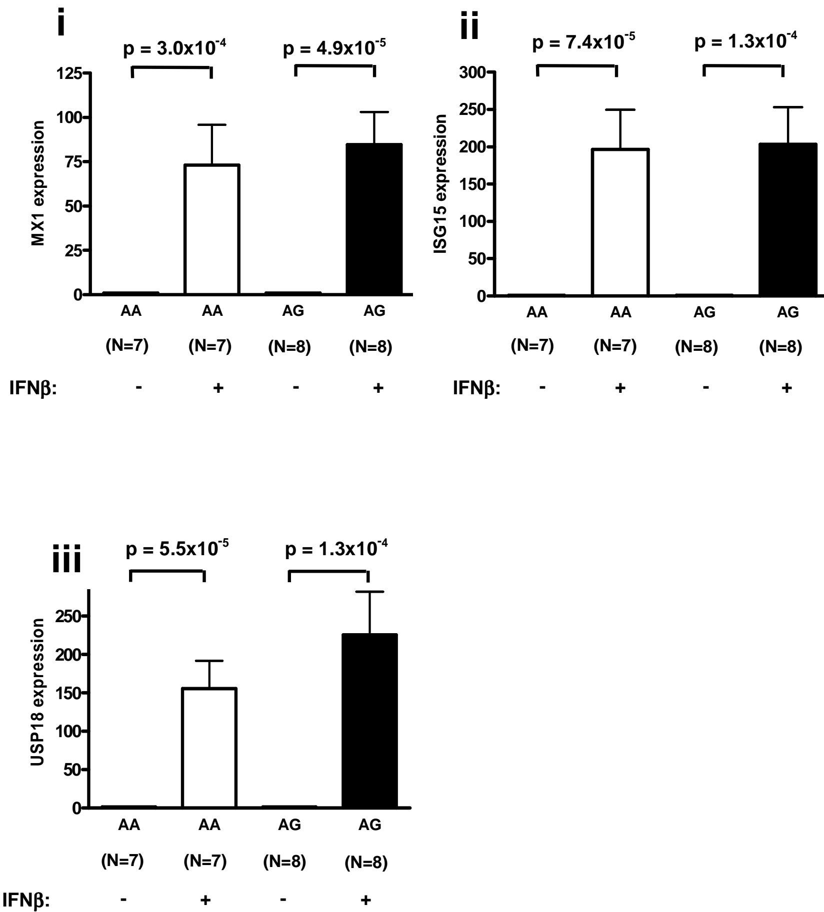


*Figure 3.C. PBMCs isolated from AA homozygous and AG heterozygous were treated with or without IFNβ for 24hrs. After incubation, cells were isolated and mRNA was extracted as mentioned in methods. Gene expression level of MX1 (i), ISG15 (ii) & USP18 (iii) was determined by real-time PCR. Results were plotted as fold change in gene expression relative to untreated condition. White bar graph represents AA homozygous, filled bar graph represents AG heterozygous.*

## 3.4. Discussion

*USP18* encodes a type I IFN-inducible cysteine protease that deconjugates ISG15 from target proteins (Malakhov et al. 2002). In a previous study by our group, USP18 gene expression was found to be lower in MS patients than in healthy controls (Malhotra et al. 2011).

Based on these findings, the first main goal of the present study was to further explore the potential role of *USP18* in disease pathogenesis by investigating first its contribution to the genetic risk for MS. For this, two polymorphisms of the *USP18* gene, one intronic and another positioned in the promoter region were genotyped in a cohort of 691 relapse-onset MS patients (which included patients with relapsing-remitting and secondary progressive MS) and 1028 healthy controls. Two haplotypes, H2 (*TG*) and H3 (*CG*) which were present in 10% and 22% of MS patients respectively, were associated with disease. Interestingly, when we tried to correlate USP18 haplotypes with gene expression levels, haplotype H3 (*CG*) was associated with a lower expression of *USP18* and its target *ISG15*. These findings are in agreement with our previous results showing a deficient expression of USP18 in MS patients (Malhotra et al. 2011). Insomuch as USP18 has been shown to negatively regulate the type I IFN signalling pathway, its deficiency results in enhanced and prolonged STAT1 phosphorylation (Malakhov et al. 2002; Malakhov et al. 2003; Malakhova et al. 2003) and subsequently overactivation of IFNβ

stimulating genes (Zou et al. 2007). Previous studies by the group have suggested an association between overexpression of type I IFN-responsive genes and both the clinical response to IFNβ and disease activity (Comabella et al. 2009a; Bustamante et al. 2011). Supporting this, the subgroup of MS patients with haplotype H3 (*CG*) and lower *USP18* expression was characterized by an increase in the clinical disease activity, as reflected by the higher relapse rate observed in these patients when compared with haplotype H3 non-carriers. In addition, neurological disability scores were significantly higher in patients with lower *USP18* expression compared to those with normal expression levels.

Given that one of the *USP18* polymorphisms associated with MS, rs9618216, was located in the promoter region of the gene, we investigated whether the differences in gene expression observed between *USP18* haplotypes could be secondary to the binding of transcription factors that may regulate *USP18* transcriptional activity. Among the transcription factors that were identified in proximity to rs9618216, we observed 8 potential binding sites for STAT1 with a high score (≥0.95). STAT1 is one of the main mediators of the signaling of the type I IFN pathway (Platanias 2005), which explains the strong induction in USP18 gene expression observed after cell stimulation with IFNβ or IFNα (Malhotra et al. 2011). These findings, however, only indicate that *USP18* gene expression is modulated by STAT1 but do not explain the repression of USP18 gene expression observed in haplotype H3 (*CG*) carriers. The relationship between other potential binding sites

for transcription factors such as AP-2, NF-AT, HSF1/HSF2 and NFE2L1 and *USP18* transcriptional regulation is less obvious.

The protein encoded by USP18 specifically binds to IFNAR2 and blocks the interaction between JAK1 and the IFN receptor thus resulting in the inhibition of the downstream phosphorylation cascade (Malakhova et al. 2006) as shown in Furthermore, in a previous study by the group (Malhotra et al. 2011), USP18 was identified as one of the most specific biomarkers of IFNβ bioactivity among a panel of 9 candidate genes which included the MX1. Based on the specific induction of USP18 by type I IFNs and its role as negative regulator of the type I IFN pathway, our second main goal in the present study was to investigate the role of USP18 in the response to IFNβ. For this, both USP18 polymorphisms, rs2542109 and rs9618216, were genotyped in a cohort of 225 patients treated with IFNβ and classified into responders and non-responders according to stringent clinical criteria which were used in previous studies by the group (Comabella et al. 2009b; Comabella et al. 2008; Malhotra et al. 2011). Interestingly, *AA* homozygosis for the intronic SNP (rs2542109) was found to be significantly more represented among IFNβ responders. However, the ability of the *USP18* gene to respond to IFNβ in vitro was fully retained in patients *AA* homozygotes for the intronic SNP, as reflected by the lack of differences observed in USP18 gene and protein expression induced by IFNβ among patients carrying rs2542109 genotypes.

In this context, additional functional studies will be needed to further implicate this gene in the response to IFNβ.

**Article 3.**

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# Chapter 4

# Exploring the role of siglecs as disease activity biomarkers in MS

# SIGLEC1 and SIGLEC7 expression in circulating monocytes of patients with multiple sclerosis

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Keywords: multiple sclerosis; SIGLEC1; SIGLEC7; monocytes; biomarkers; acute relapses; disease progression

## Abstract

**Background:** Siglecs (sialic acid binding immunoglobulin-like lectins) are cell surface receptors that recognize sialic acids and may attenuate immune responses and reduce inflammation.

**Objective:** To investigate the role of two members of the Siglec family, SIGLEC1 and SIGLEC7, in the clinical course and disease activity of patients with multiple sclerosis (MS).

**Methods:** SIGLEC1 and SIGLEC7 expression was determined by flow cytometry in blood monocytes of 16 healthy controls and 55 untreated MS patients [13 primary progressive MS patients (PPMS), 13 secondary progressive MS patients (SPMS), and 29 relapsing-remitting MS patients (RRMS) (18 during clinical remission and 11 during relapse)].

**Results:** SIGLEC1 expression by CD14+ monocytes was significantly increased in MS patients compared with controls. Stratification of patients into different clinical forms revealed increased SIGLEC1 expression in patients with progressive forms of the disease, particularly in those with PPMS. Both inflammatory and resident monocytes contributed to the increase in SIGLEC1 expression observed in PPMS patients. SIGLEC7 expression was significantly up-regulated in blood monocytes from RRMS during relapse compared with patients during clinical remission.

**Conclusions:** These findings suggest roles for SIGLEC1 in the chronic progressive phases of MS and for SIGLEC7 in acute disease activity.

# Chapter 4
# Exploring the role of Siglecs as disease activity biomarkers in MS

## 4.1 Introduction

The sialic acid binding immunoglobulin-like lectin (Siglec) family is a member of the immunoglobulin superfamily. Structurally, Siglecs contain one N-terminal variable (V)-set immunoglobulin-like domain that binds sialic acid followed by variable numbers of C2-set immunoglobulin domains in their extracellular regions. (May et al. 1998; Varki et al. 2006; Crocker et al. 1998). Siglecs are expressed by different cellular populations which include granulocytes, monocytes, B cells, NK cells, and CD8 cells. (Hartnell et al. 2001) SIGLEC1 and SIGLEC7 are two members of the Siglec family that, because of their function and involvement in other autoimmune disorders, may also play roles in the pathogenesis of multiple sclerosis (MS).

An increased expression of SIGLEC1 has been observed in the peripheral blood monocytes of patients with autoimmune diseases such as systemic sclerosis, systemic lupus erythematosus, (Biesen et al. 2008) and primary biliary cirrhosis. (Bao et al. 2010) Furthermore, in the previous study conducted by our group with microrrays, SIGLEC1 was among the top genes that followed similar expression patterns to the MxA and it was found to be

specifically induced by type I IFNs, findings that set the rationale for further investigation of SIGLEC1 as prognostic biomarker in MS (Malhotra et al. 2011).

It has been reported that SIGLEC7, another member of the Siglec family, is the target of suppressor of cytokine signaling 3 (SOCS3), a negative regulator of the JAK/STAT signaling pathway that is up-regulated during inflammation (Orr et al. 2007) and with potential roles in MS pathogenesis (Baker et al. 2009).

Based on these observations, in the present study we aimed to investigate the roles of SIGLEC1 and SIGLEC7 in MS as disease activity biomarkers by determining their expression levels in peripheral blood monocytes from patients with different clinical forms and activity phases of the disease.

## 4.2 Material and methods

### *4.2.1 Patients*

Sixteen healthy controls and 55 patients with clinically definite MS who had not received treatment with corticosteroids in the three months before blood sampling were included in the study. Patients were labeled as RRMS (n=18), SPMS (n=13), or PPMS (n=13) according to the Lublin and Reingold classification. (Lublin et al. 1996) A group of RRMS patients whose blood was drawn at the time of an acute relapse was also included in the study (n=11), and was defined by the appearance of new neurological symptoms or

worsening of pre-existing neurological symptoms attributable to MS which persisted for over 24 hours. None of these patients had ever received treatment with immunomodulatory or immunosuppressive therapies.

**Table. 4.1**

| Characteristics | PPMS | SPMS | RRMS | Relapses |
|---|---|---|---|---|
| N | 13 | 13 | 18 | 11 |
| F/M (% F) | 7/6 (53.8) | 8/5 (61.5) | 10/8 (55.6) | 8/3 (72.7) |
| Age (yrs) | 49.4 (9.3) | 47.3 (6.9) | 35.6 (8.5) | 32.7 (8.9) |
| Duration of disease (yrs)[a] | 10.2 (5.9) | 11.5 (6.2) | 6.0 (4.6) | 7.6 (5.4) |
| EDSS[b] | 6.0 (4.5 – 6.5) | 4.0 (4.0 – 4.5) | 2.0 (1.5 – 2.3) | 3.0 (2.5 – 3.5) |

*Table 4.1 Demographic and baseline clinical characteristics of MS & HC.*

[a]Data are expressed as mean (SD). [b]Data are expressed as median (interquartile range). . Relapses: RRMS patients during acute relapse.

### *4.2.2 Cell surface immunostaining to detect expression of SIGLEC1 and SIGLEC7 by flow cytometry (FACS) in blood monocytes*

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Isopaque density gradient centrifugation (Gibco BRL, Life Technologies Ltd, UK) and stored in liquid nitrogen until used. Cells were stained with allophycocyanin (APC)-conjugated mouse anti-human CD14 (BD Biosciences, California, USA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD16 (BD

Biosciences), phycoerythrin (PE)-conjugated mouse antihuman CD328/SIGLEC7 and CD169/SIGLEC1 (both from eBioscience, San Diego, USA), PE-conjugated mouse antihuman CD64 (DakoCytomation, Denmark, Europe), and isotype controls FITC- and PE-conjugated IgG1 (Biolegend, San Diego, California, USA).

Cells were acquired in a FACS LSR Fortessa (Becton Dickinson) and analyzed using FACSDiva software. The primary gate was established for the monocyte population based on the forward and side-scatter light properties of total leukocytes. A secondary gate was established by selecting CD14+ monocytes within the primary gate. Monocyte subpopulations, CD16+ monocytes (CD14+CD16+; resident monocytes) and CD16- monocytes (CD14++ CD16-; inflammatory monocytes) were selected based on CD14 and CD16 expression within the secondary gate. Results are presented as percentage of positive cells and as mean fluorescence intensity (MFI). Negative gates were established using the appropriate isotype controls prior to running each sample and substracted from the specific signals.

### *4.2.3 Determination of mRNA expression levels of SIGLEC1 in PBMC by RT-PCR*

Total RNA was extracted from PBMC using an RNeasy kit (Quiagen, Santa Clarita, USA) and cDNA synthesized using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). SIGLEC1 mRNA expression levels were determined with TaqMan® probes specific for the gene (Applied Biosystems).

The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (Applied Biosystems). Assays were run on the ABI PRISM® 7900HT system (Applied Biosystems) and data were analyzed with the $2^{\Delta\Delta CT}$ method. (Livak et al. 2001)

### *4.2.4 Statistical analysis*

Statistical analysis was performed by using the SPSS 17.0 package (SPSS Inc, Chicago, Illinois, USA) for MS-Windows. A Mann-Whitney test was used to test for significant differences in SIGLEC1 and SIGLEC7 expression between healthy controls and the whole MS group, and between RRMS patients in clinical remission and during relapses. The Kruskal-Wallis test was used to analyze differences in SIGLEC1, SIGLEC7, and CD64 expression between controls and patients with different clinical forms of MS. If significant differences were found ($p<0.05$), a Mann-Whitney test was then used respectively to test for significant differences between two groups. Bonferroni correction was used to correct the alpha level for multiple comparisons between monocyte markers and MS patients with different clinical forms of the disease (alpha=0.008). Correlations between SIGLEC1 mRNA expression levels (using the threshold cycle –CT- values, which are inversely related to quantity) and protein expression levels were assessed by the Spearman rank correlation coefficient. Partial correlations controlling for age were used to evaluate the relationship between SIGLEC1 and SIGLEC7 expression and clinical variables.

## 4.3 Results

### *4.3.1 SIGLEC1 expression is elevated in blood monocytes of MS patients*

We first compared SIGLEC1 and SIGLEC7 expression by monocytes between healthy controls and the whole MS group. As shown in Figure 4.A (i), SIGLEC1 expression in CD14+ monocytes determined by FACS was significantly increased in MS patients compared with healthy controls (p=0.025 for percentage of positive cells; p=0.007 for MFI). However, SIGLEC7 expression by monocytes did not differ between MS patients and healthy controls (p>0.05 for both percentage of positive cells and MFI; Figure 4.A (ii)).

As shown in Figure 4.A (iii), differences in SIGLEC1expression observed at the protein level were also confirmed by real time PCR, and SIGLEC1 mRNA expression levels in PBMC were significantly higher in MS patients than in healthy controls (p=0.010). Correlations between SIGLEC1 mRNA expression levels significantly correlated with the percentage of positive cells (r= -0.51; p=6.6 x $10^{-5}$) and MFI (r= -0.59; p=2.1 x $10^{-6}$).

**Fig. 4. A**

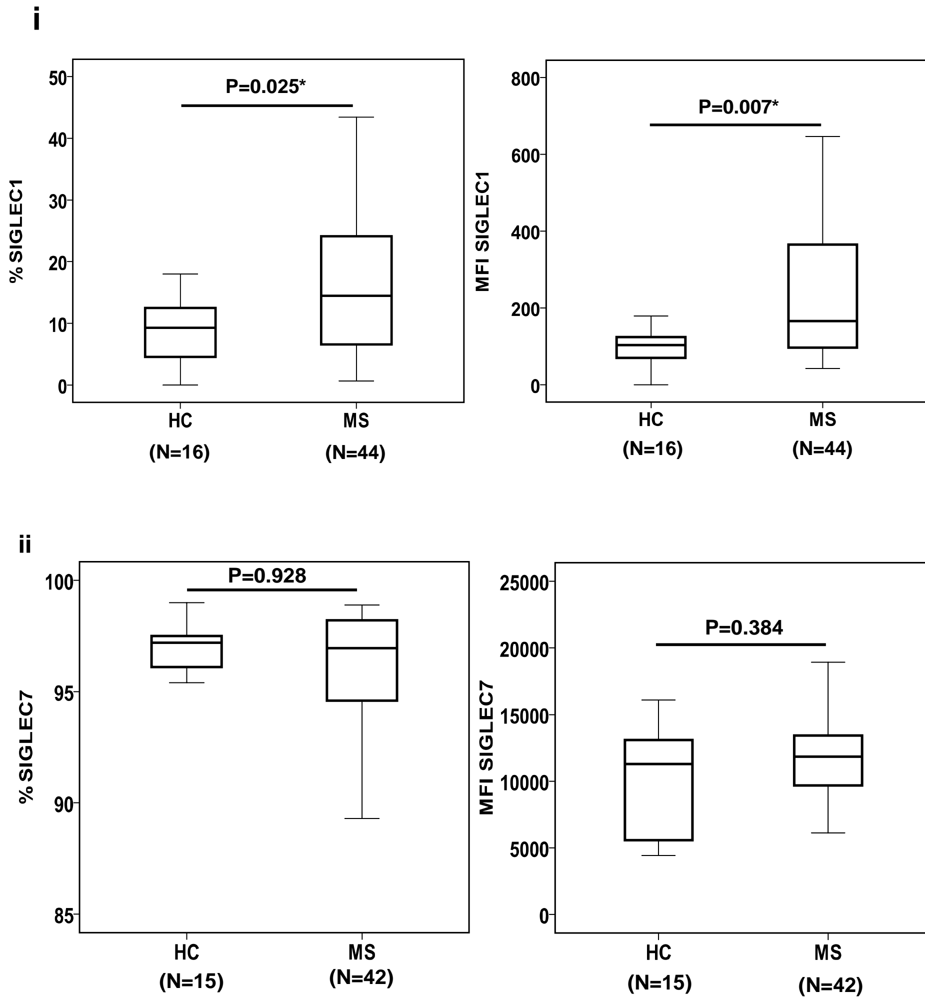



*Figure 4.A (i) Comparison of SIGLEC1 and SIGLEC7 expression between healthy controls (HC) and the whole multiple sclerosis (MS) group. (i) Boxplots showing the percentage of CD14+ monocytes expressing SIGLEC1 (%SIGLEC1; left) and mean fluorescence intensity (MFI) of SIGLEC1 expression in CD14+ monocytes (MFI SIGLEC1; right).*

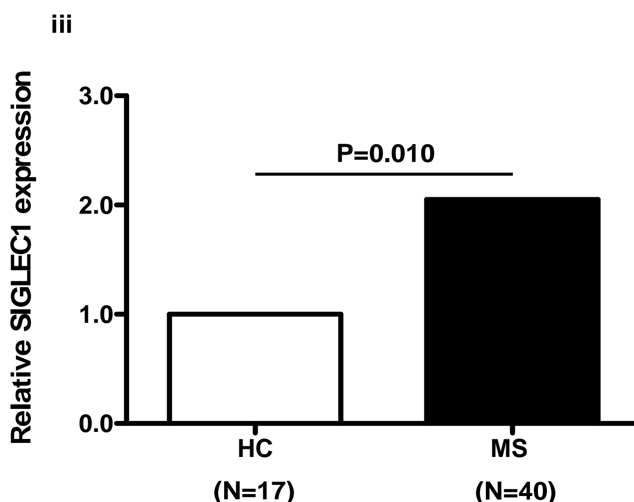



*Figure 4. (iii) Bar graph showing SIGLEC1 mRNA expression levels determined by real time PCR in peripheral blood mononuclear cells (PBMC) from MS patients and controls. Results are expressed as fold change in SIGLEC1 expression in MS patients relative to healthy controls (calibrators). SIGLEC1 expression was investigated in a group of 17 HC (includes five new healthy controls that were not stained for FACSand 40 MS patients (this group also includes five new MS patients that were not immunophenotyped).*

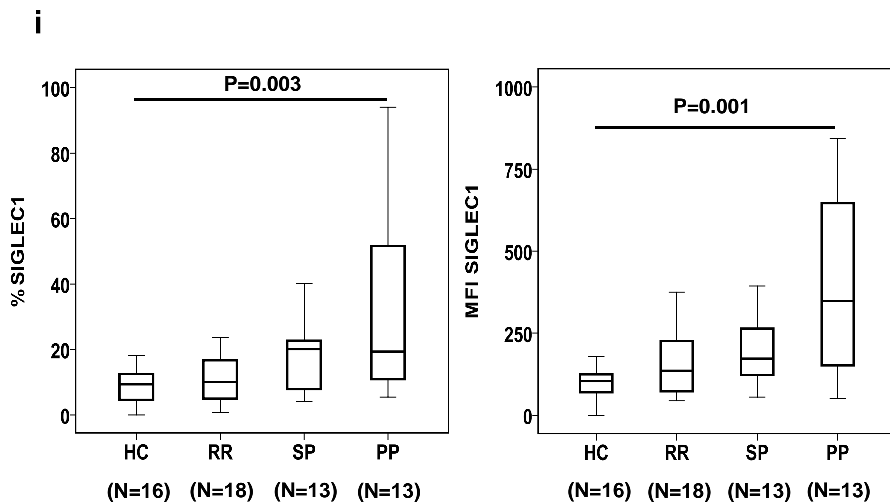
### *4.3.2 SIGLEC1 expression is increased in patients with progressive forms of the disease*

When MS patients were classified based on the clinical form, the highest SIGLEC1 expression was observed in CD14+ monocytes of patients with progressive forms of MS, and differences reached statistical significance for PPMS patients when compared with healthy controls (p=0.003 for percentage of positive cells; p=0.001 for MFI) (Figure 4.B (i)). Trends towards increased SIGLEC1 expression by blood monocytes were also observed in PPMS patients when compared with RRMS patients (p=0.031 for percentage of positive cells; p=0.056 for MFI), and SPMS patients

when compared with controls (p=0.056 for percentage of positive cells; p=0.010 for MFI) (Figure 4.B (i)).

As shown in Figure 4.B (ii), SIGLEC7 expression by blood monocytes was not significantly different between healthy controls and patients with different clinical forms of MS.

**Fig. 4.B**



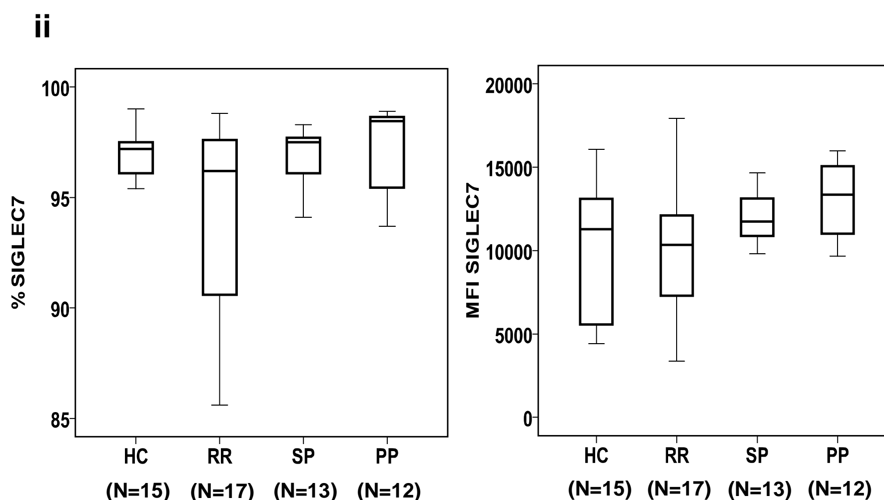



*Figure 4.B.Boxplots showing (i) SIGLEC1 in CD14+ blood monocytes from healthy controls (HC) and multiple sclerosis (MS) patients stratified into different clinical forms. Results are expressed as percentage of positive cells (left graphs) and mean fluorescence intensity (MFI) (right graphs). (ii) SIGLEC7 expression in CD14+ blood monocytes from healthy controls (HC) and multiple sclerosis (MS) patients stratified into different clinical forms. Results are expressed as percentage of positive cells (left graphs) and mean fluorescence intensity (MFI) (right graphs).*

### *4.3.3 Both inflammatory and resident monocytes contribute to the elevated SIGLEC1expression observed in patients with PPMS*

We next investigated the blood monocyte population that was associated with the increased SIGLEC1 expression observed in monocytes from PPMS patients. For this, the CD14+ monocyte population was further segregated into inflammatory and resident monocytes on the basis of CD16 positivity. As shown in Table 4.2, both monocyte populations contributed to the increased SIGLEC1 expression seen in PPMS patients, and differences reached statistical significance for the percentage of SIGLEC1 positive inflammatory monocytes when compared with healthy controls and

RRMS patients ($p=0.002$ and $p=0.005$ respectively), and a trend was observed for the MFI and percentage of SIGLEC1 positive resident monocytes when compared with controls ($p=0.022$ and $p=0.032$ respectively). Trends towards increased SIGLEC1 expression were also observed in both inflammatory and resident monocytes from SPMS patients when compared with healthy controls ($p=0.017$ and $p=0.013$ for MFI respectively) (Table 4.2).

### *4.3.4 SIGLEC7 expression by blood monocytes is increased in RRMS patients during relapse*

We also evaluated whether SIGLEC1 and SIGLEC7 expression was changed in RRMS patients at the time of acute exacerbations. The mean time (SD) between symptoms onset and blood drawing was 6.0 days (4.2). As shown in Figure 4.C(ii) SIGLEC7 expression by CD14+ blood monocytes was increased in RRMS patients during relapse, and differences reached statistical significance for the MFI ($p=0.001$). However, SIGLEC1 expression in monocytes was not significantly different between RRMS patients in clinical remission and RRMS patients during relapse ($p>0.05$ for both percentage of positive cells and MFI; Figure 4.C(i).

**Fig. 4 .C**

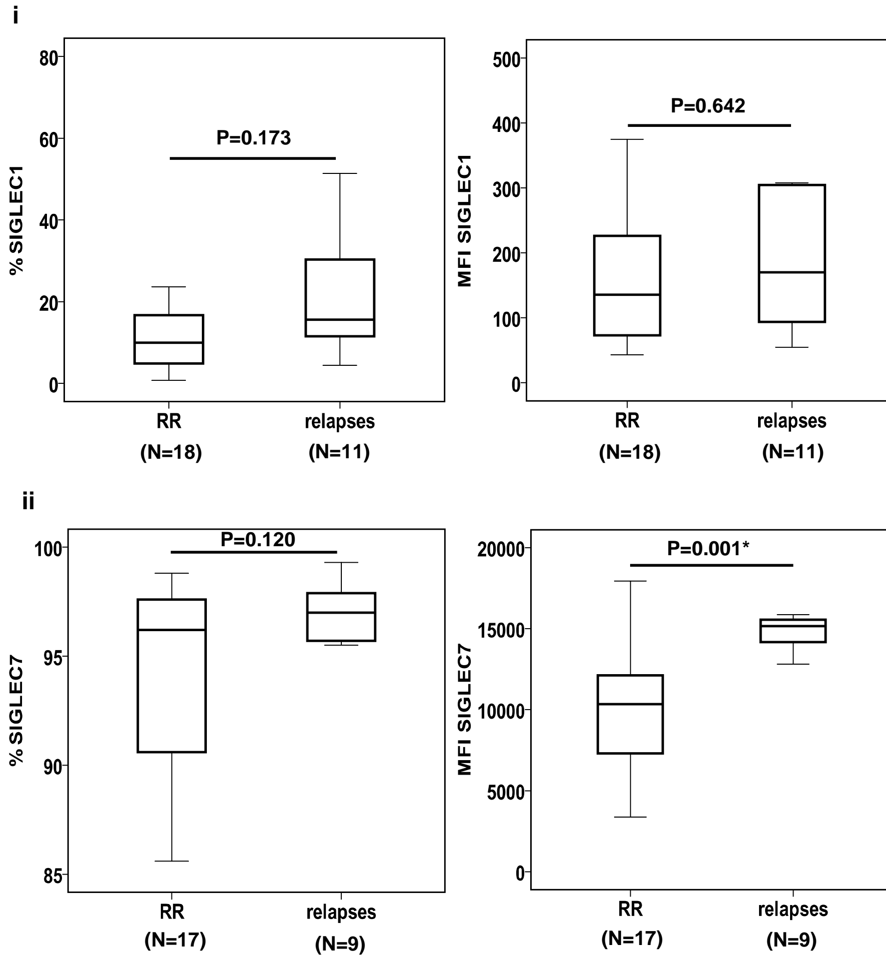



*Figure 4C. Comparison of (i) SIGLEC1 and (ii) SIGLEC7 expression between RRMS patients during clinical remission and acute relapses. Results are expressed as % of positive cells (left graphs) and mean fluorescence intensity (MFI) (right graphs).*

**Table 4.2**

| | | HC | RRMS | SPMS | PPMS |
|---|---|---|---|---|---|
| CD14++CD16- (inflammatory) | % | 7.9 (1.4) | 12.2 (4.7) | 19.2 (6.8) | 31.1 (8.5)* |
| | MFI | 98.8 (12.3) | 217.5 (87.4) | 539.8 (390.0) | 953.5 (575.4) |
| CD14+CD16+ | % | 0.4 (0.3) | 2.2 (1.2) | 0.6 (0.6) | 6.0 (4.6) |
| (resident) | MFI | 125.6 (33.7) | 498.7 (203.7) | 546.5 (292.4) | 1041.8 (573.8) |

### 4.3.5 Expression of the activation marker CD64 is similar between MS patients and healthy controls

To investigate whether the abovementioned differences observed in SIGLEC1 and SIGLEC7 expression between groups were secondary to differences in the activation status of blood monocytes, expression of the activation marker CD64 by CD14+ monocytes was compared among groups. As shown in Figure 4.D, the percentage of CD64 positive cells and MFI did not significantly differ between healthy controls, MS patients with different clinical forms of the disease, and RRMS patients during relapse ($p>0.05$ for all the comparisons).

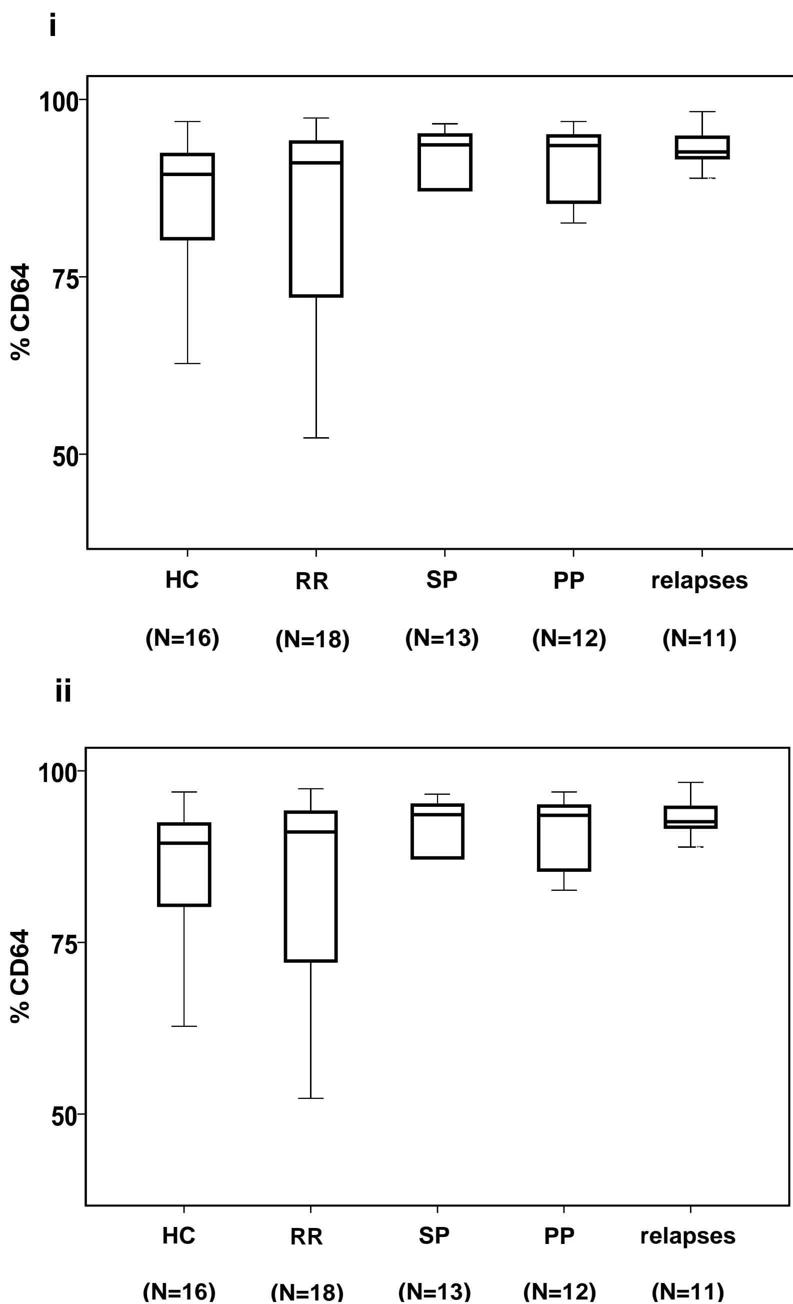



*Figure 4.D Boxplots showing expression of the activation marker CD64 in blood monocytes of healthy controls (HC) and multiple sclerosis (MS) patients. CD64 expression was determined in CD14+ monocytes by means of FACS. Results are expressed as a (i) percentage of CD14+ monocytes expressing CD64 (%CD64;*

*left) and (ii) mean fluorescence intensity (MFI) of CD64 expression in CD14+ monocytes (MFI CD64; right).*

### *4.3.6 Association between SIGLEC1 and SIGLEC7 expression and clinical variables*

No statistically significant correlations were observed between the frequency of SIGLEC1 and SIGLEC7 positive monocytes or their expression levels and clinical variables such as disease duration and number of relapses in the previous two years. In PPMS patients, a trend towards significant correlation was found between the percentage of SIGLEC1 positive monocytes and EDSS score at the time of blood collection ($r=0.71$, $p=0.010$), although the association did not reach the threshold for statistical significance after Bonferroni correction (data not shown).

## 4.4 Discussion

Siglecs are cell surface receptors with immunoglobulin domains that allow sialic acid-mediated recognition and cellular interactions. (May et al. 1998; Varki et al. 2006; Crocker et al. 1998) It is also known that some members of the Siglec family are important sources of inhibitory signals that may attenuate immune responses and reduce inflammation (Pillai et al. 2012). Of the Siglecs that have been identified thus far in humans, in the present study we focused, because of their function and involvement in other autoimmune disorders, on SIGLEC1 and SIGLEC7. We investigated the potential roles of SIGLEC1 and SIGLEC7 in MS by determining their expression in peripheral blood cells from a

cohort of patients with different clinical forms and activity phases of the disease. Insomuch as most Siglecs are expressed primarily in innate immune cells, (Pillai et al. 2012) and monocytes are among the main components of inflammatory infiltrates in MS brains and are known to contribute to the inflammatory process and to neuronal damage in the disease, (Hendriks et al. 2005) SIGLEC1 and SIGLEC7 expression was mostly determined in the population of circulating blood monocytes.

When comparing the whole MS group with healthy controls, SIGLEC1 protein expression was increased in blood monocytes from MS patients. This finding was confirmed at the SIGLEC1 gene expression level by real time PCR using PBMC. Further stratification of MS patients into clinical forms revealed that SIGLEC1 protein expression was predominantly up-regulated in patients with PPMS, and to a lesser degree in patients with SPMS. This increase was observed both in the frequency of SIGLEC1 positive monocytes and mean SIGLEC1 expression levels.

SIGLEC1 (also known as CD169) belongs to the group of Siglecs that lack inhibitory signaling cytosolic motifs in their cytoplasmic tails and primarily mediate adhesion events (Pillai et al. 2012). Previous studies have shown increased expression of SIGLEC1 at the mRNA and protein levels in the blood monocytes of patients with autoimmune disease such as systemic lupus erythematosus, (Biesen et al. 2008) systemic sclerosis (York et al. 2007) and primary biliary cirrhosis. (Bao et al. 2010) In patients with systemic lupus erythematosus, the frequency of CD14+ blood monocytes

expressing SIGLEC1 positively correlated with disease activity. (Biesen et al. 2008) In our study, SIGLEC1 expression did not correlate with acute disease activity, since its expression was similar between RRMS patients during clinical remission and acute exacerbations. In contrast, SIGLEC1 expression was increased during the chronic progressive phases of the disease.

The monocyte population in human peripheral blood is heterogeneous and can be divided into two circulating subsets based on the differential expression of CD14 and CD16, (Biesen et al. 2008; Gordon et al. 2005) inflammatory monocytes (CD14++CD16-) and resident monocytes (CD14+CD16+), which resemble mature tissue macrophages. When SIGLEC1 expression was analyzed in these two monocyte subsets, it was observed that both inflammatory and resident monocytes contributed to the SIGLEC1 up-regulation seen in PPMS patients. The similar expression observed for the activation marker CD64 between patients with different clinical forms of MS rules out an increase in SIGLEC1 expression secondary to global activation of monocytes in patients with PPMS.

Whereas the frequency of SIGLEC7 positive monocytes and mean expression levels did not significantly differ between MS patients with different clinical forms of the disease and healthy controls, it was interesting to observe that SIGLEC7 expression was clearly up-regulated in RRMS patients during clinical relapses.

SIGLEC7 belongs to the group of Siglecs containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails which are probably a source of inhibitory signals upon phosporilation of ITIM tyrosines. (Pillai et al. 2012) SIGLEC7 is the target of SOCS3, (Orr et al. 2007) a member of the SOCS family of inducible proteins that inhibit cytokine signaling in immune and central nervous system and has been implicated in MS. Binding of SOCS3 to the phosphorylated ITIM of SIGLEC7 targets it for proteasomal mediated degradation and blocks the inhibitory effect of SIGLEC7 on cytokine-induced proliferation. (Orr et al. 2007). Of note, SOCS3 expression by monocytes has been reported to decrease in RRMS patients during relapses compared with clinical remission. (Frisullo et al. 2007) Given the inverse functional relationship existing between SIGLEC7 and SOCS3, the upregulation of SIGLEC7 observed in the present study during acute relapses may well reflect the lack of the inhibitory effect that SOCS3 has on SIGLEC7 due to SOCS3 relapse associated-down-regulation.

**Article 4.**

Malhotra S, Morcillo-Suárez C, Nurtdinov R, Rio J, Sarro E, Moreno M, Castilló J, Navarro A, Montalban X, Comabella M. Roles of the ubiquitin peptidase USP18 in multiple sclerosis and the response to interferon-β treatment. Eur J Neurol. 2013 May 22. doi: 10.1111/ene.12193. [Epub ahead of print]

# Chapter 5
# Role of IL28B in the response to IFNβ treatment

# IL28B polymorphisms are not associated with the response to interferon-beta in multiple sclerosis

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## Abstract

Recent studies have revealed an association between interleukin 28B (*IL28B*) and response to IFN-alpha treatment in hepatitis C patients. Here we investigated the influence of *IL28B* polymorphisms in the response to interferon-beta (IFNβ) in multiple sclerosis (MS) patients. We genotyped two SNPs of the *IL28B* gene (rs8099917 and rs12979860) in 588 MS patients classified into responders (n=281) and non-responders (n=307) to IFNβ. Combined analysis of the study cohorts showed no significant associations between SNPs rs8099917 and rs12979860 and the response to treatment. These findings do not support a role of IL28B polymorphisms in the response to IFNβ in MS patients.

Keywords: multiple sclerosis; polymorphisms; interferon-beta; response

# Chapter 5

# Role of IL28B in the response to IFNβ treatment

## 5.1 Introduction

IFNβ is one of the most widely prescribed disease-modifying therapy for RRMS and has demonstrated a beneficial effect on disease activity (The Interferon β Multiple Sclerosis Study Group et al. 1993; Jacobs et al. 1996; PRISMS et al. 1998). IFNβ is, however, partially effective and there is a proportion of patients who will show a lack of clinical response to IFNβ (Río et al. 2002). Although several pharmacogenetic studies have aimed to identify genes that may influence the response to IFNβ (Sriram et al. 2003; Cunningham et al. 2005; Leyva et al. 2005; López-Gómez et al. 2013), to date, there is a lack of biomarkers reliably associated with the response to treatment.

Several studies have shown that the IL28B gene (Official symbol IFNL3 - interferon, lambda 3) is associated with the response to IFN-alpha (IFNα) therapy in patients suffering from hepatitis C (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009; Rauch et al. 2010; Rallón et al. 2010; Pineda et al. 2010). Furthermore, recent studies have pointed to an inverse relationship between a polymorphism located in the IL28B gene (rs12979860) and the expression of ISG15 (Abe et al. 2011), the target of the USP18.

Based on these observations, and considering that IFNα and IFNβ belong to same family of type I IFNs and both signal through the same JAK/STAT pathway inducing similar set of genes (Platanias et al. 2005), in the present study we aimed to investigate whether IL28B polymorphisms were also influencing the response to IFNβ in MS patients.

## 5.2 Materials and Methods

### *5.2.1 Patients and definition of response to IFNβ therapy*

All subjects satisfied Poser's and McDonald's criteria for clinically definite MS (Poser et al. 1983; McDonald et al. 2001). The study was approved by the local ethics committees and all patient samples were collected with written informed consent.

### *5.2.2 Genotyping of IL28B polymorphisms*

Genomic DNA from peripheral blood samples was obtained using standard methods. Genotyping of rs8099917 and rs12979860 was performed by means of the 5' nuclease assay technology for allelic discrimination using fluorogenic TaqMan® probes. SNP rs8099917 was commercially available from Applied Biosystems through the Assay-on-Demand service and rs12979860 was custom designed.

The sequence of rs12979860 primers and probes were: forward primer 5´-GCCTGTCGTGTACTGAACCA-3´, reverse primer 5´-GCGCGGAGTGCAATTCAAC-3´, probe (C allele) 5´-VIC-

TGGTTCGCGCCTTC-3´, probe (T allele) 5´-FAM-CTGGTTCACGCCTTC-3´ (Urban et al. 2010).

All cohorts used in the study were genotyped in one single center (Cemcat, Barcelona). Except for 2 cohorts (Serbia and Bochum), a number of the DNA samples included in the present study were also genotyped in previous MS case-control and pharmacogenomic studies.

### *5.2.3 Statistical analysis*

For both SNPs, allele frequencies were compared between IFNβ responders and non-responders taking into account possible stratification due to different population origin using the Cochran-Mantel-Haenszel test. Additional allele comparisons were performed in subsets of samples from the same center and in patients receiving the same type of IFNβ treatment. Data processing, missingness, Hardy-Weinberg analysis and allele association analysis were performed with SNPator (www.snpator.org) (Morcillo-Suarez et al. 2008). Cochran-Mantel-Haenszel test was performed using plink (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell et al. 2007).

## 5.3 Results

A total of 588 MS patients fulfilled the response criteria and were included in the study. Of these, 281 (47.8%) patients were classified

as responders and 307 (52.2%) classified as non-responders to IFNβ.

Table 5.1 summarizes demographic and baseline clinical details of the patient cohorts used in the present study.

The overall genotype success was 97.1%, and 34 out of 588 samples had 1 genotype missing. Inclusion or exclusion of these samples did not alter the results. Both SNPs were in Hardy-Weinberg equilibrium (p=0.891 for rs8099917; p=0.436 for rs12979860).

As shown in Table 4.1, combined analysis of all the study cohorts revealed lack of significant associations between rs8099917 [OR: 1.2 (0.9-1.6), p-value=0.1806] or rs12979860 [OR: 1.1 (0.8-1.4), p-value=0.5324] and the response to IFNβ. Individual analysis revealed that in one cohort (France), SNPs rs8099917 and rs12979860 were significantly associated with the response to IFNβ [OR: 2.8 (1.4-5.7), uncorrected p-value=0.0038, corrected p-value=0.057 for rs8099917; OR: 2.7 (1.5-4.9), uncorrected p-value=0.0014, corrected p-value=0.042 for rs12979860] (Table 5.1).

Further analysis based on the type of IFNβ that MS patients were receiving did not reveal statistically significant associations between *IL28B* polymorphisms and the response to IFNβ after p-value correction for multiple testing (data not shown).

# Table 5.1

| | N (%) | | Age (y) | | F/M (% F) | |
|---|---|---|---|---|---|---|
| **Sets** | **R** | **NR** | **R** | **NR** | **R** | **NR** |
| Newcastle | 18 (47.4) | 20 (52.6) | 41.9 (11.5) | 34.9 (10.9) | 15/3 (83.3) | 14/6 (70.0) |
| Barcelona | 107 (46.1) | 125 (53.9) | 41.0 (9.0) | 40.1 (10.1) | 87/20 (81.3) | 97/28 (77.6) |
| Bochum | 4 (50.0) | 4 (50.0) | 37.8 (4.6) | 36 (9.8) | 2/2 (50.0) | 2/2 (50.0) |
| Madrid SC | 14 (38.9) | 22 (61.1) | 34.0 (6.1) | 35.0 (8.4) | 11/3 (78.6) | 11/11 (50.0) |
| Madrid PH | 12 (60.0) | 8 (40.0) | 37.3 (8.3) | 34.8 (11.5) | 7/5 (58.3) | 7/1 (87.5) |
| Malaga | 17 (53.1) | 15 (46.9) | 38.3 (10.0) | 41.3 (8.9) | 13/4 (76.5) | 11/4 (73.3) |
| Milan | 6 (60.0) | 4 (40.0) | 36.5 (10.8) | 38.6 (19.8) | 3/3 (50.0) | 2/2 (50.0) |
| Rostock | 40 (48.2) | 43 (51.8) | 40 (9.5) | 38.3 (11.3) | 6/34 (15.0) | 7/36 (16.3) |
| San Francisco | 2 (50.0) | 2 (50.0) | 40.5 (2.1) | 41.5 (6.4) | 2/- (100.0) | 2/- (100.0) |
| Serbia | 8 (34.8) | 15 (65.2) | 30.9 (3.4) | 33.1 (6.8) | 5/3 (63.0) | 7/8 (47.0) |
| Toulouse | 53 (52.0) | 49 (48.0) | 28.7 (7.2) | 27.8 (8.4) | 44/9 (83.0) | 35/14 (71.4) |
| Total | 281 (47.8) | 307 (52.2) | 37.4 (9.8) | 36.7 (10.8) | 195/86 (69.3) | 195/112 (63.5) |

***Table 5.1*** *Summary of demographic and baseline clinical characteristics for all the cohorts of MS patients responders and non-responders to IFNβ treatment.*

*Newcastle Refer to: University Newcastle,Callaghan Campus, Australia. Barcelona refers to: Hospital Universitari Vall d´Hebron (HUVH), Barcelona. Bochum refers to St. Josef-Hospital, Ruhr-University Bochum, Germany. Madrid SC: refers to Hospital Clínico San Carlos, Madrid. Madrid PH: refers to Hospital Universitario Puerta de Hierro, Madrid. Malaga refers to HospitalRegional Universitario Carlos Haya, Málaga. Milan refers to HospitalRegional Universitario Carlos Haya, Málaga. Rostock refers to University of Rostock, Rostock, Germany. San Francisco refers to School of Medicine University of California, San Francisco, CA, USA. Serbia refers to Clinical Centre of Serbia (CCS), University of Belgrade, Serbia. Toulouse refers to INSERM U1043, Université de Toulouse III, Hopital Purpan, Toulouse, France.*

| | Disease duration (y) | | EDSS[a] | | IFNβ (1/2/3)[b] | |
|---|---|---|---|---|---|---|
| **Sets** | **R** | **NR** | **R** | **NR** | **R** | **NR** |
| Newcastle | 7.3 (11.0) | 6.6 (6.5) | NA | NA | 1/12/05 | 2/10/8 |
| Barcelona | 13.8 (6.7) | 13 (7.4) | 2.0 (2.0) | 6.0 (4.0) | 30/45/32 | 32/57/36 |
| Bochum | 3.8 (3.8) | 4.3 (3.6) | 2.0 (2.1) | 3.0 (4.1) | 1/03/- | 1/2/1 |
| Madrid SC | 5.1 (4.9) | 5 (7.1) | 2.0 (2.2) | 2.0 (2.5) | 3/5/6 | 4/10/8 |
| Madrid PH | 4.9 (3.5) | 5.7 (2.4) | 1.2 (2.4) | 2.2 (1.9) | 3/3/6 | 2/2/4 |
| Malaga | 13.3 (6.5) | 15.4 (4.1) | 0 (1.0) | 1.0 (2.0) | 5/4/8 | 5/3/7 |
| Milan | 3.7 (5.1) | 5.8 (8.5) | 2.0 (1.2) | 2.5 (2.5) | 2/-/4 | 1/-/3 |
| Rostock | 4.9 (4.9) | 4.6 (5.5) | 1.0 (1) | 3.0 (2.5) | 9/20/11 | 6/31/6 |
| San Francisco | 1.9 (0.9) | 3.7 (4.1) | 1.0 (0) | 1.5 (0) | 1/-/1 | 1/-/1 |
| Serbia | 2.5 (2.7) | 6.3 (4.5) | 1.0 (1.1) | 2.5 (1.5) | -/2/6 | -/7/8 |
| Toulouse | 4.5 (5.1) | 4.6 (5.7) | 2.0 (2) | 1.5 (2.8) | 34/8/11 | 29/11/9 |
| Total | 8.7 (7.5) | 8.8 (7.5) | 1.5 (1.5) | 3.0 (4.0) | 89/102/90 | 83/133/91 |

## 5.4 Discussion

The *IL28B* gene codes for IFN-lambda 3 (IFN-λ-3), a cytokine induced by viral infections that is related with the type I IFNs and the IL-10 family (Ank *et al.* 2006). Recently, several studies have shown that *IL28B* polymorphisms are associated with spontaneous hepatitis C virus (HCV) clearance and response to IFNα-based therapies in chronically infected HCV patients. Associations were mainly driven by two SNPs positioned near the IL28B gene on chromosome 19: rs8099917, located ~8 Kb downstream from IL28B (Suppiah et al. 2009; Tanaka et al. 2009; Rauch et al. 2010); and rs12979860, located 3 Kb upstream of the IL28B gene (Ge et al. 2009; Rallón et al. 2010; Pineda et al. 2010).

IFNβ is a common therapy for MS. However, it is only partially effective and not all patients respond to treatment. Previous studies performed either at the candidate gene level or at the genome-wide level suggest that the response to IFNβ appears to be complex and polygenic in nature (Vandenbroeck et al. 2010). IFNβ and IFNα belong to the same family of type I IFNs, which bind to the same cell surface receptor and lead to up-regulation of a similar set of genes that share IFN-stimulated response element (ISRE) promoter sequence (Platanias 2005). Furthermore, the IFN-λ proteins (also known as type III IFNs) encoded by the *IL28A*, *IL28B* and *IL29* genes, signal through a unique heterodimeric receptor consisting of IL10RB and IL28RA that share a common downstream signalling pathway with the type I IFNs (Ank et al. 2008). These observations prompted us to evaluate whether the genetic variants of the IL*28B* gene that were associated with the response to IFNα therapy in patients with HCV infection were also influencing the response to IFNβ in patients with MS. IL28B was not proposed in previous studies as a candidate gene for MS susceptibility or for the response to IFNβ. In addition, rs8099917 and rs12979860 were not included in the SNP arrays used for genotyping in the two previous genome-wide pharmacogenomic studies published in relation with the response to IFNβ (Byun et al. 2008; Comabella et al. 2009b).

As part of a multicentric collaborative study, SNPs rs8099917 and rs12979860 were genotyped in a large cohort of MS patients treated with IFNβ and classified into responders and non-responders based on stringent clinical criteria. However, combined analysis revealed

that allele and genotype frequencies were similarly distributed in IFNβ responders and non-responders, results that overall do not support a role of IL28B polymorphisms as modifiers of the response to IFNβ. However, the finding of a marginal significant association of SNPs rs8099917 and rs12979860 with the response to IFNβ in the French cohort may warrant further studies of IL28B polymorphisms in particular populations.

In patients infected with HCV, the beneficial effects of IFN-based therapies are most likely related with their antiviral properties. In contrast, in patients with MS the mechanisms whereby IFNβ produces its positive effects remain unknown. Furthermore, although several viruses have been implicated as potential etiologic factors in MS (Giovannoni et al. 2006), studies relating IFNβ efficacy with its antiviral properties are scarce (Hong et al. 2002; García-Montojo et al. 2011; Vandenbroeck et al. 2010). These observations may explain that polymorphisms in the *IL28B* gene, which are directly related with clearance of the etiologic factor, i.e. HCV, positively influence the response to IFN-based therapies. However, *IL28B* polymorphisms do not appear to influence the response to IFNβ in MS, a disorder for which there is no conclusive evidence yet of a viral etiologic factor.

# PART III

# DISCUSSION AND CONCLUSIONS

# Chapter 6

# Discussion

# Chapter 6

# Discussion

MS is a heterogeneous disease in different aspects: disease severity, clinical course, neuroradiological findings, histopathological characteristics of CNS lesions, and response to treatment. In this scenario, there is a strong need for the identification of biomarkers that reflect these different aspects of the disease and may help to predict disease course or the response to treatment. Bearing this in mind, the main objective of the first study included in this dissertation was to identify prognostic biomarkers in MS. To achieve this, we used gene expression microarrays as one of the most developed technologies for biomarker discovery. In this regard, microarrays were performed in PBMC from MS patients who developed NAB in response to IFNβ at 12 and/or 24 months of treatment and patients who remained NAB-negative. We identified 9 biomarkers (IFI6, IFI27, IFI44L, IFIT1, HERC5, LY6E, RSAD2, SIGLEC1, and USP18) that followed changes in gene expression over time similar to the *MX1*, which was used as the gold standard gene. While some of these biomarkers were used in previous studies to evaluate the biological response to IFNβ (Sellebjerg et al. 2009; Pachner et al. 2009), others had not been tested yet. Although *MX1* induction was highly selective for type I IFNs, dose- and time-dependent induction experiments were compared for the 9 selected genes. From dose- and time-dependent experiments we evaluated sensitivity and selectivity of the genes. Sensitivity was defined as

the minimum IFNβ concentration that induced a statistically significant increase in gene expression when compared with the untreated condition. We compared sensitivity of the gold standard, MX1, and found five genes to be more sensitive than MX1: RSAD2, HERC5, IFI6, IFI27, and USP18. On the other hand, selectivity was defined as the difference observed in gene expression between different concentrations of type I and type II IFNs, and it was calculated by comparing the AUC obtained for IFNβ and IFNγ. Two genes were found to be more selective than the MX1: HERC5 and USP18. Combined evaluation pointed to USP18 and HERC5 as the most sensitive and selective genes. Following, we compared basal gene expression between untreated MS patients and controls. We compared all the genes against the gold standard and found 3 genes, USP18, HERC5, and LY6E which were differentially expressed between both groups, with lower expression in MS patients. However, USP18 was the only gene that survived bonferroni correction. It is important to mention that the decrease in USP18 expression in MS patients may result in an overactivation of the type I IFN pathway, considering that USP18 acts as a negative regulator of the IFNβ-related pathways (Fig 6A).

**Fig. 6.A**

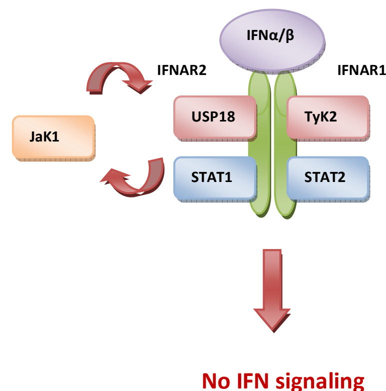

*Figure 6 A USP18 functions as negative regulator of type 1 IFN signalling by interfering with binding of Jak1 to IFNAR2.*

The findings with the USP18 gene in this initial study prompted us to further investigate its potential role as MS disease activity biomarker and IFNβ-response biomarker. USP18 encodes a type I IFN-inducible cysteine protease that deconjugates ISG15 from target proteins (Malakhov et al. 2002) as shown in Fig 6.B.

**Fig. 6.B**

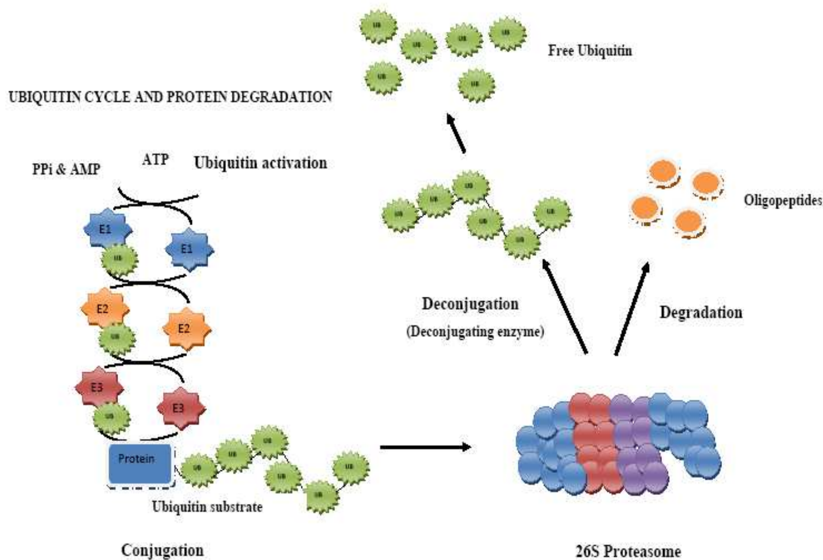


*Figure 6.B Ubiquitinating pathway involving ATP dependent activation, conjugation by ubiquitin carrier, followed by ligation with protein substrate, which finally results in degradation by 26S proteasome or lead to deconjugation by deconjugating enzymes such as USP18.*

To investigate the role of USP18 as disease activity biomarker, two USP18 polymorphisms, rs2542109 (intronic) and rs9618216 (promoter), were genotyped in relapse-onset (relapsing-remitting and secondary progressive) MS patients and healthy controls. Two haplotypes, (TG and CG) were significantly associated with MS. The CG haplotype acted as a disease protective haplotype whereas the TG haplotype acted as a risk haplotype. On the basis of the haplotypic analysis, we compared the basal gene expression among the normal, protective and risk haplotypes. We found that USP18 expression was significantly reduced in patients carrying the protective CG haplotype. Similarly, lower expression of ISG15, the USP18 target, was observed in CG carriers. These findings are in line with our previous results showing a deficient USP18 expression in MS patients compared with controls. On further analysis, patients carrying the protective CG haplotype had higher disease activity as reflected by the increased relapse rate, and also higher neurological disability as shown by the higher EDSS scores observed in these patients.

To investigate the role of USP18 as IFNβ response biomarker, these two polymorphisms were also investigated in a cohort of MS patients responders and non-responders to IFNβ classified according to stringent clinical criteria. Notably, AA homozygosis for the intronic polymorphism rs2542109 was associated with the responder phenotype, and may be used to predict the responder status in MS patients receiving treatment with IFNβ.

Altogether, these results pointed to a role of USP18 in MS pathogenesis and the therapeutic response to IFNβ.

Another finding from the initial microarray study that motivated further investigation related to genes belonging to the Siglec family. SIGLEC1 was found to be more selective and equally sensitive to MX1, thus becoming an attractive candidate biomarker. We also decided to include SIGLEC7 in the study, taking into account that SIGLEC7 is the target of SOCS3, a negative regulator of the JAK/STAT signaling pathway and, consequently, a biomarker IFNβ-related.

It is relevant to mention that SIGLEC1 and SIGLEC7 were previously known to play roles in inflammatory conditions and autoimmune diseases, but their potential implication in MS had not been investigated. Hence, we aimed to evaluate SIGLEC1 and SIGLEC7 as disease activity biomarkers in MS.

SIGLEC1 (also known as CD169) acts as a macrophage-restricted sialic acid receptor that interacts with lymphoid and myeloid cells. It belongs to the group of Siglecs that lack inhibitory signaling cytosolic motifs in their cytoplasmic tails and primarily mediate adhesion events (Pillai et al. 2012). SIGLEC1 has been found up-regulated in patients with other autoimmune disease such as systemic sclerosis and systemic lupus erythematosus (Biesen et al. 2008; Feng et al. 2006; Tang et al. 2008; York et al. 2007). On the other hand, SIGLEC7 belongs to ITIM which are responsible for

sending inhibitory signals upon phosphorylation and has been found up-regulated during inflammation (Orr et al. 2007).

SIGLEC1 and SIGLEC7 expression was compared between MS patients and healthy controls. SIGLEC1 protein expression was higher in peripheral blood monocytes from MS patients compared to healthy controls, a findings that was replicated at the mRNA level. Further stratification of the MS population suggested that the increase in SIGLEC1 expression was mainly driven by patients with progressive forms of MS, particularly patients with PPMS. After investigating the subtypes of monocytes that could potentially be involved in these findings, we observed that both inflammatory and resident monocytes contributed to the heightened SIGLEC1 expression in PPMS patients. The other candidate, SIGLEC7, had similar expression levels between MS patients and healthy control. Therefore, these results point to a role of SIGLEC1 in the progressive phases of the disease.

When the expression levels for SIGLEC1 and SIGLEC7 were compared between patients with RRMS in relapses and remission, SIGLEC7 but not SIGLEC1 was significantly increased during acute exacerbations, suggesting a role of SIGLEC7 in acute disease activity. Considering that SIGLEC7 is the target of SOCS3, which has been found decreased in RRMS patients at the time of relapses (Frisullo et al. 2007), the up-regulation of SIGLEC7 observed during acute relapses may be due to the lack of an inhibitory effect of SOCS3 on SIGLEC7.

The last study belonging to this dissertation focused on role of IL28B in the response to IFNβ treatment. Recent studies pointed to an inverse relationship between a polymorphism located in the IL28B gene (interleukin-28B; official symbol IFNL3 - interferon, lambda 3) and the expression of ISG15 (Abe et al. 2011), the target of the USP18, and several studies reported an association between IL28B and the response to IFN-alpha (IFNα) therapy in patients suffering from hepatitis C (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009; Rauch et al. 2010; Rallón et al. 2010; Pineda et al. 2010). Based on these observations, and considering that IFNα and IFNβ belong to same family of type I IFNs and both signal through the same JAK/STAT pathway inducing similar set of genes (Platanias et al. 2005), we decided to investigate the role of the IL28B gene in IFNβ response. Two SNPs of the IL28B gene (rs8099917 and rs12979860) were genotyped in a relatively large cohort of responders and non-responders to IFNβ. The negative results obtained in the analysis, with lack of significant associations between these two polymorphisms and IFNβ response precluded a potential role of IL28B as biomarker of response to IFNβ in MS patients.

# Chapter 7
# Conclusions

# Chapter 7

# Conclusions

In this dissertation, we searched for prognostic biomarkers in MS. Our investigation led to the identification of MS biomarkers with potential roles in disease activity and the response to IFNβ treatment. We now summarize the main conclusions derived from these studies:

1. We identified prognostic biomarkers that may be considered in addition to the MxA to measure the biological response to IFNβ and the in vivo effects of NABs. Findings from this initial study further suggested that some of the selected biomarkers might also be playing roles in MS disease activity (USP18 and SIGLEC1) and the therapeutic response to IFNβ (USP18).
2. Two polymorphisms, one intronic and another located in the promoter region of the *USP18* gene, were associated with MS susceptibility. Haplotypic analysis revealed one haplotype (*CG*) that correlated with lower *USP18* gene expression and higher clinical disease activity. The intronic polymorphism was found to be associated with the IFNβ response. Altogether, these findings point to a prognostic role of USP18 in MS as both disease activity and IFNβ response biomarker.
3. We investigated the role of two members of the Siglec family, SIGLEC1 and SIGLEC7, in MS as disease activity

biomarkers. SIGLEC1 expression was increased in monocytes from MS patients, particularly patients with PPMS, and hence SIGLEC1 may be playing roles in the progressive forms of MS. Further experiments led us to conclude that both inflammatory and resident monocytes were responsible for the elevated SIGLEC1 expression observed in monocytes from PPMS patients. In contrast, analysis of the monocyte expression levels of our second Siglec candidate, SIGLEC7, did not discriminate between healthy controls and patients with different clinical forms of the disease; however, SIGLEC7 expression was significantly up-regulated in RRMS patients at the time of clinical exacerbations, suggesting that SIGLEC7 may be playing a more important role as biomarker for acute disease activity in MS patients.

4. IL28B polymorphisms are not associated with the response to IFNβ and exclude a role of IL28B as response biomarker in MS.

# PART IV
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# PART V

# APPENDIX

# Appendix

***Appendix Table .1*** *Canonical pathways up-regulated with IFNβ*

| Pathways | P-values | Ratio* |
|---|---|---|
| Molecular Mechanisms of Cancer | 0.0016 | 0.0603 |
| Interferon Signaling | 0.0037 | 0.147 |
| Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses | 0.0102 | 0.0854 |
| Starch and Sucrose Metabolism | 0.0110 | 0.0882 |
| Activation of IRF by Cytosolic Pattern Recognition Receptors | 0.0138 | 0.0923 |
| ERK5 Signaling | 0.0138 | 0.0952 |
| Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency | 0.0263 | 0.0702 |
| Notch Signaling | 0.0324 | 0.093 |
| Sphingolipid Metabolism | 0.0331 | 0.0759 |
| PPARα/RXRα Activation | 0.0355 | 0.0575 |
| T Cell Receptor Signaling | 0.0355 | 0.068 |
| Protein Ubiquitination Pathway | 0.0380 | 0.0519 |
| Glioma Invasiveness Signaling | 0.0389 | 0.0847 |

| Pathways | P-values | Ratio* |
|---|---|---|
| Cyclins and Cell Cycle Regulation | 0.0417 | 0.0682 |
| Galactose Metabolism | 0.0417 | 0.0889 |
| Purine Metabolism | 0.0427 | 0.053 |
| Colorectal Cancer Metastasis Signaling | 0.0427 | 0.0526 |
| Role of JAK1, JAK2 and TYK2 in Interferon Signaling | 0.0437 | 0.115 |
| Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis | 0.0457 | 0.0464 |
| Role of PI3K/AKT Signaling in the Pathogenesis of Influenza | 0.0479 | 0.0714 |
| Role of JAK family kinases in IL-6-type Cytokine Signaling | 0.0479 | 0.115 |

*Refers to the ratio between the number of genes found up-regulated at any time point during IFNβ treatment compared with the untreated condition and the number of genes belonging to the corresponding pathway.

***Appendix Table 2.*** *Canonical pathways down-regulated with IFN$\beta$*

| Pathways | P-values | Ratio* |
|---|---|---|
| Thrombin Signaling | 0.0054 | 0.0408 |
| Airway Pathology in Chronic Obstructive Pulmonary Disease | 0.0055 | 0.2500 |
| Colorectal Cancer Metastasis Signaling | 0.0063 | 0.0364 |
| Regulation of Actin-based Motility by Rho | 0.0066 | 0.0568 |
| Gα12/13 Signaling | 0.0066 | 0.0480 |
| Integrin Signaling | 0.0066 | 0.0385 |
| TNFR2 Signaling | 0.0074 | 0.0909 |
| Glioma Invasiveness Signaling | 0.0091 | 0.0678 |
| SAPK/JNK Signaling | 0.0095 | 0.0495 |
| Acute Phase Response Signaling | 0.0102 | 0.0407 |
| Role of MAPK Signaling in the Pathogenesis of Influenza | 0.0107 | 0.0645 |

| **Pathways** | **P-values** | **Ratio*** |
|---|---|---|
| Relaxin Signaling | 0.0117 | 0.0414 |
| Hypoxia Signaling in the Cardiovascular System | 0.0120 | 0.0588 |