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Genetic Susceptibility and Immune Dysfunction in Multiple Sclerosis

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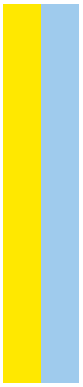

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GENETIC SUSCEPTIBILITY AND IMMUNE DYSFUNCTION IN MULTIPLE SCLEROSIS

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Paper 1. A Bettencourt, C Carvalho, B Leal, S Brás, D Lopes, AM Silva, E Santos, T Torres, I Almeida, F Farinha, P Barbosa, A Marinho, M Selores, J Correia, C Vasconcelos, PP Costa, B Martins Silva. "The protective role of HLA-DRB1*13 in Autoimmune Diseases" J Immunol Res. 2015;2015:948723. doi: 10.1155/2015/948723.

Paper 2. Bettencourt A**,** Silva AM, Carvalho C, Leal B, Santos E, Costa PP, Silva BM. "The role of KIR2DS1 in multiple sclerosis - KIR in Portuguese MS patients." J Neuroimmunol. 2014 Apr 15;269(1-2):52-5. doi: 10.1016/j.jneuroim.2014.01.009.

Paper 3. A Bettencourt, B Leal, C Carvalho, E Santos, M Soares, PP Costa, B Silva, AM Silva "Nrf2 polymorphisms and Multiple Sclerosis" (In preparation).

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Paper 6. Bettencourt A, Boleixa D, Reis J, Oliveira JC, Mendonça D, Costa PP, Silva BM, Marinho A, Silva AM. "Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal." J Steroid Biochem Mol Biol. 2016 Nov 5. doi: 10.1016/j.jsbmb.2016.11.005.

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Às Marias da minha vida…

À minha Mãe a quem devo tudo aquilo que sou, ou pretendo ser…

e à minha filha a quem devo tudo o que serei…

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TABLE OF CONTENTS

Abbreviations

APC	Antigen-Presenting Cell
ARE	Antioxidant Response Element
BBB	Blood Brain Barrier
CLEC16A	C-type Lectin domain family 16
CNS	Central Nervous System
CSF	CerebroSpinal Fluid
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
CYP27B1	Cytochrome P450, family 27, subfamily b, polypeptide 1
DCs	Dendritic Cells
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphate
$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
EDSS	Extended Disability Status Scale
EDTA	EthyleneDiamineTetraacetic acid
Foxp3	Forkhead box P3
FRET	Fluorescence Resonance Energy Transfer
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GWAS	Genome-Wide Association Studies
HC	Healthy Controls
HFE	Hemocromatosis Gene
HHV-6	Human HerpesVirus-6
HLA	Human Leukocyte Antigen
HWE	Hardy-Weinberg Equilibrium
25(OH)D	25-hydroxyvitamin D
IFN-γ	Interferon Gamma
IL2RA	Interleukin-2 Receptor alpha chain
IL7R	Interleukin-7 Receptor
IMSGC	International Multiple Sclerosis Genetics Consortium
IRF8	Interferon Regulatory Factor 8
KIF1B	Kinesin-like protein KIF1B
KIF21B	Kinesin family member 21B
KIR	Killer cell Immunoglobulin like Receptors
LD	Linkage Disequilibrium
MgCl2	Magnesium chloride
MHC	Major Histocompatibility Complex
miRNAs	MicroRNAs
MRI	Magnetic Resonance Imaging

MS	Multiple Sclerosis
MSSS	Multiple Sclerosis Severity Score
NaCL	Sodium chloride
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
Nrf2	Nuclear factor [erythroid-derived 2]-like 2
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PCR-SSP	Polymerase Chain Reaction with Sequence Specific Primers
PMS	Progressive Multiple Sclerosis
PPMS	Primary-Progressive Multiple Sclerosis
PTH	ParaThyroid Hormone
PTPN22	Protein Tyrosine Phosphatase Non-receptor 22
RA	Rheumatoid Arthritis
RFLP	Restriction Fragment Length Polymorphism
RMS	Relapsing Multiple Sclerosis
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RRMS	Relapsing-Remitting Multiple Sclerosis
RT	Reverse Transcription
RT-PCR	Real Time Polimerase Chain Reaction
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphisms
SPMS	Secondary-Progressive Multiple Sclerosis
STAT3	Signal transducer and activator of transcription 3
STR	Short Tandem Repeat
Taq	Thermus aquaticus
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor beta
TNF-α	Tumor Necrosis Factor alpha
TNF-β	Tumor Necrosis Factor beta
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A
Tregs	Regulatory T cells
TYK2	Tyrosine kinase 2
VDBP	Vitamin D Binding Protein
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Elements
UVR	Ultraviolet Radiation
VZV	Varicella-Zoster Virus

Prologue

This work has been carried out in the setting of the long standing interest for Multiple Sclerosis (MS) of the Autoimmunity & Neurosciences research group at Unit for Multidisciplinary Research in Biomedicine - UMIB/ICBAS/UP. This group develops bidirectional translational research and bridges basic research with clinical care, by taking advantage of the possibilities provided by well characterized patient cohorts at the associated Centro Hospitalar do Porto. Its major aim is to go beyond the traditional immunogenetics of complex disease and uncover new biomarkers for disease susceptibility, progression and response to treatment, and to characterize relative risk in genetically defined subpopulation groups, with an emphasis in autoimmune and inflammatory diseases [e.g. Multiple Sclerosis, Systemic Lupus Erythematosus, and Behçet Disease].

The study of the role of genetic polymorphisms in Portuguese MS patients has started more than ten years ago, and our group was able to show that some genetic variants are correlated with increased/decreased risk of MS. The present thesis is a *continuum* of the initial work, and intends to further explore some genetic and non-genetic factors involved in Multiple Sclerosis immune dysfunction.

The thesis is organized in four main chapters: General Introduction, Study Design, Results, and Discussion.

In **Chapter 1,** a general introduction, reviewing the main topics addressed in this thesis, is presented. It gives the state of the art concerning Multiple Sclerosis, namely its epidemiology, clinical presentation and immunopathology. The current knowledge about the disease etiology, including genetic, epigenetic and environmental factors is also described.

The aim of the study and the description of the cohorts and methodologies used in the different studies will be presented in **Chapter 2.**

Chapter 3 (Results) compiles a set of studies presented in the form of eight final manuscripts. Four focus on genetic and epigenetic factors and the other four concerns environmental factors.

The general discussion and conclusion of the work are presented in **Chapter 4**.

Abstract

Multiple sclerosis (MS) is a chronic disease in which both genetic and environmental factors contribute to disease susceptibility and outcome. MS patients suffer from inflammatory lesions in the central nervous system which results in demyelination, reduced neuronal activity and neurodegeneration.

The immune system has a central role in MS pathogenesis. Genetic variants within the Human Leukocyte Antigen (HLA) region, namely HLA-DRB1*15 allele, are the strongest genetic risk factors associated with MS. We confirmed the association of HLA-DRB1*15 and MS susceptibility in a large group of Portuguese patients. As HLA genotype is estimated to account only for 10 to 40% of the genetic risk, we investigated other immune-relevant genes that could also influence MS development. The influence of Killer Immunoglobulin-like Receptor (KIR) genes, and their HLA class I ligands, on MS susceptibility was studied. A negative association between the activating KIR2DS1 gene and MS, independent from the presence of HLA-DRB1*15 allele was observed. This activating receptor seems to confer protection against the development of MS, possibly through modulation of autoreactive T cells by natural killer cells.

Inflammation and oxidative stress are thought to promote tissue damage in Multiple Sclerosis. Being Nrf2 a central transcription factor for the antioxidant response, we investigated the association of two functional single nucleotide polymorphisms in the promoter region of this gene (-653A/G and -617C/A) with MS susceptibility, disease form and progression. Patients with the -653GG genotype, and consequently with low Nrf2 expression, seem to be more prone to develop a relapse-remitting course of the disease.

MicroRNAs (miRNAs) are a class of small non-coding RNAs, which regulate gene expression post-transcriptionally by binding to mRNA targets. One of the best-characterized miRNAs is miR-155, which has pleiotropic functions being involved in inflammation, autoimmunity, and cell plasticity. We have shown that patients with MS have higher circulating levels of miR-155. These observations are in accordance with other studies and implicate a deregulation of inflammation in MS.

The influence of migration and latitude on MS prevalence strongly suggests a role for the environment in MS pathogenesis. Recent studies highlighted that vitamin D deficiency is widespread across Europe, even in countries with abundant sunlight, and at prevalence rates that meet the criteria of a pandemic. The results presented in this thesis demonstrate that vitamin D deficiency is also prevalent in the North of Portugal, affecting almost half of the population. Body mass index and season/sunlight exposure,

are predictors for lower 25-hydroxyvitamin D [25(OH)D] levels and vitamin D status. There is compelling evidence indicating that lower levels of vitamin D are associated with an increased risk and disease activity in MS. We have observed that Portuguese MS patients have lower levels of serum 25(OH)D compared with healthy individuals, even patients with recent disease onset. If we assume that vitamin D status is associated with MS risk the literature shows that the risk may start as early as the prenatal period. Month of birth has been described as a risk factor for Multiple Sclerosis susceptibility and disease phenotype in different studies. Individuals from the north of Portugal, born between January to June, are more prone to develop Multiple Sclerosis. In patients disease progression is independent of birth timing.

Calcitriol, the biologically active metabolite of vitamin D, initiates its signalling cascade by binding to the vitamin D receptor (VDR). Results of previous VDR gene association studies in MS are conflicting. We report an association between FokI ff genotype and MS susceptibility, but no associations with disease forms or progression were found.

Our results confirm that Multiple Sclerosis is a complex disease with high heterogeneity, resulting from interactions between genetic and environmental factors. This thesis contributes with several pieces of information to the large puzzle of MS etiopathogenesis, and suggests important roles for several genes and molecules in inflammation and immune deregulation.

Resumo

A Esclerose Múltipla (EM) é uma doença crónica em que fatores genéticos e ambientais contribuem para a susceptibilidade e prognóstico. A doença cursa com lesões inflamatórias do sistema nervoso central com desmielinização, diminuição da atividade neuronal e neurodegeneração.

O sistema imune desempenha um papel central na etiopatogenese da Esclerose Múltipla. Variantes genéticas na região HLA, nomeadamente o alelo HLA-DRB1*15, são os fatores de risco mais fortemente associados à EM. Neste trabalho confirmamos a associação do alelo HLA-DRB1*15 com a suscetibilidade à EM num grupo alargado de doentes Portugueses. Sendo que os genes da região HLA representam apenas cerca de 10-40% do risco genético para o desenvolvimento da EM, outros genes "imuno-relevantes" foram investigados. A influência dos genes KIR e dos seus ligandos HLA de classe I na suscetibilidade à MS foi investigada. Foi encontrada uma associação negativa entre o gene ativatório KIR2DS1 e a EM, associação essa independente da presença do alelo HLA-DRB1*15. A presença deste recetor de ativaçao parece conferir proteção contra o desenvolvimento da EM, possivelmente pela modulação de células T auto reativas por células natural killer.

A inflamação e o stresse oxidativo poderão estar subjacentes ao dano tecidular observado na Esclerose Múltipla. Sendo o Nrf2 um fator de transcrição central na resposta antioxidante, a associação de polimorfismos de nucleótido simples funcionais na região promotora deste gene (-653A/G e -617C/A) com a suscetibilidade à MS, formas de doença e progressão foi investigada. Verificou-se que os doentes com o genótipo -653GG, e consequentemente com baixa expressão de Nrf2, parecem ser mais suscetíveis a desenvolver um curso de doença do tipo surto-remissão.

Os microRNAs (miRNAs) são uma classe de pequenos RNAs não codificantes que regulam a expressão génica pós-transcricionalmente por ligação a mRNAs alvo. Um dos miRNAs mais bem caracterizados é o miR-155, que tem funções pleiotrópicas na inflamação, autoimunidade e plasticidade celular. Neste estudo demonstramos que os doentes com Esclerose Múltipla apresentam níveis circulantes elevados do miR-155. Esta observação, que está de acordo com outros estudos já publicados na literatura, implica uma desregulação do controlo da inflamação na EM.

A influência da migração e da latitude na prevalência da EM sugere um papel do ambiente na etiopatogenia da doença. Estudos recentes sugerem que a deficiência de vitamina D está difundida por toda a Europa, mesmo em países com abundância de luz solar, podendo atingir prevalências próximas de pandemia. Os resultados obtidos

na presente tese indicam que a deficiência de vitamina D é prevalente no Norte de Portugal, afetando quase metade da população. Foi ainda observado que, o índice de massa corporal e a estação do ano são preditores para níveis mais baixos de 25-hidroxivitamina D [25(OH)D] e *status* de vitamina D. Nesta tese observamos níveis séricos reduzidos de 25(OH)D, mesmo em doentes com um início recente de doença, o que suporta a evidência de que níveis baixos de vitamina D estão associados ao aumento da suscetibilidade e de atividade da doença. O mês de nascimento foi descrito como um fator de risco para a suscetibilidade à Esclerose Múltipla e o fenótipo da doença, em diferentes estudos. A Esclerose Múltipla parece ser mais frequente em indivíduos do norte de Portugal nascidas entre janeiro e junho, mas o mês de nascimento não parece influenciar a progressão da doença.

O calcitriol, o metabolito biologicamente ativo da vitamina D, inicia a sua cascata de sinalização ao ligar-se ao recetor da vitamina D (VDR). Reportamos nesta tese uma associação entre o genótipo ff do polimorfismo FokI e a suscetibilidade à EM, mas não com a forma ou progressão da doença.

O trabalho desenvolvido confirma que a Esclerose Múltipla é uma doença complexa e heterogénea, resultante de interações entre fatores genéticos e ambientais. Os resultados obtidos nesta tese acrescentam várias peças ao puzzle proposto para explicar a etiopatogenia da EM e sugerem um importante papel de alguns genes e moléculas na inflamação e desregulação imune.

CHAPTER 1

General Introduction

1.1 - Multiple Sclerosis

Multiple sclerosis (MS) is the most common inflammatory demyelinating disease of the Central Nervous System (CNS) and the leading cause of non-traumatic neurological disability in young adults in the United States and Europe (Browne et al. 2014). Strong evidences suggest that MS is an autoimmune disease directed against CNS myelin (insulating layer surrounding neurons in the brain and spinal cord) or oligodendrocytes (responsible for the formation of myelin) (Podbielska et al. 2013). Myelin helps electrical signals pass quickly and smoothly between the brain and the rest of the body. When the myelin is destroyed, nerve messages are sent more slowly and less efficiently, causing a variety of symptoms including muscular weakness, loss of coordination and speech as well as visual disturbances (Compston et al. 2008). Pathologically, it is characterized by perivascular infiltrates of mononuclear inflammatory cells, demyelination, axonal loss and gliosis mainly in the white matter, with the formation of multiple plaques in the brain and spinal cord (Popescu et al. 2013).

Diagnostic criteria for Multiple Sclerosis include clinical and paraclinical laboratory assessments emphasizing the need to demonstrate dissemination of lesions in space and time and to exclude alternative diagnoses (Polman et al. 2011).

1.1.1 - Epidemiology

The estimated number of people diagnosed with MS has increased from 2.1 million in 2008 to 2.3 million in 2013 (Browne et al. 2014). Europe is considered a high prevalence region for this pathology, enclosing more than half of the global population of people diagnosed with MS. It is also common in the United States, Canada, New Zealand, and southern Australia; conversely this pathology is rare in Asia, and in the tropics and subtropics (Kingwell et al. 2013). A decreasing north-to-south gradient in the distribution of MS prevalence rates is observed (Figure 1).

Portugal was considered to be a low-medium prevalence zone for MS (Kurtzke 1980), but following epidemiological studies suggest that it should be considered a medium prevalence zone (De Sa et al. 2006). In Portugal, data on MS prevalence points for a prevalence of 47 per 100,000 inhabitants in Santarem, Central Region of Portugal (De Sa et al. 2006; de Sa 2010). In 2015, a study from the District of Braga, north of Portugal, found a prevalence of 39.82/100,000 inhabitants (Figueiredo et al. 2015). Similar risks were described in Spain where surveys revealed rates ranging from 32 per 100,000

inhabitants in the province of Teruel (Modrego Pardo et al. 1997) to 65 in the Gijon health district (Uria et al. 1997).

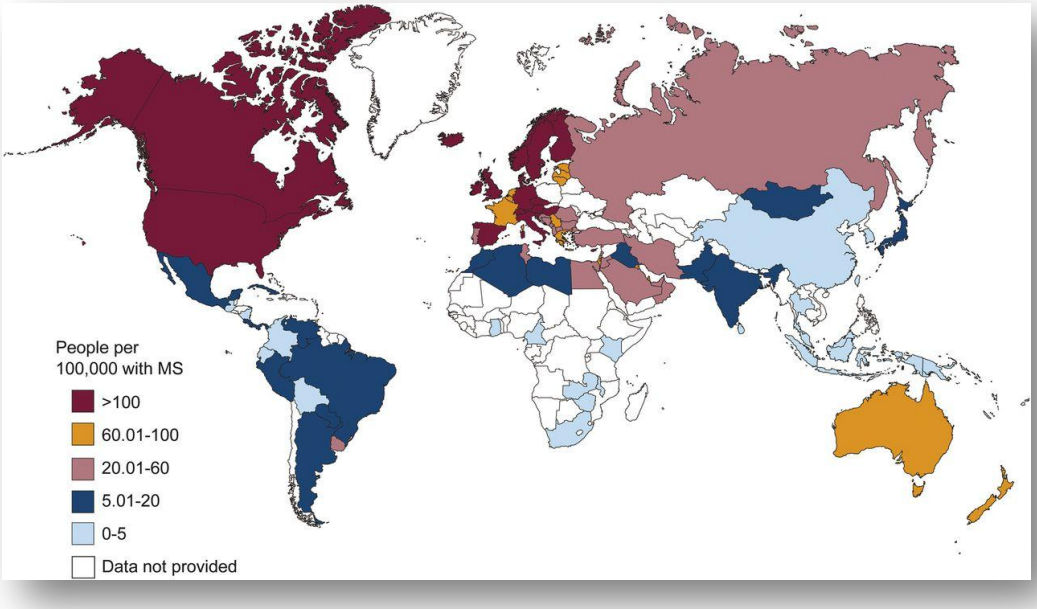

Figure 1. Global prevalence of Multiple Sclerosis in 2013 (www.atlasofms.org)

A recent review of MS incidence surveys conducted in the European Economic Area in the period of 1985 to 2009 revealed that, after 1985, MS incidence ranged from just over 1 to almost 7 per 100,000 inhabitants, was higher in females, tripled with latitude, and doubled with midpoint year of study period (Alcalde-Cabero et al. 2013). Multiple Sclerosis incidence, in the northern Lisbon, was similar to or moderately lower than that in other European populations (de Sa et al. 2014), in the period of 1998 to 2007, the rate per 100,000 inhabitants was 3.16. In the north of Portugal the annual incidence was 2.74/100,000 inhabitants in 2009 (Figueiredo et al. 2015).

The mean age of onset of patients diagnosed with MS is approximately 30 years. Usually symptoms appear between ages of 20 and 40 years (in ~70% of patients). Disease onset rarely occurs prior to 10 or after 60 years of age. However, patients as young as 3 and as old as 67 years of age have been described. As in other auto-immune diseases there is a clear gender difference, with females being more frequently affected than men (2:1) (Noseworthy et al. 2000).

1.1.2 - Clinical presentation

Clinically, MS patients show a variety of neurological signs and symptoms attributed to white matter lesions disseminated in time and space. It may occur in sudden attacks or be insidious/progressive (Lublin et al. 1996). Common presenting symptoms include paresthesia or numbness, motor weakness, monocular visual disturbances (optic neuritis), incoordination, diplopia, dizziness and vertigo. Other accompanying symptoms and signs may include fatigue, urinary urgency or retention, sexual dysfunction, depression, heat intolerance, pain, cognitive dysfunction, spasticity, ataxia and nystagmus (Compston et al. 2008).

Clinical and paraclinical evidences are essential to define the phenotype of MS because no reliable specific laboratory tests exist (Polman et al. 2011). A definite diagnosis of MS requires that two different areas of the CNS are affected by inflammation, in the form of lesion or plaque formation, and observation of neurological dysfunction expressed by two separate occurrences or relapses (Polman et al. 2011). In addition to this broad definition, some criteria are required for a definitive diagnosis, namely the exclusion of other pathologies that mimic MS (Miller et al. 2008). New diagnostic criteria for Multiple Sclerosis, integrating Magnetic Resonance Imaging (MRI) assessment, with clinical and other paraclinical methods, have been introduced in recent years (Polman et al. 2005; Polman et al. 2011; Milo et al. 2014).

In 1996, a consensus paper was published in which three clinical courses of disease were defined: Relapsing-Remitting MS (RRMS), Primary Progressive MS (PPMS) and Secondary Progressive MS (SPMS) (Lublin et al. 1996) (Figure 2). It is estimated that about 85% of the patients initially present a relapsing-remitting course characterized by acute relapses of new or recurrent neurological signs and symptoms, followed by complete or partial recovery, lasting from a few days to several months. These relapses are separated by variable periods of stable neurological condition without clinical disease activity. It is known, from the natural history of the disease, that approximately 60-80% of RRMS patients convert to a secondary-progressive course, usually after 10 years of disease onset - neurological disability accumulates progressively between relapses. With time, relapses become less frequent (Compston et al. 2006). About 10-15% of MS patients have a primary-progressive course, characterized by a steady accumulation of neurological disability (mainly progressive myelopathy) from disease onset (Compston et al. 2006).

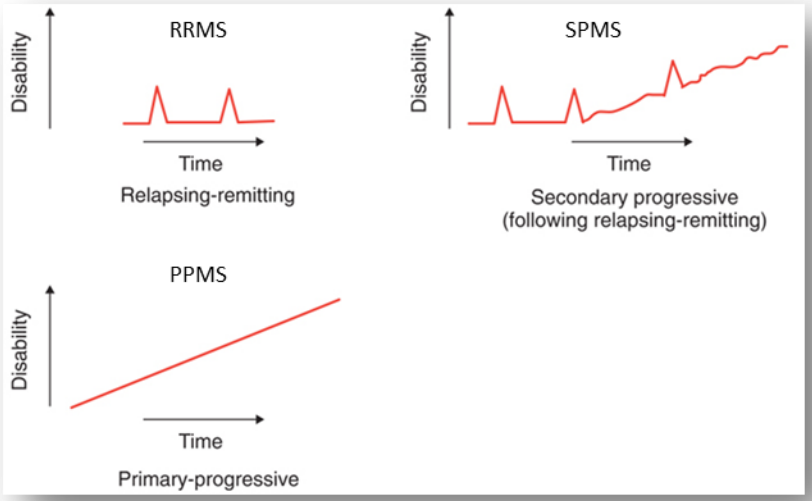

Figure 2. Clinical courses of Multiple Sclerosis. [Adapted from (Lublin et al. 1996)].

A re-examination of MS disease phenotypes was performed in the last years, based in the increased understanding of MS and its pathology (Lublin et al. 2014). This new characterization of MS phenotypes is based on disease activity (clinical relapse rate and MRI findings) and disease progression. Now, two major groups are considered: relapsing MS (RMS) and progressive MS (PMS), where the secondary progressive form was included. Both relapsing and progressive forms are categorized as showing or not showing evidence of inflammation, which is indicative of new active relapses. This is in contrast to chronic, long-term lesions. Patients with progressive forms of MS are also characterized as having or not having progression. The absence of inflammation and the absence of disease progression indicate a state called “stable disease” in which the patient’s condition does not worsen (Lublin et al. 2014).

Clinical relapses or progressive course may result in accumulating neurological impairment that is typically quantified in practice and clinical trials with the Kurtzke Extended Disability Status Scale (EDSS). The scale ranges from 0.0 (normal neurological exam) to 10.0 (death due to MS) (Figure 3) (Kurtzke 1983). Nevertheless, the EDSS, while an excellent attempt at quantifying MS disability, has its weak points. It is a subjective measurement and do not assess disease duration or the difference in rates of disease progression. To overcome these limitations an algorithm was developed to create a new severity score, the Multiple Sclerosis Severity Score (MSSS), which provides a more accurate estimate of future disability. This method compares the distribution of disability in individuals with comparable durations. The model was tested, for its stability over time and its ability to predict future disability with a single EDSS measurement, in a

collection of 9,892 patients from 11 countries, including Portugal (n=215) (Roxburgh et al. 2005).

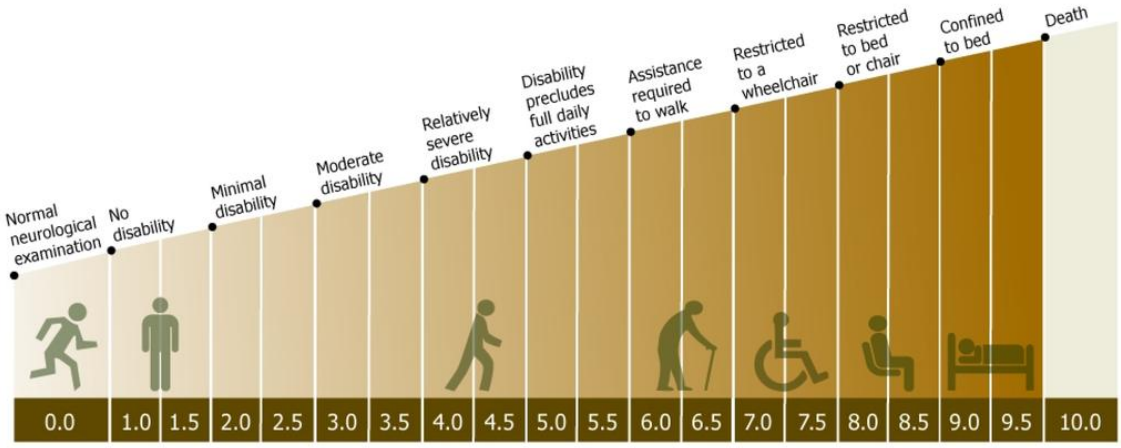

Figure 3. Expanded Disability Status Scale (Buzzard et al. 2012).

1.1.3 - Immunopathology

Immune deregulation in MS

The immune deregulation in MS is a consequence of abnormalities in the 'crosstalk' between the innate and adaptive immune systems (Dendrou et al. 2015).

During the establishment of central tolerance in the thymus, most autoreactive T cells are deleted; however, this process is imperfect, and some autoreactive T cells are released into the periphery. In health, peripheral tolerance mechanisms control these cells. If this tolerance fails — through the reduced function of regulatory T cells (Tregs) and/or the increased resistance of effector B cells and T cells to suppressive mechanisms — CNS-directed autoreactive B cells and T cells can be activated in the periphery and become aggressive effector cells (Sprent et al. 2001a).

In Multiple Sclerosis, CD4+ T cells can be activated in the periphery by molecular mimicry, novel autoantigen presentation, recognition of sequestered CNS antigen released into the periphery or bystander activation. Genetic and environmental factors contribute to these events (Sospedra et al. 2005). These pro-inflammatory CD4+ T cells cross the Blood Brain Barrier (BBB), penetrate the CNS and, in response to CNS antigens, are re-activated locally in contact with Antigen Presenting Cells (APCs), including dendritic cells (DCs). Antigen-presenting cells can promote the secretion of cytokines that creates a cytokine milieu that, in turn, influences naive CD4+ T cells to follow distinct pathways. In

the presence of interleukin-12 (IL-12), naive CD4+ T cells differentiate into interferon-gamma (IFN-γ) secreting Th1 helper cells. In the presence of IL-23, naive CD4+ T cells differentiate into IL-17-secreting Th17 cells. Under normal physiological conditions, Th1 cells mediate defences against intracellular pathogens, whereas Th17 cells are implicated, for example, in the defence against fungal infections. However, when these T helper cells are activated within the setting of an autoimmune disease, the production of pro-inflammatory effector cytokines is deleterious (Dendrou et al. 2015). This resulting inflammatory response leads to monocyte recruitment into the CNS, as well as naive CD4+ T cell activation through epitope spreading that further boost the inflammation. Pro-inflammatory cytokines induce macrophage and microglial activation which, in turn, produces other pro-inflammatory mediators and oxygen and nitric oxide radicals, ultimately leading to demyelination and axonal loss (Friese et al. 2014; Heneka et al. 2014).

Animal models have demonstrated that CD8+ T cells also play a role in MS pathophysiology (Salou et al. 2015; Sinha et al. 2015). These cells are the most abundant T cells in CNS lesions of MS patients (Booss et al. 1983), this indicates an important role for CD8+ T cells in the target organ. They produce pro-inflammatory mediators (lymphotoxin and IL-17) (Buckle et al. 2003) and their presence in the brain and Cerebrospinal Fluid (CSF) has been correlated with acute axonal damage (Bitsch et al. 2000). CD8+ T cells have been shown to be involved in several disease-driving mechanisms, ranging from cytotoxicity and demyelination to pro-inflammatory cytokine production; nevertheless several lines of evidence exist demonstrating the regulatory mechanisms performed by CD8+ T cells in the context of MS (Sinha et al. 2015).

It has been shown that regulatory T cells take part in the immunopathogenesis of the disease as well. Tregs are known to limit the inflammatory reactions using different mechanisms, including direct inhibition of autoreactive T cell activation, by secreting immunosuppressive mediators or cell-to-cell contact, or indirectly via inhibition of the stimulatory capacity of antigen-presenting cells (Schmidt et al. 2012). They are characterized by surface CD4 and CD25 expression and the transcription factor forkhead box P3 (Foxp3), which is essential for phenotypic and functional development of this cell lineage. They produce immunosuppressive cytokines, including IL-10, IL-35, and Transforming Growth Factor beta (TGF-β) (Buc 2013). IL-2 and TGF-β have been reported as crucial to induce the differentiation of naive CD4+ T cells into Tregs (Kleinewietfeld et al. 2013a). In mice with relapsing–remitting Experimental Autoimmune Encephalomyelitis (EAE), depletion of Tregs increases acute-phase severity, as well as prevents remission (Zhang et al. 2006). Although the number of Tregs is not decreased in

MS patients (Noori-Zadeh et al. 2016), their capability to suppress polyclonal or antigen-specific proliferation of T cells *in vitro* has been shown to be compromised (Viglietta et al. 2004; Haas et al. 2005; Venken et al. 2006).

Natural Killer (NK) cells are innate lymphocytes known for their cytolytic activity function, which controls tumor growth and microbial infection. The NK cells can produce pro-inflammatory cytokines IFN-γ and Tumor Necrosis Factor-alpha (TNF-α), as well as the immunosuppressive cytokine IL-10 and the growth factor Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) in response to IL-12, IL-15, or IL-18 (Vivier et al. 2011). Human NK cells can be broadly separated into two types on the basis of their expression of CD16 and CD56. $CD16^{+}CD56^{dim}$ cells express more intracellular perforin and are more efficient killers, whereas the $CD16^{dim/-}CD56^{bright}$ subset produce greater amounts and a wider variety of cytokines, and are naturally more regulatory (Vivier et al. 2008). The general consensus in the literature is that the $CD56^{bright}$ NK cells play a protective role in MS, controlling T cell responses and maintaining homeostasis (Gross et al. 2016). NK cells are normally restrained by inhibitory receptors that recognize target-cell-expressed Major Histocompatibility Complex (MHC) class I molecules; they recognize and interact with MHC class I antigens through Killer cell Immunoglobulin-like Receptors (KIR). While clear association with genes of the HLA region has been established in MS, there has been limited examination of the role of NK cells or their receptors, including KIR. However, because HLA class I molecules serve as the primary ligand for many KIR, it is likely that the association signals observed for many diseases may be related to KIR function. In MS, alleles of HLA-A, -B and -C that are known to serve as ligands for KIR have been implicated in disease. Given that KIR may also be expressed by CD4 T cells (van Bergen et al. 2004) it is conceivable that KIR diversity can influence specific antibody production and thus also explain some class II associations in MS. Importantly, both underlying immunoregulatory dysfunction and inflammatory processes have been proposed for MS (Hartung et al. 2014).

B lymphocytes have essential roles in the autoimmune pathogenesis of Multiple Sclerosis (Wekerle 2017). B cells, plasma cells and their products have a fundamental contribution in immune responses, and have a central role in the pathogenesis of the disease: plasmablasts and plasma cells can produce autoantibodies recognizing surface myelin antigens, which can be pathogenic and initiate an acute inflammatory cascade by complement activation (Hoffman et al. 2016). Antibodies can also induce tissue injury by a mechanism that involves the binding to Fc receptors on macrophages, neutrophils and NK cells, and attack their targets via an antibody-dependent cell-mediated cytotoxic process.

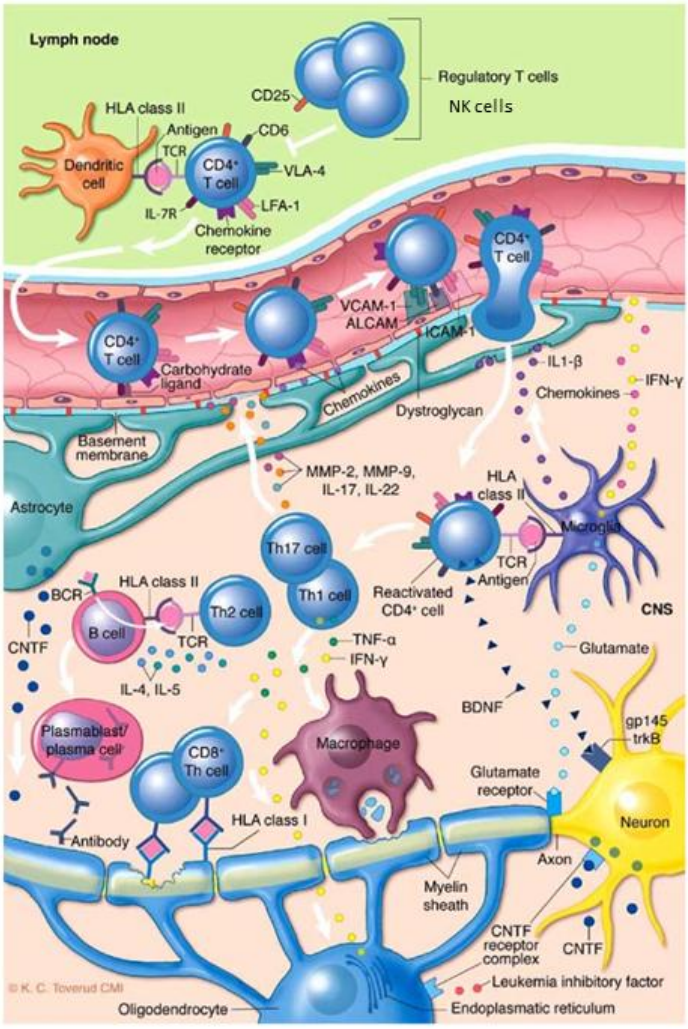

Figure 4. Schematic overview of immunopathogenesis of MS. Naive CD4+ T cells and B cells are activated in secondary lymphoid organs by unknown antigens. T cell activation depends on signalling through cytokine receptors including IL-7R and IL-2R/CD25, and is neutralized by different subsets of regulatory T cells and NK cells. Upon activation, T cells express chemokine receptors and adhesion molecules needed for attraction and adhesion to brain endothelia and subsequent penetration of the BBB. Within the brain, CD4+ T cells are reactivated upon encounter with their cognate antigen, or an antigen with sufficient homology to be cross-recognized in the context of appropriate HLA class II molecules. Depending on their phenotype, the reactivated CD4+ T cells initiate a variety of effector functions, including activation of CD8+ cytotoxic T cells, macrophages, microglia and B cells, leading to production of IL, antibodies, chemokines, reactive oxygen species and glutamate which damage neurons and oligodendrocytes, breach the BBB and attract additional leukocytes. [Adapted from (Holmoy 2007)].

Autoreactive B cells can function as effective and specific APC and activate their cognate autoreactive T cells through the trimolecular complex and costimulatory molecules. Such B cell–T cell interactions result in simultaneous expansion of antigen-specific B and T cells that enhance the immune response and promote disease (Hampe 2012). B cells from MS patients show exaggerated pro-inflammatory response to activating stimuli and may contribute to abnormal T cell activation and autoimmunity through “bystander activation”, by secreting pro-inflammatory cytokines. Regulatory B cells secreting IL-10, which normally maintain homeostasis and protect from autoimmunity, are deficient in MS, and thus contribute to unchecked autoimmunity (Milo 2016). Finally, lymphogenesis supported by B cell cytokines and chemokines in the brain may promote on-going local immune injury (Dalakas 2008; Barun et al. 2012). The possible contribution of B cells to MS pathogenesis is also supported by the beneficial effect of B cell targeted therapies in MS (Alexopoulos et al. 2016; Gasperi et al. 2016).

Inflammation and Neurodegeneration

Although MS was traditionally considered to be an inflammatory demyelinating disease of the CNS, which leaves the axons largely intact at least at onset of the disease, recent studies have shown that neurodegenerative processes also play an important role early in the pathogenesis of MS (Ellwardt et al. 2014). Inflammation and focal demyelination, with break-down of the BBB, are prominent features in relapsing MS and consequently relapses are considered to be its clinical manifestation. In progressive MS focal disruption of the BBB is less common and widespread degeneration of the white and grey matter, with resultant atrophy, are pathological hallmarks (Lassmann et al. 2012). MS may be seen as a spectrum with an intense focal inflammatory component in Relapsing MS and more neurodegenerative features with concomitant chronic inflammation and axon loss in Progressive MS (Lassmann 2007) (Figure 5).

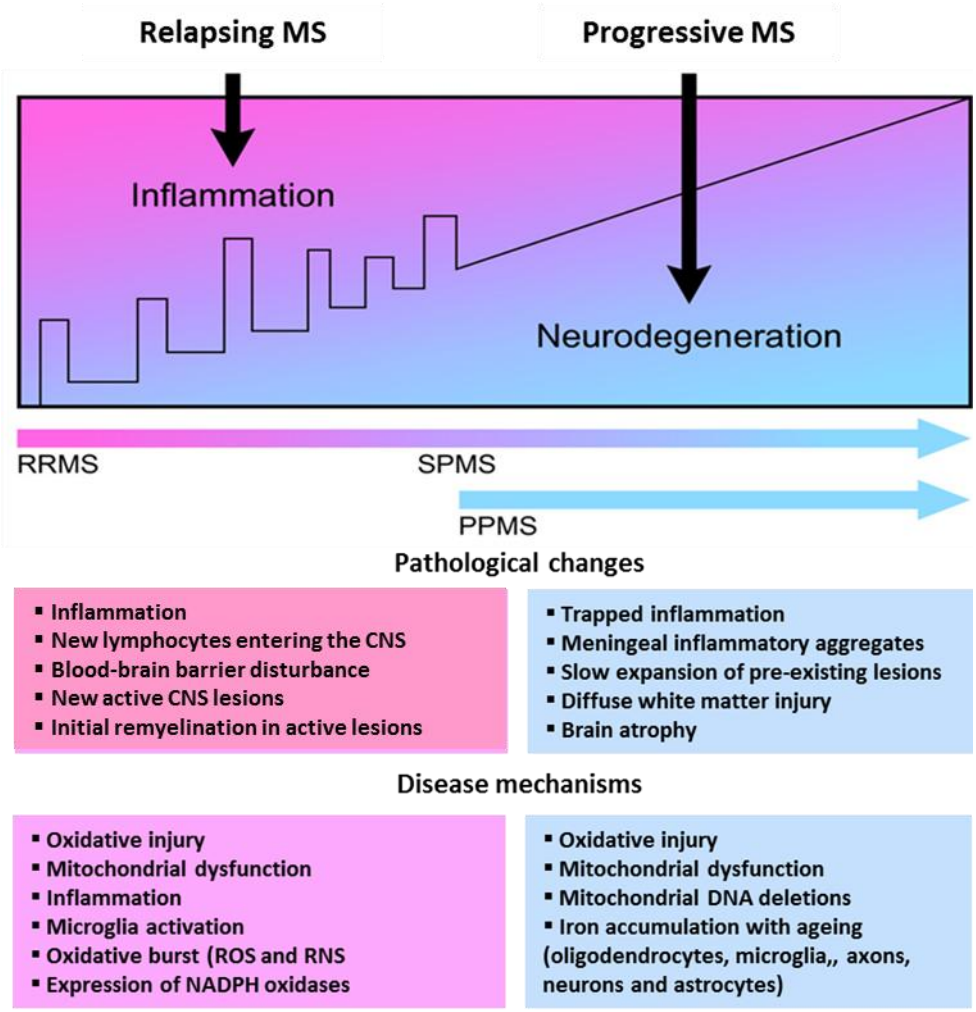

Figure 5. Pathological changes and disease mechanisms in Relapsing MS and Progressive MS [Adapted from (Lassmann et al. 2012)]

Neuroinflammation is mediated primarily by the resident macrophages of the nervous system, the microglia. Microglia have been viewed as a “double-edged sword” (Suzumura 2013) or as “friend or foe” (Block et al. 2007). When activated, microglia secretes cytotoxic reactive oxygen (ROS) and nitrogen (RNS) species, contributing to the deleterious effects on neurons, especially in cases of microglial over activation and deregulation (Du et al. 2016). Furthermore, components of dead or damaged neurons also activate microglia (reactive microgliosis) via pattern recognition receptors (e.g. toll-like receptors), resulting in a perpetuating cycle of neuronal cell death (Block et al. 2007). The eventual fate of microglial cells is tightly controlled by their gene expression profile and by two ROS-regulated transcription factors, namely nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and Nuclear factor [erythroid-derived 2]-like 2 (NRF2).

Nuclear factor-kB is a transcriptional factor that regulates a battery of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development (Caamano et al. 2002). This molecule is the master regulator of the inflammatory responses to brain infections and environmental and cellular damage. It is the redox state of microglia that controls regulatory kinases up stream of NF-kB, as well as NF-kB nuclear levels (Rojo et al. 2014). It is expressed in neurons and microglia and exhibits a dual role in neurodegenerative diseases (Mattson 2005). Thus, activation of NF-kB in neurons promotes their survival; whereas activation in microglia may lead to pathological neuroinflammation. Microglia functions normally when basal levels of ROS are managed by antioxidant machinery; however, persistent ROS or RNS continuously activates the NF-kB signalling pathway, amplifying the pro-inflammatory function of microglia and thus contributing to microglial over activation and neurotoxic consequences (Figure 6).

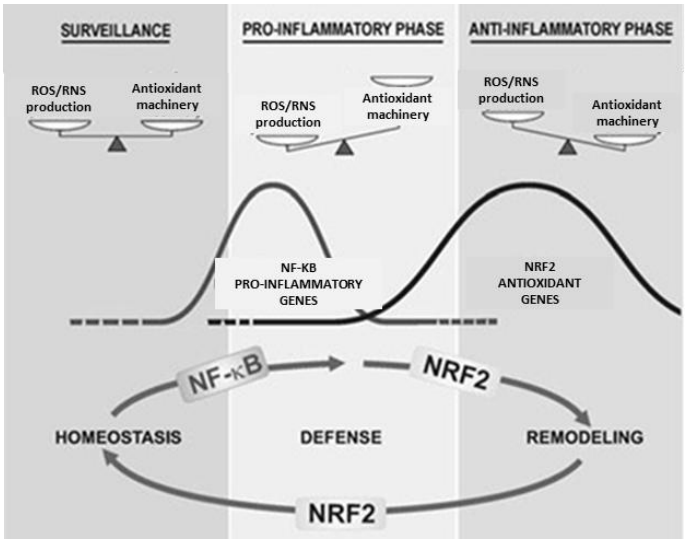

Figure 6. Inflammation is tightly controlled by NF-kB and NRF2 [Adapted from (Rojo et al. 2014)].

Redox homeostasis is tightly controlled by the transcription factor NRF2, which is a crucial player in the antioxidant protection of microglial cells, thus avoiding pernicious effects of oxidative stress. NRF2 is a member of the Cap'n'Collar family of basic region-leucine zipper transcription factors and is the master regulator of the antioxidant response. NRF2 binds to the Antioxidant Response Element (ARE), a cis-acting enhancer sequence in the promoter region of Nrf2-regulated genes, and modulates transcription of these target genes (Nguyen et al. 2003). This cis-acting element mediates a transcriptional response to reactive chemical stress inducing the production of many cytoprotective enzymes (ex. Heme oxygenase-1 and superoxide dismutase). Presently, there is available a disease modifying treatment for MS, dimethyl fumarate, that is known to activate the NRF2 transcriptional pathway, which reduces oxidative cell stress, and also to modulate NF-kB, which have anti-inflammatory effects (Albrecht et al. 2012; Fox et al. 2014).

Neuronal and axonal degeneration in MS is a slow process initiated by acute lymphocytic inflammation, and subsequently driven by chronicall, diffuse parenchymal myeloid and meningeal lymphocytic inflammation. Inflammation in the CNS leads to innumerable molecular changes. Immune cells secrete neurotoxic products - including ROS, glutamate, cytokines and chemokines - that direct the evolution of immune responses and also alter cellular metabolism in neurons and their axons. Oxidative stress, mitochondrial injury (damage and dysfunction) and subsequent ion channel dysfunction, secondary to chronic inflammation, have a constant impact on neurons and axons, leading to their death during progressive MS (Mahad et al. 2015). Also, several ion channels show compensatory changes in response to the inflammatory stimulus by altering their relative distribution in the neuron—a process that eventually becomes maladaptive and perpetuates neuroaxonal injury. Eventually, most of the above-described neurodegenerative pathways, combined with a lack of neuroprotective support, result in a common final pathway of neuronal and axonal demise, most probably mediated by the initiation of apoptosis and Wallerian degeneration - an active process that is similar to apoptosis. The balance between these continuous stressors and intrinsic defending mechanisms can depend partly on age, sex and genetic factors, which eventually determine the clinical course of the disease (Lassmann et al. 2011).

1.1.4 - Treatment

There is no curing therapy for MS. The goal of treatment in patients with relapsing-remitting MS is to reduce the frequency and severity of relapses (and thereby prevent exacerbations) and to prevent or postpone the onset of the progressive phase of the disease.

Based on the evolving understanding of disease mechanisms in MS there are currently more and more effective treatments, although with potential long term risks. This knowledge has led to effective anti-inflammatory and immunomodulatory treatments that reduce the severity and frequency of new demyelinating episodes (Hohlfeld et al. 2004). The introduction of immunomodulatory drugs in the 1990s transformed the treatment of relapse remitting MS. Presently approved approaches derive from a general shaping of the immune system towards anti-inflammatory immune responses. This is accomplished by non-cell-selective immune modulation (e.g., interferons, glatiramer acetate, teriflunomide and dimethyl fumarate), a selectively depletion of immune cells (T and/or B cells) considered important for initiating or perpetuating MS [e.g., alemtuzumab (anti-CD52), ocrelizumab (anti-CD20)], or a selective inhibition of distinct molecular pathways in order to sequester leucocytes (e.g., natalizumab, fingolimod (Meuth et al. 2012; Wingerchuk et al. 2014).

Progressive Multiple Sclerosis is the greatest therapeutic challenge facing the Multiple Sclerosis community today, but over the last few years ocrelizumab, a humanized monoclonal antibody that selectively depletes CD20-expressing B cells was studied in primary progressive patients and was associated with lower rates of clinical and MRI progression than placebo (Montalban et al. 2017). Also, among patients with relapsing Multiple Sclerosis, ocrelizumab was associated with lower rates of disease activity and progression than interferon beta-1a over a period of 96 weeks (Hauser et al. 2017). These observations led to the US Food and Drug Administration approval of ocrelizumab for RRMS and PPMS. Also, data from a Phase III study indicated that siponimod, a selective sphingosine-1-phosphate receptor, delayed disability progression in patients with secondary progressive form of MS (Correale et al. 2016).

1.2 - The etiology of Multiple Sclerosis

1.2.1 – Genetic and Epigenetic factors

Genetic contribution to the development of the disease has been demonstrated in numerous twin, sibling and adoption studies comparing the recurrence rates in relatives of MS patients with the disease prevalence in the general population. The prevalence of this disease among first-degree relatives of affected individuals is 20 to 40 times higher than in the overall population (Sadovnick 1993; Mumford et al. 1994; Sadovnick et al. 1995). The recurrence risk is dependent of the degree of relationship, being the highest values for identical twins (~25-34%), followed by full siblings (20-30%) and half siblings (10-16%) (Sadovnick et al. 1999; O'Gorman et al. 2013). However, a recent study from Switzerland points to a lower risk ratios of familial recurrence in MS than previous studies, with a lower prevalence among dizygotic twins (17%) (Westerlind et al. 2014). Adoptees have a comparable risk to the general population indicating that living with an affected individual has little or no effect on one‘s susceptibility in the absence of biological relatedness.

1.2.1.1 - The HLA region

Genetic studies pointed the MHC region, on chromosome 6p21.3, as the only region consistently associated with MS (2001; 2003). The MHC region, denominated Human Leucocyte Antigen (HLA) in humans, compasses approximately 4 Mb of DNA and it is the most polymorphic region of the human genome, comprising approximately 300 loci and over 160 protein-coding genes. The region is divided into three regions based on the functional characteristics of genes within each class, named class I, class III and class II (Figure 7).

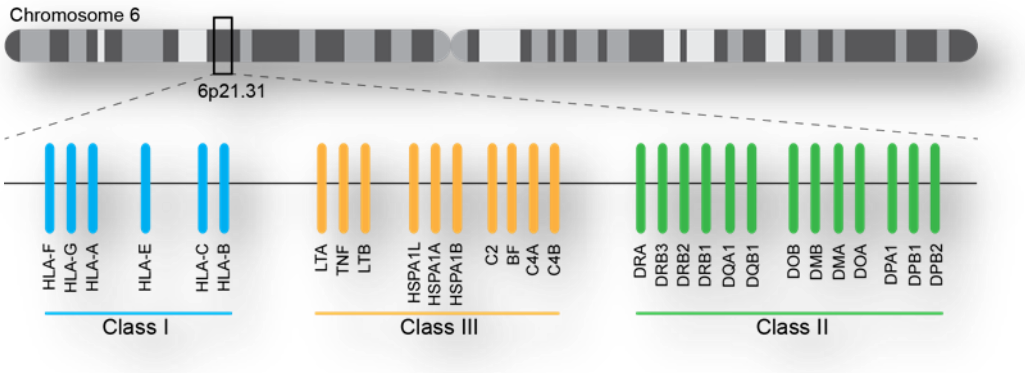

Figure 7. Simplified map of the HLA region.

The class I and class II genes encode molecules that bind and present peptide fragments to T lymphocytes via the antigen binding groove of the mature HLA cell surface protein (Trowsdale et al. 2013). Class I molecules are found on most cell types and present endogenous peptides derived from the intracellular environment of cancer cells, infected cells (viruses), or cells that are damaged in other ways. The bound peptide in the class I molecules is presented to CD8+ T cells, which kill the infected/damaged cells. The class I molecules act also as ligands for the KIR molecules, which are important receptors balancing the activation of NK cells (Flodstrom-Tullberg et al. 2009) and some subset of T cells (van Bergen et al. 2004). Class II molecules are mainly found on APCs and present the antigen to CD4+ T helper cells. These molecules are specialized in presenting peptides derived from outside the host cells to the T Cell Receptor (TCR) on the CD4+ T cells, such as bacterial fragments and other extracellular antigens. The class III region contains genes for cytokines and the components of the complement system that also play important roles in the immune response.

Both HLA class I and class II genes are fundamental to the body's recognition of self and non-self. In the thymus, a developing thymocyte first encounters a highly heterogeneous array of endogenous (self) peptide-MHC (pMHC) complexes on thymic APCs. Combinations of self-peptides and MHC molecules instruct thymocytes to either survive or perish, based on the affinity/avidity of their TCRs to the pMHC. Through processes referred to as thymic positive and negative selection, thymocytes that bind self pMHCs with either intermediate-to-low or high avidity are instructed to survive or perish, respectively (Palmer 2003). *Negative selection* deletes potentially self-reactive thymocytes, thereby generating a repertoire of peripheral T cells that is largely self-tolerant (Sprent et al. 2001b; Starr et al. 2003).

The first HLA-association with MS was reported for the HLA class I alleles; HLA-A3 (Naito et al. 1972) and HLA-B7 (Jersild et al. 1972). Later, it was shown that the HLA class I associations were mainly secondary to an association in the HLA class II region due to strong linkage disequilibrium (LD) in the region (Compston et al. 1976; de Moerloose et al. 1979; Olerup et al. 1991). Within this region, a strong association with the specific HLA class II DR2 haplotype (HLA-DRB1*1501-DQB1*0602) was repeatedly observed (Barcellos et al. 2002). Associations between HLA-DRB1*1501 allele and MS have been observed across virtually all populations that have been studied including the Portuguese (Oksenberg et al. 2005; Silva et al. 2007a). The exact mechanisms whereby HLA-DRB1 influences MS susceptibility remains undetermined, but is almost certainly related to the physiological function of HLA molecules in various immunological processes, such as antigen binding and presentation, and T cell repertoire determination. Allelic

heterogeneity, as well as copy number and cis–trans regulatory effects have been reported for HLA-DRB1 (Barcellos et al. 2003; Dyment et al. 2005). Such findings also suggest an allelic gradient of disease association related to the presence of this gene, ranging from a phenotype of high vulnerability (HLA-DRB1*15 homozygotes and HLA-DRB1*15–HLA-DRB1*08 heterozygotes) to a moderate susceptibility (HLA-DRB1*03 homozygotes and heterozygotes); a resistance state (HLA-DRB1*15–HLA-DRB1*14 heterozygotes) is also reported, and it is hypothesized that HLA-DRB1*14 alleles would abrogate the susceptibility effect of HLA-DRB1*15 (Barcellos et al. 2006). It has also been reported that genes located in the MHC, outside the class II region, are independently associated with MS. Emerging data suggest protective influences of HLA class I alleles in various studies (Brynedal et al. 2007; Yeo et al. 2007; Friese et al. 2008; Silva et al. 2009).

1.2.1.2 – Other susceptibility genes

The HLA genotype is estimated to account for between 10 and 40% of the genetic risk (Hillert 2006). Therefore, although this region plays a significant role in MS susceptibility, much of the remaining 60-90% of the genetic effect in MS remains to be explained.

Attributable risk to MS of other alleles is very small, the populations studied worldwide are quite heterogeneous and, in addition, the lack of statistical power are the reasons why, until very recently, no other risk genes besides HLA gene have been detected. To overcome the problem of statistical power, the International MS Genetics Consortium (IMSGC) performed a Genome-Wide Association Study (GWAS) using 500,000 Single Nucleotide Polymorphisms (SNPs) arrays in 931 families. Subsequently they performed a combined analysis of more than 12,000 samples. Genome wide association studies examine single nucleotide variation across genomes to identify genetic factors that are associated with a quantifiable trait. Even though it is a recent experimental approach, GWAS have identified hundreds of polymorphisms that are associated with disease and other traits and, hence, have provided new knowledge and important biological insights. Most of these associated variants conferred small increments risk (1.2- fold–1.5- fold).
The results of the GWAS, performed by IMSGC, revealed the existence of 16 non-MHC susceptibility SNPs in 13 gene *loci*, all of them with modest effect. The SNP rs3118470, in the IL2RA gene, achieved genome-wide significant association ($p=2.96\times10^{-8}$) (Hafler et al. 2007). For rs6897932 of IL7R gene, functional support was obtained (Gregory et al. 2007; Lundmark et al. 2007). Follow-up studies and further genome-wide analyses have now provided genome-wide significant support for several non-HLA MS risk genes

(Oksenberg et al. 2010), amongst which are IL7R, IL2R, CLEC16A (Rubio et al. 2008; 2009a; Hoppenbrouwers et al. 2009), CD58 (Hoppenbrouwers et al. 2009), CD226 (2009a), KIF1B (Aulchenko et al. 2008), KIF21B (2010; Goris et al. 2010), CD6, IRF8 and TNFRSF1A (De Jager et al. 2009), and two *loci* on chromosomes 12 and 20, which have been suspected to relate to the genes CD40 and CYP27B1 (2009b; Sundqvist et al. 2010), TYK2 and STAT3 (Jakkula et al. 2010). The overrepresentation of genes that influence T cell maturation provides independent and compelling evidence that the critical disease mechanisms primarily involve immune deregulation (Sawcer et al. 2011). Some MS risk genes and respective functions are presented in Table 1.

Table 1. Different MS risk genes and corresponding functions.

Gene	Chromosome	Function
KIF21B	1	• Is a member of the kinesin superfamily but its function is still unknown
K1F1B	1	• Encodes a kinesin superfamily member believed to be responsible for axonal transport of mitochondria and synaptic vesicle precursors
CD58	1	• Influences T-cell proliferation and differentiation
IL7R	5	• Homeostasis of the memory T-cell pool
IL2R	10	• Regulation of T-cells
CD6	11	• Is a pattern recognition receptor and influence circulating levels of TNFα
CYP27B1	12	• Hydroxylates 25-hydroxyvitamin D into the bioactive form
TNFRSF1A	12	• Influences the TNFα pathway
CLEC16A	16	• Provides signals for decisions between tolerance and immunity
IRF8	16	• Interferon regulatory factor 8, is associated with higher mRNA expression of interferon response pathway genes in subjects with MS
STAT3	17	• Involved in multiple pathways and functions, including the Jak-STAT pathway, neuron axonal guidance, apoptosis, activation of immune responses and Th17 cell proliferation
CD226	18	• Adhesion and co-stimulation T-cells
CD40	20	• Regulator of humoral and cellular immunity

The overlap in the genetic architecture underlying susceptibility to autoimmune diseases prompted the collaborative construction of the “ImmunoChip”, an efficient genotyping platform designed to deeply investigate 184 non-MHC *loci*, with genome-wide significant associations to at least one autoimmune disease, and provide lighter coverage of other genomic regions with suggestive evidence of association. In 2013, using the “ImmunoChip” custom genotyping array, 14,498 subjects with Multiple Sclerosis and 24,091 healthy controls were analysed for 161,311 autosomal variants and 135 potentially associated regions were identified (Beecham et al. 2013). In a replication phase, these observations were combined with previous GWAS data from an independent 14,802

subjects with Multiple Sclerosis and 26,703 healthy controls. In these 80,094 individuals of European ancestry, 48 new susceptibility variants were identified, 3 of which were found after conditioning on previously identified variants. Thus, 110 established Multiple Sclerosis risk variants at 103 discrete *loci* outside of the major histocompatibility complex (Beecham et al. 2013) are now described. The authors estimate that these 110 non-MHC established risk variants explain 20% of the sibling recurrence risk; this value increases to 28% if we include the already identified MHC effects (Sawcer et al. 2011).

1.2.1.3 - Genetic studies in the Portuguese population

Genetic Analysis of Multiple sclerosis in EuropeanS (GAMES)

In order to take advantage of common heritage, groups researching Multiple Sclerosis in various native and migrant European populations, met in April 2000 and established a collaborative network designated as "GAMES", the Genetic Analysis of Multiple Sclerosis in EuropeanS, to identify MS susceptibility genes. Two groups of Portuguese MS patients were included in this whole genome association study that happened in 2003, a ground-breaking event in the study of MS genetics (Martins Silva et al. 2003; Santos et al. 2003). Our group contributed with 200 unrelated MS Portuguese patients. An equal control population, also from the north of Portugal was employed. A total of 3,974 markers were successfully typed, from which a list of 46 markers showing strong evidence of association was singled out. When compared to a physical map, three regions were noted, where two or more of these markers could be found less than 1.5 Mb apart: 6p21.3 (the MHC region), 6q14.1 and 7q34 (Martins Silva et al. 2003). The 7q34 region has also been implicated in MS susceptibility in a linkage study from Sweden, from which it was concluded that 7q34-36 may harbour genes with importance for MS (Xu et al. 2001).

Human Leukocyte Antigen Studies

As in other complex diseases with autoimmune features, a genetic association with the HLA complex is well documented in MS (Sawcer 2008). Initial associations with MS were observed in the HLA Class I region (HLA-A3 and HLA-B7 alleles) and later with polymorphisms in the HLA class II region, namely the HLA-DRB1*15 allele (Schmidt et al. 2007).

After confirming that HLA class II region (HLA-DRB1*15) was implicated in the susceptibility to MS in Portuguese patients, we investigated the role of other HLA-DRB1 alleles, as well as their association with the clinical course of the disease. HLA-DRB1 alleles were analysed in 248 patients and 282 healthy controls. In order to relate HLA-DRB1 alleles to disease aggressiveness, patients with RRMS and SPMS were subdivided into 3 groups: 'benign' MS patients who maintain an Extended Disability Status Scale (EDSS) score of ≤ 3 at least 10 years after disease onset; non benign MS patients with EDSS>3 after the same period and 'aggressive' MS those with EDSS≥6 within 15 years of disease onset. As expected, a higher frequency of HLA-DRB1*15 allele was found in MS patients [29.8% vs. 19.9%, p=0.008, OR=1.72 (1.15-2.56)]. The HLA-DRB1*03 allele was also positively associated with MS [22.6% vs. 15.6%, p=0.040, OR=1.58(1.02-2.45)].Concerning disease aggressiveness, HLA-DRB1*15 occurred more frequently both in the group with benign disease [42.6% vs. 19.9%, OR=2.99(1.56-5.72)] and in the group with non-benign disease [34.1% vs. 19.9%, OR=2.09(1.05-4.16)] compared with controls. When time to reach an EDSS=3 or EDSS=6 was considered as end point, HLA-DRB1*15 negative patients were found to have a worse prognosis. So we concluded that, in this group of Portuguese MS patients, the HLA-DRB1*15 allele is an established genetic marker for susceptibility to MS and is also associated with a better outcome (Silva et al. 2007a). This observation is in agreement with studies carried out in other populations (Madigand et al. 1982; de la Concha et al. 1997; Weatherby et al. 2001).

In 2009, the influence of HLA-A*02 and HLA-A*03 class I alleles, also described as associated with MS in several European populations (Fogdell-Hahn et al. 2000; Brynedal et al. 2007), was examined in 342 patients using a logistic regression model. HLA-DRB1*15 allele increased the risk of developing MS, HLA-A*02 decreased the risk and HLA-A*03 had no effect. The lack of association with this allele is not surprising, as the reported association, in higher latitudes, have been attributed to LD with HLA-DRB1*15. To analyse if the HLA-A*02 association was independent of HLA-DRB1*15, an interaction between these two alleles was introduced in the model; no significant results were found. This study, as other reports, supports the idea that genes located in the HLA complex, outside class II, may contribute to genetic susceptibility to MS independently of the HLA-DRB1 *locus* (Silva et al. 2009).

Hemochromatosis Gene

The hemochromatosis gene (HFE), responsible for iron accumulation in patients with hereditary hemochromatosis, is also located in the MHC region. Several observations suggest that iron may have an active role in the pathogenesis of MS (LeVine 1997; Minagar et al. 2004) and there is also some evidence that the hemochochromatosis-associated allele C282Y is implicated in MS susceptibility and disease course (Rubio et al. 2004; Ristic et al. 2005; Kotze et al. 2006). Recently, Zamboni and colleagues described the existence of chronic cerebrospinal venous insufficiency in patients with MS (Zamboni et al. 2009) and explored the possibility that chronic insufficiency of venous drainage leads to increased iron stores in the affected tissue (Bergan et al. 2006; Zamboni 2006).

We analysed whether HFE gene variants contribute to MS susceptibility and/or severity in Portuguese patients with MS. The C282Y and H63D HFE variants frequencies were determined in 373 patients. Despite the suggestion that iron deposition could influence MS pathogenesis, no significant association was found between HFE polymorphisms and disease susceptibility, in accordance with previously published reports (Ristic et al. 2005; Kotze et al. 2006; Ramagopalan et al. 2008). An analysis of the association of genotypes with disease severity was carried out, and the C282Y allele was found to be more frequent in the aggressive group. Kaplan–Meier survival analysis of the distribution of time from onset of MS to reach mild disability (EDSS=3) and severe disability (EDSS=6) was also performed, and suggests that mutation carriers reach an EDSS of 6 five years sooner that non-carriers. Therefore, the HFE C282Y polymorphism may be implicated in MS aggressiveness, and could be a marker of worse prognosis (Bettencourt et al. 2011).

Tumor Necrosis Factor-α

TNF-α is a pro-inflammatory cytokine that has been implicated in the pathogenesis of CNS damage, and it is found in high levels both in the serum of patients and also in MS lesions (Selmaj et al. 1991). Given that the TNF-α gene is located between disease associated HLA class I and class II *loci*, it is possible that polymorphisms within this gene could be in linkage disequilibrium (LD) with haplotypes associated with MS. Therefore, several groups have investigated the relevance of the TNF -308 and -238 gene polymorphisms in MS, but with conflicting results (de Jong et al. 2002; Mihailova et al. 2005; Kamali-Sarvestani et al. 2007).

We have typed 195 MS patients and 222 controls for the -308G>A and -238G>A polymorphisms. Also, a linkage disequilibrium analysis was performed between HLA-DRB1*15 and the TNF-308G>A and -238G>A alleles.

The TNF-238 G/A genotype and the TNFA-238A allele was underrepresented in the MS group (5.2% vs. 11.3%, p=0.027, OR=0.43; 2.6% vs. 5.6%, p=0.031, OR=0.45, respectively). Stratification according to HLA-DRB1*15 did not confirm that this effect is independent of the HLA-DRB1*15 allele (OR=0.46, p=0.06). However, our analysis revealed that this allele is not in LD with HLA-DRB1 susceptibility alleles. We observed a weak association of TNF-238G>A polymorphism with our MS population, that may be, in part, explained by LD with HLA. Further studies are warranted to clarify this issue. The TNF-308A allele was not associated with the disease in this group of patients (Pereira et al. 2005) as in other reported studies (Kamali-Sarvestani et al. 2007).

Cytotoxic T Lymphocyte Antigen 4

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is an important molecule involved in the down-regulation of T-cell activation. It recognizes the co-stimulatory molecules CD80 and CD86 and promotes cell anergy upon ligation, shutting off T cell responses. This molecule has been suggested to have a considerable impact on the biology of T-cells in MS (Oliveira et al. 2003; Kosmaczewska et al. 2007). In fact, CTLA-4 is critical for the induction of peripheral tolerance and for the deletion of self-reactive T cells.

Portuguese and Italian patients and families were used, as an independent cohort, for a replication study on a CTLA-4 polymorphism (-651A/C) (Alizadeh et al. 2003). Using a transmission disequilibrium test, a family-based study in a French cohort had strongly suggested that this SNP, region could be linked to and associated with MS, particularly but not only in patients carrying the HLA-DRB1*15 allele. The replication study confirmed these findings.

Protein Tyrosine Phosphatase Non-receptor 22

The PTPN22 1858T variant (620W allele of the Lyp protein phosphatase), is known to modulate T lymphocyte effector function, by diminishing the threshold for T-cell activation. This variant has been found to be over represented in patients with autoimmune diseases (Lee et al. 2007), and it is considered to be the most consensual non-HLA genetic factor involved in the development of tan “autoimmune phenotype” in

these patients. Previously, the 1858T allele was not found to be associated with MS (Hinks et al. 2005; Matesanz et al. 2005; Harbo et al. 2006).

To evaluate the role of the PTPN22 1858T variant in the susceptibility to MS in a Portuguese population and to study this variant in subgroups of patients characterized according to disease aggressiveness, 285 patients with MS and 279 ethnically-matched controls were studied.

The 1858T variant frequency in the whole MS population did not show differences from controls (6.5% vs. 6.9%, p=0.792), which confirms the results described for other populations. Interestingly, this autoimmunity risk allele was present in 10.0% of benign MS patients (OR=1.95, p=0.05), approaching the frequencies described in other autoimmune diseases in which the 1858T allele is a predisposing factor. This variant may be implicated in the genetic susceptibility to benign forms of MS (Bettencourt et al. 2008), since its frequency was higher than in the overall MS group and similar to those described in other autoimmune diseases (Criswell et al. 2005).

Apolipoprotein E

The APOE gene is located on chromosome 19q13, a region suspected to be linked to MS (Haines et al. 2002). In the CNS, ApoE is synthesized and secreted by glial cells, particularly astrocytes; it serves as a ligand mediating the uptake of plasma lipoproteins, which are vital for membrane repair, and may have neurotrophic, anti-oxidant and immunomodulatory effects as well (Beffert et al. 1998). The immune related properties of ApoE include modulation of inflammation and oxidation, suppression of T cell proliferation, regulation of macrophage functions, and facilitation of lipid antigen presentation by CD1 molecules to natural killer T cells (Zhang et al. 2010). The APOE gene is polymorphic with three common alleles, ε2, ε3 and ε4, producing three isoforms of the protein, designated E2, E3 and E4. ApoE genotype may influence clinical progression of MS, although there is conflicting evidence regarding the role of ApoE polymorphisms in disease outcome (Kantarci et al. 2004; Pinholt et al. 2005; Ramagopalan et al. 2007; van der Walt et al. 2009).

A total of 278 Portuguese patients with MS were compared with a previously studied cohort used as control population (Rodrigues et al. 2005). ApoE ε2 and ε4 alleles frequencies were similar in patients and controls (Silva et al. 2007b), which is in accordance with previous reports (Santos et al. 2004; Burwick et al. 2006). A recent study by Sena and collaborators (Sena et al. 2009) suggests that ApoE ε4 interacts with cigarette smoking, modulating the progression of MS.

1.2.1.4 - Epigenetic factors

Cumulatively, the genetic *loci* discovered by genome-wide association studies can explain about 28% of the perceived heritability of MS (Lin et al. 2013). The exact component of MS risk that is heritable is difficult to determine because of the impact of environmental factors on the risk of MS, though estimates based on twin studies and MS families suggest that the risk may be between 15% and 40% (Compston et al. 2008). Therefore, our current level of genetic knowledge can explain only 5%–8% of the overall risk of MS, depending on ethnicity and environment.

Recently, research has suggested that epigenetic mechanisms may contribute to the pathophysiology of MS and explain a portion of the 'missing heritability' (Lill 2014). Epigenetics represents all heritable or non-heritable changes that are not related to modified DNA sequences and lead to altered expression or translation of the genome (Felsenfeld 2014). Epigenetic mechanisms can change the expression of genes and may also modulate the response to many environmental factors thus potentially modifying MS susceptibility (Aslani et al. 2017). It operates using many mechanisms including DNA methylation, histone modifications and RNA interference.

DNA methylation

DNA methylation involves the addition of a methyl group to the carbon-5 of a cytosine residue in DNA and is carried out by one of several DNA methyltransferase enzymes. This occurs in special genomic regions called CpG islands that contain greater than 50% cytosine and guanine nucleotides. CpG islands are 300–3,000 bp in length and are located in or near promoters in approximately 40% of mammalian genes. The methylation or hypermethylation of CpG islands in promoter regions usually prevents the expression of the associated gene. DNA methylation is currently the best studied epigenetic mechanism, and it is known to have a crucial role in normal development, cell proliferation and genome stability (Weber et al. 2007).

Histone modifications

In mammalian cells, histone proteins interact with DNA to form chromatin, the packaged form of DNA. Histones are octamers consisting of two copies of each of the four histone proteins: H2A, H2B, H3 and H4. Each histone octamer has 146 bp of the DNA strand wound around it to make up one nucleosome, which is the basic unit of chromatin. Histones undergo many types of reversible modifications such as acetylation,

methylation, phosphorylation, ubiquitination and ribosylation. These histone modifications induce changes to the structure of chromatin and thereby affect the accessibility of the DNA strand to transcriptional enzymes, resulting in activation or repression of genes associated with the modified histone (Dieker et al. 2010). The best understood histone modification is acetylation, which is mediated by histone acetyltransferases and deacetylases. Acetylation of histones is usually associated with up regulated transcriptional activity of the associated gene, whereas deacetylation of histones contributes to transcriptional silencing (Brooks et al. 2010).

MicroRNA

MicroRNAs (miRNAs) are a class of small noncoding RNAs, which have recently been discovered to be regulatory modulators of gene expression post-transcriptionally, either by targeting mRNA degradation or by inhibition of protein translation. miRNA are predicted to regulate 30% of all coding genes (Rajewsky et al. 2004)

They are implicated in almost every biological process, including pathways involved in immune homeostasis, such as immune cell development, central and peripheral tolerance, and T helper cell differentiation. (O'Connell et al. 2010). MiRNAs play a role at multiple checkpoints in both central and peripheral lymphoid organs to maintain immune tolerance and prevent autoimmunity. As already mentioned, autoreactive T and B cells are either deleted in the primary lymphoid organs - which prevents newly generated autoreactive T cells from escaping elimination - or silenced in the periphery - which suppresses the activation of already circulating autoreactive T cells (Bluestone 2011). During the establishment of central tolerance, the affinity between TCR and peptide/MHC complexes is central to T cell selection. Some miRNAs are abundantly expressed at this stage of T cell development (Li et al. 2007) and are involved in this well-orchestrated process. These molecules also act as potent regulators of the establishment and maintenance of B cell–tolerance mechanisms by controlling the expression of several molecules directly involved in the silencing of developing autoreactive B cells (Gonzalez-Martin et al. 2016). They lay also a major role in the peripheral tolerance mechanisms that exist to control the self-reactive T cells that escape negative selection and enter in the periphery (Simpson et al. 2015).

Involvement of miRNAs in the pathogenesis of various diseases such as cancer, heart failure, viral infections, and neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease has been demonstrated (Hebert et al. 2009; Chang et al. 2013; Ji et al. 2017). Alterations in miRNA expression and function can lead to major dysfunction of

the immune system and mediate susceptibility to autoimmune disease (Garo et al. 2016). Deregulated miRNAs, such as miR-155, can be found in different autoimmune diseases including Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), and systemic sclerosis (Dai et al. 2011). MicroRNA-155 is one of the miRNAs particularly well studied. Recent data reveal that miR-155 can be localized in immune responses, including B and T cell differentiation and development. MiR-155 overexpression results in human chronic inflammatory condition (O'Connell et al. 2012). Inflammation is a protective response requiring the mobilization of immune cells and molecular mediators. However, excessive inflammation is detrimental to the host and can lead to tissue damage or even cell death.

miRNAs and MS

Several studies performed miRNA profiling in MS patients and control subjects using Peripheral Blood Mononuclear Cells (PBMC) (Otaegui et al. 2009; Fenoglio et al. 2011; Martinelli-Boneschi et al. 2012), whole blood (Keller et al. 2009; Cox et al. 2010), brain lesions (Junker et al. 2009) and serum (Zhang et al. 2014). All reports showed altered miRNA expression profiles in MS patients compared to control subjects. Some discrepancies, however, were observed between the miRNAs that were identified as deregulated in these studies. This could be partly attributed to differences in the studied material, or to differences in the miRNA level quantification methods (mainly qRT-PCR or microarray). Moreover, patients under different treatment conditions were often included, and this could have influence in the results.

MiRNA-155 is the only miRNA consistently increased in in a variety of tissues including whole blood, CD4+and CD8+ T cells, serum and brain lesions of MS patients (Jagot et al. 2016).

1.2.2 – Environmental factors

Infectious and non-infectious causes are under discussion for MS pathogenesis since genetic susceptibility can only partially explain the geographic and epidemiological distribution of MS. For example, the fact that there is a change in MS risk combined with migration at early ages, cannot be explained by genetic factors (Ascherio et al. 2007).

1.2.2.1 - Infection

In order to explain the non-genetic phenomena, the “hygiene hypothesis” postulates that exposure to varying infectious agents during early development may be protective for MS and that the disease would be triggered by multiple infective microorganisms with risk increasing with age at infection (Fleming et al. 2007). In contrast the “prevalence hypothesis” claims that MS may be caused by a pathogen, which is more common in regions of higher MS prevalence, and leads to asymptomatic persistent infections that result in MS manifestation in a few cases after several years (Ascherio et al. 2007). From the very early days of MS discovery, infections have been proposed to be the underlying cause of disease initiation (Kakalacheva et al. 2011). A long list of potential infectious triggers of MS has been investigated since then. Viruses that have received most attention during the past years are presented in Table 2.

Table 2. Viral agents suspected to be triggers of Multiple Sclerosis

Viruses	Evidence for association
Epstein–Barr virus	• Near absolute seropositivity in children and adults with MS • Increased risk of MS in individuals with history of infectious mononucleosis • Virus reactivation linked to disease activity in early MS • Increased EBNA1 specific antibodies before MS onset • Cross-reactivity of clonally expanded EBNA1 specific T cells with myelin antigens • Enrichment of EBV-infected B cells in MS brain tissues
Human herpesvirus -6	• Isolated from blood, CSF and brain tissue • Presence of antiviral antibodies in blood and CSF (inconsistent observations) • Increased viral load linked to MS exacerbation • Cross-reactivity between virus-specific T cells and myelin antigens
Varicella zoster virus	• Acquired earlier in life by MS patients • Reactivation linked to MS exacerbation • Viral DNA isolated from blood and CSF • Virions observed by electron microscopy in CSF (inconsistent observations)

Several mechanisms have been described to explain how viruses might trigger autoimmune diseases, including virus-induced general activation of the immune system

and the provision of viral antigens that specifically stimulate immune responses that cross-react with self-antigens and therefore cause auto reactive immunopathology's (Munz et al. 2009).

After a microbial infection, activated microbe-specific Th1 cells migrate to the CNS. *Molecular mimicry* describes the activation of cross-reactive Th1 cells that recognize both the microbial epitope and the self-epitope. Activation of the cross-reactive T cells results in the release of cytokines and chemokines that recruit and activate monocytes and macrophages, which mediate self-tissue damage. The subsequent release of self-tissue antigens and their uptake by APCs perpetuate the autoimmune disease. *Epitope spreading* involves a persistent microbial infection that causes the activation of microorganism-specific Th1 cells, which mediate self-tissue damage. This results in the release of self-peptides, which are engulfed by APCs and presented to self-reactive Th1 cells. Continual damage and release of self-peptides results in the spread of the self-reactive immune response to multiple self-epitopes. *Bystander activation* is the nonspecific activation of self-reactive Th1 cells. Activation of microorganism-specific Th1 cells leads to inflammation and results in the increased infiltration of T cells at the site of infection and the activation of self-reactive Th1 cells by TCR-dependent and -independent mechanisms. Self-reactive T cells activated in this manner mediate self-tissue damage and perpetuate the autoimmune response (Munz et al. 2009).

1.2.2.2 - Smoking

Smoking is prevalent throughout the world, but rates are highly variable; smoking rates have changed over the last decades and in many regions more women than men smoke (making it a candidate environmental factor that could potentially cause the increasing female-to-male incidence ratio) (Palacios et al. 2011).

Cigarette smoking has emerged as a potential environmental risk factor for MS and may also influence disease course. Several case-control studies and prospective studies revealed an association between cigarette smoking and MS (Ghadirian et al. 2001; Riise et al. 2003; Hedstrom et al. 2009; Hedstrom et al. 2013; Ramagopalan et al. 2013). A meta-analysis from 2016 concluded that smoking habits are significantly associated with MS, although the association is not very strong (OR=1.46). However, there is a dose-response relationship between the smoking habits and MS (Poorolajal et al. 2016). In addition to being a risk factor for MS, cigarette smoking modify disease course. It is associated with more rapid conversion from Clinically Isolated Syndrome (ClS) to confirmed MS, increased risk of conversion from RRMS to SPMS, and more rapid

neurological worsening with greater clinical disability in the progressive phase (Hernan et al. 2005; Sundstrom et al. 2008; Healy et al. 2009; Pittas et al. 2009; Manouchehrinia et al. 2013; Briggs et al. 2017). Smokers with MS also have increased MRI lesion burden, greater brain atrophy compared with non-smokers (Zivadinov et al. 2009) and premature mortality (Manouchehrinia et al. 2014). In 2017, a study by Briggs et al. concluded that smokers with MS have greater decrements in quality of life and disability than non-smokers (Briggs et al. 2017).

The possible mechanisms that relate cigarette smoking to MS risk or progression have been poorly investigated. These could include neurotoxic effects (Mikaeloff et al. 2007) of cigarette smoke components, as indirectly supported by the association of cigarette smoking with optic neuropathy (Healy et al. 2009), or immunomodulatory effects as suggested by the association of cigarette smoking with an increased risk for development of other autoimmune diseases, including RA, SLE, autoimmune thyroid diseases, and inflammatory bowel disease (Arnson et al. 2010).

1.2.2.4 – Vitamin D

Vitamin D is a fat-soluble vitamin and a steroid hormone that plays a central role in maintaining calcium phosphorus and bone homeostasis in close interaction with parathyroid hormone, acting on its classical target tissues, namely, bone, kidney, intestine, and parathyroid glands. Vitamin D endocrine system regulates several genes (about 3 % of the human genome) involved in cell differentiation, cell-cycle control, and cell function and exerts noncalcemic/pleiotropic effects on extraskeletal target tissues, such as immune and cardiovascular system, pancreatic endocrine cells, muscle, and adipose tissue. Several studies have demonstrated the role of vitamin D supplementation in the prevention/treatment of various autoimmune diseases and improvement of glucose metabolism, muscle, and adipose tissue function (Wimalawansa 2016).

Synthesis, sources and metabolism

Vitamin D is found in two forms: vitamin D_2 and vitamin D_3. Vitamin D_3 is synthetized in the skin by Ultraviolet Radiation (UVR) or consumed in the diet through products like eggs and milk. On the contrary, vitamin D_2 cannot be formed by UVR; it is only acquired through food. Nevertheless exposure to sunlight is the major source of vitamin D. Sunlight mediates the conversion of 7-dehydrocholesterol (pre-vitamin D_3) to

cholecalciferol (vitamin D_3) in the skin which is then hydroxylated in the liver to form metabolic inactive 25-hydroxyvitamin D [25(OH)D] or calcidiol, known to be the major circulating metabolite of vitamin D. Measurement of the 25(OH)D concentration is therefore used as an indicator for the vitamin D status (Lips 2007). In the kidney, 25(OH)D is further hydroxylated, mainly by 1 α-hydroxylase, to 1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$], or calcitriol, the biologically active metabolite of vitamin D. This second hydroxylation is carefully regulated by Parathyroid Hormone (PTH), phosphate and vitamin D itself (Bikle 2014). The parathyroid hormone stimulates hydroxylation, whereas phosphate and vitamin D inhibit it. The fat solubility of vitamin D and its metabolites makes it necessary to be transported in blood bound to vitamin D-Binding Proteins (*DBP*s), furthermore makes it also possible to leave DBP and freely diffuse across cell membranes and bind to cytosolic Vitamin D Receptors (VDRs) (Bikle 2014). Vitamin D metabolism is outlined in Figure 8.

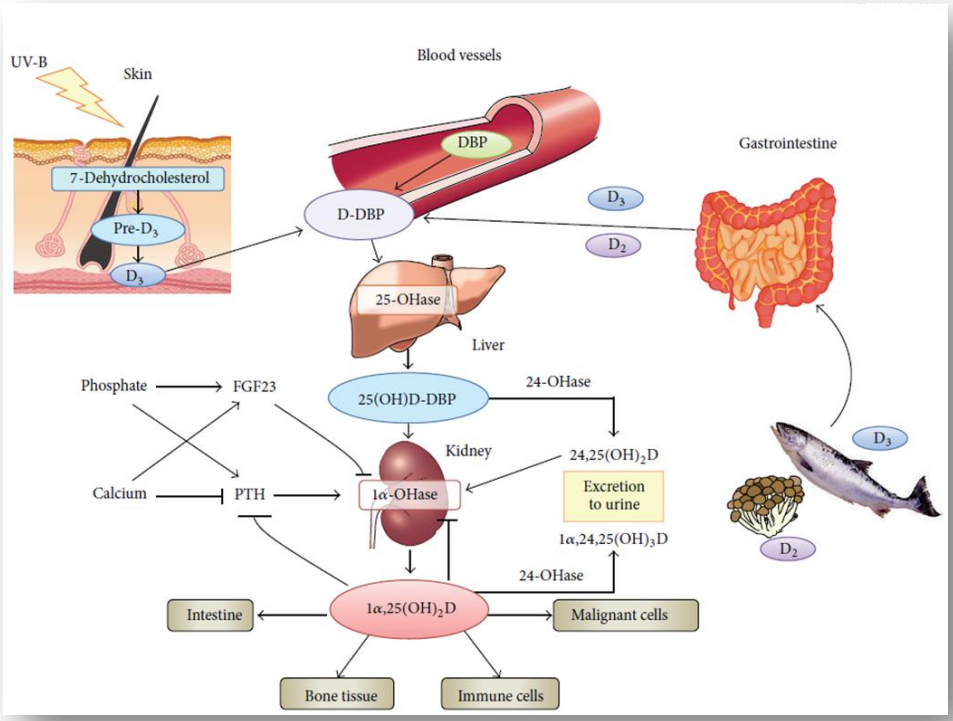

Figure 8. Vitamin D metabolism (Obi et al. 2015).

Immune function

Vitamin D is a potent immune modulator actively involved in the regulation of innate and adaptive immune responses.(Provvedini et al. 1983; Takahashi et al. 2002; Adams et al. 2008; Fernandes de Abreu et al. 2009) . Most of the biological effects of $1,25(OH)_2D_3$ are mediated by the VDR, a nuclear receptor member of the steroid receptor super-family

that influences the rate of transcription of vitamin D responsive genes by binding to vitamin D response elements (VDREs) – a short stretch of DNA that is a signature of genes regulated by vitamin D – in the genome, regulating a multitude of different genes (Carlberg et al. 2001; Smolders et al. 2008a). Direct genomic signalling by $1,25(OH)_2D_3$ occurs through the VDR, which is present in multiple cells of the immune system including activated CD4+ and CD8+ T cells, B cells, neutrophils and APCs, such as macrophages and DCs. Active vitamin D, i.e., $1,25(OH)_2D_3$, is recognised by target tissues that possess VDR. This receptor is expressed widely in the intestine, skin, bone, kidneys, pituitary, parathyroid, pancreatic beta cells, gonads, skeletal muscles, circulating monocytes, activated T and B lymphocytes as well as in neurons and glial cells in the human brain (Smolders et al. 2011).

Whereas naive T cells only display very low VDR levels, this receptor is present in large quantities upon T cell activation (Provvedini et al. 1983; Baeke et al. 2010). By contrast, differentiation of monocytes, either into macrophages or DCs, is accompanied by a decrease in VDR-expression, making these cells less sensitive to $1,25(OH)_2D_3$ when they mature. The consequence of $1,25(OH)_2D_3$ action on T cells is to block the induction of Th1 cell cytokines, particularly IFN-γ, while promoting Th2 cell responses, an effect mediated both indirectly by decreasing IFN-γ production and directly by enhancing IL-4 production (van Etten et al. 2005). The activity of $1,25(OH)_2D_3$ on effector T cell differentiation is further enhanced by its effect on antigen-presenting DCs, in which it suppresses the synthesis of IL-12, a cytokine that promotes Th1 cell responses (D'Ambrosio et al. 1998; Penna et al. 2000). Furthermore, $1,25(OH)_2D_3$ also inhibits Th17 cell responses, probably owing in part to its capacity to inhibit IL-6 and IL-23 production, and induces the reciprocal differentiation and/or expansion of Tregs (Penna et al. 2005; Gorman et al. 2007). Proliferation assays show an association of high 25(OH)D levels with an improved regulatory T cell function in patients with MS (Correale et al. 2009; Smolders et al. 2009d).

In addition to its inhibitory effects on T cells, $1,25(OH)_2D_3$ decreases B cell proliferation, plasma cell differentiation and Immunoglobulin G secretion (Chen et al. 2007) . It has been suggested that the effect of $1,25(OH)_2D_3$ on B cells might be indirectly mediated through the effect it has on APCs function and/or T helper cell (Muller et al. 1991). Indeed, there are conflicting reports concerning the expression of VDR by B cells (Veldman et al. 2000; Chen et al. 2007; Shirakawa et al. 2008), leaving it unclear whether $1,25(OH)_2D_3$ can act directly on B cells.

Cells of the innate immune system can also be inhibited by $1,25(OH)_2D_3$, which is known to inhibit the differentiation, maturation and immunostimulatory capacity of DCs by

decreasing the expression of MHC class II molecules as well of CD40, CD80 and CD86 (Penna et al. 2000; Fritsche et al. 2003; van Etten et al. 2005). In addition, $1,25(OH)_2D_3$ decreases the synthesis of IL-12 (D'Ambrosio et al. 1998; Penna et al. 2000) and simultaneously increases the production of IL-10 by DCs (Penna et al. 2000). Although $1,25(OH)_2D_3$ primarily has inhibitory effects on the adaptive immune response, some of its effects on innate immune cells are stimulatory. For example, $1,25(OH)_2D_3$ can stimulate human monocyte proliferation *in vitro* (Ohta et al. 1985) and has been shown to increase the production of both IL-1 and the bactericidal peptide cathelicidin by monocytes and macrophages (Holick 2007). The effects of vitamin D on cells of the immune system are summarized in Figure 9.

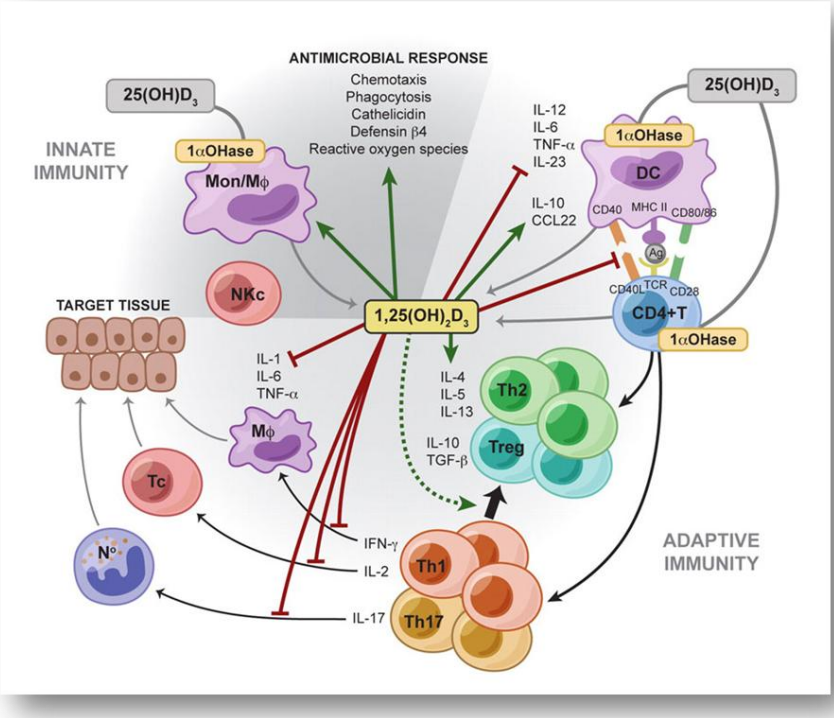

Figure 9. Effects of vitamin D on cells of the immune system []Adapted from (Verstuyf et al. 2010)].

Compared to the knowledge of vitamin D in calcium homeostasis and skeletal growth, very little is known about its role in the central nervous system. The evidence for a neurobiological effect of vitamin D came in 1991 when the regulatory effect of 1,25 dihydroxyvitamin D3 on nerve growth factor was first reported (Wion et al. 1991). Studies have since shown that bioactive vitamin D may modulate the production of neurotrophins, growth factors and neurotransmitters in the mammalian brain (Garcion et al. 2002).

Month of birth

The month of birth effect was first proposed in 1987 as part of a study examining different neurological diseases. It was suggested that those who were born in winter months have a reduced risk of developing MS, whilst people born in spring are at a higher risk (Shimura et al. 1987). A possible month of birth effect for MS has been reported in studies from Canada, northern Europe, and Australia (Templer et al. 1992; Willer et al. 2005; Sadovnick et al. 2007; Staples et al. 2010; Torkildsen et al. 2012). Combining patients from the northern hemisphere (Canada, Denmark, and Sweden), a study show that significantly more MS patients were born in May and fewer were born in November, compared to other months of the year (Willer et al. 2005). Another study found more RRMS patients born in May than in November (Sadovnick et al. 2007). Finally, in 67 Canadian patients, born in the southern hemisphere, this month of birth effect seemed reversed (Willer et al. 2005). Recently, this reversal association has been fully documented in Australia, where MS risk peaks for babies born in November/December and has its nadir for children born in May/June (Staples et al. 2010). A study from Barros et al. (Barros et al. 2013) does not support the hypothesis of month of birth as risk factor for Multiple Sclerosis in Portugal. There has been some controversy regarding the possibility that this month of birth effect may be due to an analytic bias (Fiddes et al. 2013; Fiddes et al. 2014; Torkildsen et al. 2014). Nevertheless, a recent meta-analysis and systematic review found that the month of birth effect was latitude dependent, and only significant in places that were located in regions over 52ºN (Dobson et al. 2013).

To explain this association it has been hypothesized that low maternal vitamin D levels, due to reduced sun exposure in the winter months, play a significant role in this phenomenon. Several recent observational studies strongly suggest that vitamin D deficiency during pregnancy and/or the early neonatal period is a strong cause for the development of MS in later life (Munger et al. 2016; Nielsen et al. 2017). One possible explanation is that reduced vitamin D levels may affect the immunological development of the fetus in a critical time during pregnancy, resulting in an increased risk of MS (Ebers 2008; Wagner et al. 2012).

Serum 25-hydroxyvitamin D level and Multiple Sclerosis

The two forms of vitamin D metabolite, $1,25(OH)_2D_3$ and 25(OH)D are commonly measured in serum and have been the target of most studies focusing on vitamin D metabolism. However, 25(OH)D serum concentration is widely accepted as the best

indicator of the vitamin D status of an individual in vivo. It covers not only the endocrine but also the paracrine biological pathways of vitamin D, whereas the active hormone $1,25(OH)_2D_3$ does not provide information on the vitamin D status and is often normal or increased as a result of secondary hyperthyroidism associated with vitamin D deficiency (Holick 2009). Calcidiol has an almost 1000-fold greater concentration than 1,25(OH)2D; also, 25(OH)D has a longer half-life (20 days) and hence is more stable in the circulation. Calcitriol has a half-life of only 4–6 hours. Therefore, total-body vitamin D stores are best measured by assessing circulating levels of 25(OH)D (Lips 2007).

Different studies have analysed the serum concentrations of vitamin D metabolites in patients with MS. These studies showed that patients with MS had significantly lower 25-hydroxyvitamin D levels than controls (Nieves et al. 1994; Cosman et al. 1998; Soilu-Hanninen et al. 2005; Kragt et al. 2009). There are also two longitudinal studies based on 25(OH)D concentrations before the onset of MS. One of the studies used a nested case-control design and comprised over 7 million individuals who served in the US military and had at least two serum samples stored in the US department of defence serum repository. The study concluded that serum concentration of 25(OH)D, in healthy young white adults, is an important risk predictor for MS, independently from their place of birth and latitude of residence during childhood (Munger et al. 2006). The other study was a prospective study performed in Nurses, where dietary vitamin D intake was assessed at baseline and updated every 4 years thereafter. During the follow-up, 173 cases of MS with onset of symptoms after baseline were confirmed. They concluded that women who took vitamin D (≥400 international units/day) had a 40% lower risk of MS than women who did not use vitamin D supplements (Munger et al. 2004). Also there are some studies that found a negative correlation between serum 25(OH)D and MS clinical disease activity (van der Mei et al. 2007; Smolders et al. 2008b; Soilu-Hanninen et al. 2008).

VDR gene polymorphisms

The VDR gene is located on chromosome 12 and various single nucleotide polymorphisms have been described (Whitfield et al. 2001). The most frequently studied in MS are usually referred to by the names of the digestion enzymes used for genotyping. The FokI polymorphism, found in the coding region of the VDR gene, is caused by a T to C substitution in the translation initiation site in exon 2. It leads to the production of a VDR protein that differs in length by three amino acids. Individuals with the C allele ('F') initiate translation at the second ATG site, thereby lacking the three NH_2-terminal amino acid of the full length VDR protein (424 amino acid). In contrast,

individuals with the T ('f') allele initiate translation at the first ATG site and synthesize the full length VDR protein (427 amino acid) (Smolders et al. 2009c). No differences in DNA binding, ligand binding, and heterodimer formation have been reported for the different VDR FokI isoforms (Gross et al. 1998). Although the short 'F' isoform has been associated with a higher transcriptional activity (Arai et al. 1997; Jurutka et al. 2000), not all studies could reproduce these results (Gross et al. 1998). Therefore, this polymorphism seems to have consequences for both VDR protein structure and transcriptional activity (Jurutka et al. 2000).

Concerning the functional impact of this polymorphism on the immune system no definitive conclusion can be drawn since the few studies that have been performed so far were contradictory. It was shown, in an *in vitro* system, that the addition of $1,25(OH)_2D_3$ dose-dependently decreased the degree of peripheral blood mononuclear cell proliferation (Colin et al. 2000). Carriers of an 'F' allele achieved 50% inhibition of proliferation at lower doses of $1,25(OH)_2D_3$ than their 'f' allele counterparts. However, another study could not confirm these findings (van Etten et al. 2007). In another *in vitro* study the effect of the FokI polymorphism on the $1,25(OH)_2D_3$ mediated suppression of IL-12 transcription and protein production by monocytes and DCs has been assessed. Although both alleles presented the same rate of suppression on $1,25(OH)_2D_3$, cells with a homozygous short FF genotype had a higher expression of IL-12 (mRNA and protein) than cells with a long ff genotype (van Etten et al. 2007).

The BsmI and ApaI polymorphisms are located in intron 8, and are in LD with each other and with the TaqI polymorphism found in exon 9. Although the BsmI and ApaI *locus* is intronic, a number of mechanisms have been invoked to explain how these polymorphisms might influence VDR expression. One of these explanations includes the disruption of a splice site for VDR mRNA transcription, which may result in truncated or alternatively spliced protein products. Another explanation involves the changes in mRNA stability speculating that these introns might influence the level of mRNA product (Nesic et al. 1993). The TaqI polymorphism results in a silent mutation in exon 9, with both ATT and ATC coding for isoleucine. Given that these polymorphisms do not seem to result in structural changes of the VDR protein, it is very unlikely that they have functional consequences. However, LD with other polymorphism(s) within VDR gene might underlie a potential effect. An example is the known LD with the 3'untranslated region of the VDR gene, a region which is involved in the regulation of mRNA stability and, in consequence, gene expression (Ingles et al. 1997).

VDR gene polymorphisms in MS

Patient-control and transmission studies have been used to study the association of VDR gene polymorphisms and MS. The first studies were from Japan and reported an association with BsmI and ApaI (Fukazawa et al. 1999; Niino et al. 2000). The association with ApaI was reproduced in an Australian population and an association with TaqI was also found (Tajouri et al. 2005). Although a British study showed a trend towards an underexpression of the ff FokI genotype (Partridge et al. 2004), and a Canadian study showed marginally distorted transmission in HLA-DR15-negative patients (Orton et al. 2011), no other study reported associations of these polymorphisms and MS (Steckley et al. 2000; Yeo et al. 2004; Smolders et al. 2009b; Smolders et al. 2009a; Simon et al. 2010; Sioka et al. 2011). A possible hypothesis to explain the lack of association in studies performed in areas more remote from the equator is that an association of a VDR gene polymorphism with MS might be only penetrant in a population with sufficient vitamin D status (Smolders et al. 2009b).

In addition to the potential effect of these polymorphisms on MS susceptibility they may also influence the course and severity of disease. In the UK, the 'f' allele was described as associated with a decreased level of disability 10 years after disease onset (Mamutse et al. 2008).

VDR gene polymorphisms have also been related to vitamin D metabolites levels in MS patients. Only FokI polymorphism was associated with 25(OH)D levels and the 'F' allele was correlated with significantly lower 25(OH)D levels (Orton et al. 2008; Smolders et al. 2009b).

Vitamin D and HLA-DRB1*15

It was established long ago that vitamin D modulates MHC class II gene expression, namely HLA-DR antigen expression and presentation (Rigby et al. 1990). When trying to correlate genetics with functional studies, Ramagopalan et al. (Ramagopalan et al. 2009) identified a single VDRE in the HLA-DRB1 promoter region. When they examined DNA from 322 individuals with two copies of HLA-DRB1*15 (including people with and without MS), they found the same VDRE sequence in all of them, but found different VDRE sequences in DNA samples from 168 study participants without HLA-DRB1*15. Functional assays were also used to demonstrate that this VDRE influenced gene expression, thereby conferring $1,25(OH)_2D_3$ sensitivity to HLA-DRB1*15. The variant VDRE present on other, non-MS-associated HLA-DRB1 haplotypes, were not responsive to $1,25(OH)_2D_3$. This study supports the existence of a direct biological interaction

between HLA-DRB1, the main MS susceptibility *locus*, and vitamin D, a key candidate environmental factor. The hypothesis presented is that low levels of vitamin D *in utero* or early childhood can affect the expression of HLA-DRB1 in the thymus, enabling "self-directed" T cells to escape thymic deletion (loss of central tolerance) and trigger an autoimmune response in later life (Ramagopalan et al. 2009).

Vitamin D and miRNAs

Given the strong genomic actions of $1,25(OH)_2D_3$ it is likely that it also regulates the expression of some of the remaining genome which includes microRNAs. Recently, a number of miRNAs have been identified as $1,25(OH)_2D_3$ targets (Chen et al. 2013). Calcitriol regulates the expression of miRNAs through both direct mechanism that involves VDRE and indirect mechanism that affects the genesis of mature miRNA. Through the regulation of miRNA expression, $1,25(OH)_2D_3$ modulates cell proliferation, differentiation, apoptosis, and migration in many cancer cell types (Giangreco et al. 2013; Campbell 2014; Ma et al. 2014).

1.2.2.4 – Other environmental factors

Multiple Sclerosis seems to occur in genetically predisposed individuals who are exposed to certain environmental factors, especially during childhood (Ascherio et al. 2012). Among these are the well-studied factors as EBV infection, smoking and vitamin D levels (Ascherio et al. 2012), that were already described in this thesis. Nevertheless, recent studies are evaluating other factors, including sun exposure, sodium intake, obesity during adolescence, alcohol and coffee consumption, and microbiota.

As stated before, conversion of vitamin D to its active metabolite is dependent on ultraviolet radiation, making it very difficult to distinguish between the effects of UVR and vitamin D, or determine whether they are mutually non-exclusive. Both UVR and vitamin D have been shown to be important factors in protecting against MS (Lucas et al. 2015) Increased UVR exposure is related to a decreased risk of MS and, even after correcting for vitamin D levels, UVR exposure habits are associated with the risk of MS (Baarnhielm et al. 2012). The physiological basis of a potential protective effect of UVR is not completely understood. In the EAE, UVR exposure protects against neuroinflammation independently of vitamin D (Becklund et al. 2010).

High intake of sodium is currently considered to be a potentially factor influencing the onset of MS (Zostawa et al. 2017). Also, in a study from Argentina, individuals with MS,

who had high salt intake, had markedly more relapses and MRI-evidenced disease activity than did those with a low salt consumption (Farez et al. 2015). *In vitro* experiments showed that high salt conditions promotes T cell differentiation into pathogenic Th17 cells, and mice who consumed a diet very high in salt developed a more severe course of EAE (Kleinewietfeld et al. 2013b; Wu et al. 2013).

Studies in the past few years strongly supports a role for obesity in the risk of MS (Gianfrancesco et al. 2016). Large cohort studies have associated obesity during adolescence with future risk of MS (Munger et al. 2013; Gianfrancesco et al. 2014). Obesity is also associated with an increased risk of paediatric-onset MS (Langer-Gould et al. 2013; Munger et al. 2013). Three mechanistic pathways through which obesity is involved in MS pathogenesis have been proposed; these pathways are mutually non-exclusive and partly overlap with each other. First, obesity is characterized by a low-grade inflammation in which increased levels of pro-inflammatory mediators are produced in the fat tissue (Lumeng et al. 2007); also decreased function of Tregs cells, have been described in obesity (Matarese et al. 2005). Second, obesity is associated with increased levels of leptin, a mediator connected to pro-inflammatory processes (Matarese et al. 2010). Third, obesity also leads to decreased bioavailability of vitamin D, which further promotes pro-inflammatory processes (Wortsman et al. 2000).

Studies assessing the role of alcohol and coffee consumption in MS have generated inconsistent results. In one prospective study, no impact of caffeine or alcohol on MS risk was found (Massa et al. 2013). Other large case–control study suggested a dose-dependent, inverse association between MS and alcohol use (Hedstrom et al. 2014). Interestingly, a dose-dependent inverse association has also been described between alcohol use and rheumatoid arthritis (Kallberg et al. 2009). Coffee consumption and MS risk was recently investigated in two independent population-based case–control cohorts and high consumption was associated with decreased Multiple Sclerosis risk (Hedstrom et al. 2016). In animal models of MS, caffeine decreases the risk of developing neuroinflammation, and has neuroprotective (Chen et al. 2010) and anti-inflammatory properties (Horrigan et al. 2006).

Also, there is evidence to suggest that the intestinal microflora (microbiota) could be important in the pathogenesis of MS (Bhargava et al. 2014; Colpitts et al. 2017). The gut bacteria play a role in educating the immune system and hence may be a player in the development of Multiple Sclerosis. Several studies described the differential abundance of intestinal bacteria between individuals with MS and healthy controls and tried to found a common MS microbiota signature (Miyake et al. 2015; Chen et al. 2016; Jangi et al. 2016; Tremlett et al. 2016).

1.3 - References

(2001). "A meta-analysis of genomic screens in multiple sclerosis. The Transatlantic Multiple Sclerosis Genetics Cooperative." Mult Scler **7**(1): 3-11.

(2003). "A meta-analysis of whole genome linkage screens in multiple sclerosis." J Neuroimmunol **143**(1-2): 39-46.

(2009a). "The expanding genetic overlap between multiple sclerosis and type I diabetes." Genes Immun **10**(1): 11-14.

(2009b). "Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20." Nat Genet **41**(7): 824-828.

(2010). "Comprehensive follow-up of the first genome-wide association study of multiple sclerosis identifies KIF21B and TMEM39A as susceptibility loci." Hum Mol Genet **19**(5): 953-962.

Adams, J. S. and M. Hewison (2008). "Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity." Nat Clin Pract Endocrinol Metab **4**(2): 80-90.

Albrecht, P., I. Bouchachia, N. Goebels, et al. (2012). "Effects of dimethyl fumarate on neuroprotection and immunomodulation." J Neuroinflammation **9**: 163.

Alcalde-Cabero, E., J. Almazan-Isla, A. Garcia-Merino, et al. (2013). "Incidence of multiple sclerosis among European Economic Area populations, 1985-2009: the framework for monitoring." BMC Neurol **13**: 58.

Alexopoulos, H., A. Biba and M. C. Dalakas (2016). "Anti-B-Cell Therapies in Autoimmune Neurological Diseases: Rationale and Efficacy Trials." Neurotherapeutics **13**(1): 20-33.

Alizadeh, M., M. C. Babron, B. Birebent, et al. (2003). "Genetic interaction of CTLA-4 with HLA-DR15 in multiple sclerosis patients." Ann Neurol **54**(1): 119-122.

Arai, H., K. Miyamoto, Y. Taketani, et al. (1997). "A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women." J Bone Miner Res **12**(6): 915-921.

Arnson, Y., Y. Shoenfeld and H. Amital (2010). "Effects of tobacco smoke on immunity, inflammation and autoimmunity." J Autoimmun **34**(3): J258-265.

Ascherio, A. and K. L. Munger (2007). "Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors." Ann Neurol **61**(6): 504-513.

Ascherio, A., K. L. Munger and J. D. Lunemann (2012). "The initiation and prevention of multiple sclerosis." Nat Rev Neurol **8**(11): 602-612.

Aslani, S., N. Jafari, M. R. Javan, et al. (2017). "Epigenetic Modifications and Therapy in Multiple Sclerosis." Neuromolecular Med **19**(1): 11-23.

Aulchenko, Y. S., I. A. Hoppenbrouwers, S. V. Ramagopalan, et al. (2008). "Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis." Nat Genet **40**(12): 1402-1403.

Baarnhielm, M., A. K. Hedstrom, I. Kockum, et al. (2012). "Sunlight is associated with decreased multiple sclerosis risk: no interaction with human leukocyte antigen-DRB1*15." Eur J Neurol **19**(7): 955-962.

Baeke, F., H. Korf, L. Overbergh, et al. (2010). "Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D3 in the immune system." J Steroid Biochem Mol Biol **121**(1-2): 221-227.

Barcellos, L. F., J. R. Oksenberg, A. B. Begovich, et al. (2003). "HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course." Am J Hum Genet **72**(3): 710-716.

Barcellos, L. F., J. R. Oksenberg, A. J. Green, et al. (2002). "Genetic basis for clinical expression in multiple sclerosis." Brain **125**(Pt 1): 150-158.

Barcellos, L. F., S. Sawcer, P. P. Ramsay, et al. (2006). "Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis." Hum Mol Genet **15**(18): 2813-2824.

Barros, P., J. M. de Sa and M. J. Sa (2013). "Month of birth and risk of multiple sclerosis in a Portuguese population." Clin Neurol Neurosurg **115**(9): 1762-1765.

Barun, B. and A. Bar-Or (2012). "Treatment of multiple sclerosis with anti-CD20 antibodies." Clin Immunol **142**(1): 31-37.

Becklund, B. R., K. S. Severson, S. V. Vang, et al. (2010). "UV radiation suppresses experimental autoimmune encephalomyelitis independent of vitamin D production." Proc Natl Acad Sci U S A **107**(14): 6418-6423.

Beecham, A. H., N. A. Patsopoulos, D. K. Xifara, et al. (2013). "Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis." Nat Genet **45**(11): 1353-1360.

Beffert, U., M. Danik, P. Krzywkowski, et al. (1998). "The neurobiology of apolipoproteins and their receptors in the CNS and Alzheimer's disease." Brain Res Brain Res Rev **27**(2): 119-142.

Bergan, J. J., G. W. Schmid-Schonbein, P. D. Smith, et al. (2006). "Chronic venous disease." N Engl J Med **355**(5): 488-498.

Bettencourt, A., A. M. Silva, C. Pereira, et al. (2008). Benign course in Multiple Sclerosis: Association with autoimmunity and the Protein Tyrosine Phosphatase (PTPN22) 1858C>T gene polymorphism. 9th International Congress of NeuroImmunology, Fort Worth, Texas. USA, Journal of Neuroimmunology

Bettencourt, A., A. M. Silva, E. Santos, et al. (2011). "HFE gene polymorphisms and severity in Portuguese patients with multiple sclerosis." Eur J Neurol **18**(4): 663-666.

Bhargava, P. and E. M. Mowry (2014). "Gut microbiome and multiple sclerosis." Curr Neurol Neurosci Rep **14**(10): 492.

Bikle, D. D. (2014). "Vitamin D metabolism, mechanism of action, and clinical applications." Chem Biol **21**(3): 319-329.

Bitsch, A., J. Schuchardt, S. Bunkowski, et al. (2000). "Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation." Brain **123 (Pt 6)**: 1174-1183.

Block, M. L., L. Zecca and J. S. Hong (2007). "Microglia-mediated neurotoxicity: uncovering the molecular mechanisms." Nat Rev Neurosci **8**(1): 57-69.

Bluestone, J. A. (2011). "Mechanisms of tolerance." Immunol Rev **241**(1): 5-19.

Booss, J., M. M. Esiri, W. W. Tourtellotte, et al. (1983). "Immunohistological analysis of T lymphocyte subsets in the central nervous system in chronic progressive multiple sclerosis." J Neurol Sci **62**(1-3): 219-232.

Briggs, F. B., D. D. Gunzler, D. Ontaneda, et al. (2017). "Smokers with MS have greater decrements in quality of life and disability than non-smokers." Mult Scler: 1352458516685169.

Brooks, W. H., C. Le Dantec, J. O. Pers, et al. (2010). "Epigenetics and autoimmunity." J Autoimmun **34**(3): J207-219.

Browne, P., D. Chandraratna, C. Angood, et al. (2014). "Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity." Neurology **83**(11): 1022-1024.

Brynedal, B., K. Duvefelt, G. Jonasdottir, et al. (2007). "HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis." PLoS ONE **2**(7): e664.

Buc, M. (2013). "Role of regulatory T cells in pathogenesis and biological therapy of multiple sclerosis." Mediators Inflamm **2013**: 963748.

Buckle, G. J., P. Hollsberg and D. A. Hafler (2003). "Activated CD8+ T cells in secondary progressive MS secrete lymphotoxin." Neurology **60**(4): 702-705.

Burwick, R. M., P. P. Ramsay, J. L. Haines, et al. (2006). "APOE epsilon variation in multiple sclerosis susceptibility and disease severity: some answers." Neurology **66**(9): 1373-1383.

Buzzard, K. A., S. A. Broadley and H. Butzkueven (2012). "What do effective treatments for multiple sclerosis tell us about the molecular mechanisms involved in pathogenesis?" Int J Mol Sci **13**(10): 12665-12709.

Caamano, J. and C. A. Hunter (2002). "NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions." Clin Microbiol Rev **15**(3): 414-429.

Campbell, M. J. (2014). "Vitamin D and the RNA transcriptome: more than mRNA regulation." Front Physiol **5**: 181.

Carlberg, C., M. Quack, M. Herdick, et al. (2001). "Central role of VDR conformations for understanding selective actions of vitamin D(3) analogues." Steroids **66**(3-5): 213-221.

Chang, S. T., M. J. Thomas, P. Sova, et al. (2013). "Next-generation sequencing of small RNAs from HIV-infected cells identifies phased microrna expression patterns and candidate novel microRNAs differentially expressed upon infection." MBio **4**(1): e00549-00512.

Chen, G. Q., Y. Y. Chen, X. S. Wang, et al. (2010). "Chronic caffeine treatment attenuates experimental autoimmune encephalomyelitis induced by guinea pig spinal cord homogenates in Wistar rats." Brain Res **1309**: 116-125.

Chen, J., N. Chia, K. R. Kalari, et al. (2016). "Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls." Sci Rep **6**: 28484.

Chen, S., G. P. Sims, X. X. Chen, et al. (2007). "Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation." J Immunol **179**(3): 1634-1647.

Chen, Y., W. Liu, T. Sun, et al. (2013). "1,25-Dihydroxyvitamin D promotes negative feedback regulation of TLR signaling via targeting microRNA-155-SOCS1 in macrophages." J Immunol **190**(7): 3687-3695.

Colin, E. M., A. E. Weel, A. G. Uitterlinden, et al. (2000). "Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1, 25-dihydroxyvitamin D3." Clin Endocrinol (Oxf) **52**(2): 211-216.

Colpitts, S. L. and L. H. Kasper (2017). "Influence of the Gut Microbiome on Autoimmunity in the Central Nervous System." J Immunol **198**(2): 596-604.

Compston, A. and A. Coles (2008). "Multiple sclerosis." Lancet **372**(9648): 1502-1517.

Compston, A., C. Confavreux, H. Lassmann, et al. (2006). McAlpine's multiple sclerosis. Philadelphia, Livingstone/Elsevier.

Compston, D. A., J. R. Batchelor and W. I. McDonald (1976). "B-lymphocyte alloantigens associated with multiple sclerosis." Lancet **2**(7998): 1261-1265.

Correale, J., M. I. Gaitan, M. C. Ysrraelit, et al. (2016). "Progressive multiple sclerosis: from pathogenic mechanisms to treatment." Brain.

Correale, J., M. C. Ysrraelit and M. I. Gaitan (2009). "Immunomodulatory effects of Vitamin D in multiple sclerosis." Brain **132**(Pt 5): 1146-1160.

Cosman, F., J. Nieves, L. Komar, et al. (1998). "Fracture history and bone loss in patients with MS." Neurology **51**(4): 1161-1165.

Cox, M. B., M. J. Cairns, K. S. Gandhi, et al. (2010). "MicroRNAs miR-17 and miR-20a inhibit T cell activation genes and are under-expressed in MS whole blood." PLoS One **5**(8): e12132.

Criswell, L. A., K. A. Pfeiffer, R. F. Lum, et al. (2005). "Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the PTPN22 620W allele associates with multiple autoimmune phenotypes." Am J Hum Genet **76**(4): 561-571.

D'Ambrosio, D., M. Cippitelli, M. G. Cocciolo, et al. (1998). "Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene." J Clin Invest **101**(1): 252-262.

Dai, R. and S. A. Ahmed (2011). "MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases." Transl Res **157**(4): 163-179.

Dalakas, M. C. (2008). "Invited article: inhibition of B cell functions: implications for neurology." Neurology **70**(23): 2252-2260.

De Jager, P. L., X. Jia, J. Wang, et al. (2009). "Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci." Nat Genet **41**(7): 776-782.

de Jong, B. A., T. W. Huizinga, E. Zanelli, et al. (2002). "Evidence for additional genetic risk indicators of relapse-onset MS within the HLA region." Neurology **59**(4): 549-555.

de la Concha, E. G., R. Arroyo, J. B. Crusius, et al. (1997). "Combined effect of HLA-DRB1*1501 and interleukin-1 receptor antagonist gene allele 2 in susceptibility to relapsing/remitting multiple sclerosis." J Neuroimmunol **80**(1-2): 172-178.

de Moerloose, P., M. Jeannet, B. Martins-da-Silva, et al. (1979). "Increased frequency of HLA--DRw2 and DRw3 in multiple sclerosis." Tissue Antigens **13**(5): 357-360.

de Sa, J. (2010). "[Epidemiology of multiple sclerosis in Portugal and Spain]." Rev Neurol **51**(7): 387-392.

de Sa, J., E. Alcalde-Cabero, J. Almazan-Isla, et al. (2014). "Incidence of multiple sclerosis in Northern Lisbon, Portugal: 1998-2007." BMC Neurol **14**: 249.

De Sa, J., A. Paulos, H. Mendes, et al. (2006). "The prevalence of multiple sclerosis in the District of Santarem, Portugal." J Neurol **253**(7): 914-918.

Dendrou, C. A., L. Fugger and M. A. Friese (2015). "Immunopathology of multiple sclerosis." Nat Rev Immunol **15**(9): 545-558.

Dieker, J. and S. Muller (2010). "Epigenetic histone code and autoimmunity." Clin Rev Allergy Immunol **39**(1): 78-84.

Dobson, R., G. Giovannoni and S. Ramagopalan (2013). "The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude." J Neurol Neurosurg Psychiatry **84**(4): 427-432.

Du, L., Y. Zhang, Y. Chen, et al. (2016). "Role of Microglia in Neurological Disorders and Their Potentials as a Therapeutic Target." Mol Neurobiol.

Dyment, D. A., B. M. Herrera, M. Z. Cader, et al. (2005). "Complex interactions among MHC haplotypes in multiple sclerosis: susceptibility and resistance." Hum Mol Genet **14**(14): 2019-2026.

Ebers, G. C. (2008). "Environmental factors and multiple sclerosis." Lancet Neurol **7**(3): 268-277.

Ellwardt, E. and F. Zipp (2014). "Molecular mechanisms linking neuroinflammation and neurodegeneration in MS." Exp Neurol **262 Pt A**: 8-17.

Farez, M. F., M. P. Fiol, M. I. Gaitan, et al. (2015). "Sodium intake is associated with increased disease activity in multiple sclerosis." J Neurol Neurosurg Psychiatry **86**(1): 26-31.

Felsenfeld, G. (2014). "A brief history of epigenetics." Cold Spring Harb Perspect Biol **6**(1).

Fenoglio, C., C. Cantoni, M. De Riz, et al. (2011). "Expression and genetic analysis of miRNAs involved in CD4+ cell activation in patients with multiple sclerosis." Neurosci Lett **504**(1): 9-12.

Fernandes de Abreu, D. A., D. Eyles and F. Feron (2009). "Vitamin D, a neuro-immunomodulator: implications for neurodegenerative and autoimmune diseases." Psychoneuroendocrinology **34 Suppl 1**: S265-277.

Fiddes, B., J. Wason, A. Kemppinen, et al. (2013). "Confounding underlies the apparent month of birth effect in multiple sclerosis." Ann Neurol **73**(6): 714-720.

Fiddes, B., J. Wason and S. Sawcer (2014). "Confounding in association studies: month of birth and multiple sclerosis." J Neurol **261**(10): 1851-1856.

Figueiredo, J., A. Silva, J. J. Cerqueira, et al. (2015). "MS Prevalence and Patients' Characteristics in the District of Braga, Portugal." Neurol Res Int **2015**: 895163.

Fleming, J. and Z. Fabry (2007). "The hygiene hypothesis and multiple sclerosis." Ann Neurol **61**(2): 85-89.

Flodstrom-Tullberg, M., Y. T. Bryceson, F. D. Shi, et al. (2009). "Natural killer cells in human autoimmunity." Curr Opin Immunol **21**(6): 634-640.

Fogdell-Hahn, A., A. Ligers, M. Gronning, et al. (2000). "Multiple sclerosis: a modifying influence of HLA class I genes in an HLA class II associated autoimmune disease." Tissue Antigens **55**(2): 140-148.

Fox, R. J., M. Kita, S. L. Cohan, et al. (2014). "BG-12 (dimethyl fumarate): a review of mechanism of action, efficacy, and safety." Curr Med Res Opin **30**(2): 251-262.

Friese, M. A., K. B. Jakobsen, L. Friis, et al. (2008). "Opposing effects of HLA class I molecules in tuning autoreactive CD8+ T cells in multiple sclerosis." Nat Med **14**(11): 1227-1235.

Friese, M. A., B. Schattling and L. Fugger (2014). "Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis." Nat Rev Neurol **10**(4): 225-238.

Fritsche, J., K. Mondal, A. Ehrnsperger, et al. (2003). "Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha,25-dihydroxyvitamin D3 by human dendritic cells." Blood **102**(9): 3314-3316.

Fukazawa, T., I. Yabe, S. Kikuchi, et al. (1999). "Association of vitamin D receptor gene polymorphism with multiple sclerosis in Japanese." J Neurol Sci **166**(1): 47-52.

Garcion, E., N. Wion-Barbot, C. N. Montero-Menei, et al. (2002). "New clues about vitamin D functions in the nervous system." Trends Endocrinol Metab **13**(3): 100-105.

Garo, L. P. and G. Murugaiyan (2016). "Contribution of MicroRNAs to autoimmune diseases." Cell Mol Life Sci **73**(10): 2041-2051.

Gasperi, C., O. Stuve and B. Hemmer (2016). "B cell-directed therapies in multiple sclerosis." Neurodegener Dis Manag **6**(1): 37-47.

Ghadirian, P., B. Dadgostar, R. Azani, et al. (2001). "A case-control study of the association between socio-demographic, lifestyle and medical history factors and multiple sclerosis." Can J Public Health **92**(4): 281-285.

Gianfrancesco, M. A., B. Acuna, L. Shen, et al. (2014). "Obesity during childhood and adolescence increases susceptibility to multiple sclerosis after accounting for established genetic and environmental risk factors." Obes Res Clin Pract **8**(5): e435-447.

Gianfrancesco, M. A. and L. F. Barcellos (2016). "Obesity and Multiple Sclerosis Susceptibility: A Review." J Neurol Neuromedicine **1**(7): 1-5.

Giangreco, A. A. and L. Nonn (2013). "The sum of many small changes: microRNAs are specifically and potentially globally altered by vitamin D3 metabolites." J Steroid Biochem Mol Biol **136**: 86-93.

Gonzalez-Martin, A., B. D. Adams, M. Lai, et al. (2016). "The microRNA miR-148a functions as a critical regulator of B cell tolerance and autoimmunity." Nat Immunol **17**(4): 433-440.

Goris, A., S. Boonen, B. D'Hooghe M, et al. (2010). "Replication of KIF21B as a susceptibility locus for multiple sclerosis." J Med Genet **47**(11): 775-776.

Gorman, S., L. A. Kuritzky, M. A. Judge, et al. (2007). "Topically applied 1,25-dihydroxyvitamin D3 enhances the suppressive activity of CD4+CD25+ cells in the draining lymph nodes." J Immunol **179**(9): 6273-6283.

Gregory, S. G., S. Schmidt, P. Seth, et al. (2007). "Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis." Nat Genet **39**(9): 1083-1091.

Gross, C., A. V. Krishnan, P. J. Malloy, et al. (1998). "The vitamin D receptor gene start codon polymorphism: a functional analysis of FokI variants." J Bone Miner Res **13**(11): 1691-1699.

Gross, C. C., A. Schulte-Mecklenbeck, H. Wiendl, et al. (2016). "Regulatory Functions of Natural Killer Cells in Multiple Sclerosis." Front Immunol **7**: 606.

Haas, J., A. Hug, A. Viehover, et al. (2005). "Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis." Eur J Immunol **35**(11): 3343-3352.

Hafler, D. A., A. Compston, S. Sawcer, et al. (2007). "Risk alleles for multiple sclerosis identified by a genomewide study." N Engl J Med **357**(9): 851-862.

Haines, J. L., Y. Bradford, M. E. Garcia, et al. (2002). "Multiple susceptibility loci for multiple sclerosis." Hum Mol Genet **11**(19): 2251-2256.

Hampe, C. S. (2012). "B Cell in Autoimmune Diseases." Scientifica (Cairo) **2012**.

Harbo, H. F., P. O. Ekstrom, A. R. Lorentzen, et al. (2006). "Coding region polymorphisms in T cell signal transduction genes. Prevalence and association to development of multiple sclerosis." J Neuroimmunol **177**(1-2): 40-45.

Hartung, H. P., O. Aktas, T. Menge, et al. (2014). "Immune regulation of multiple sclerosis." Handb Clin Neurol **122**: 3-14.

Hauser, S. L., A. Bar-Or, G. Comi, et al. (2017). "Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis." N Engl J Med **376**(3): 221-234.

Healy, B. C., E. N. Ali, C. R. Guttmann, et al. (2009). "Smoking and disease progression in multiple sclerosis." Arch Neurol **66**(7): 858-864.

Hebert, S. S. and B. De Strooper (2009). "Alterations of the microRNA network cause neurodegenerative disease." Trends Neurosci **32**(4): 199-206.

Hedstrom, A. K., M. Baarnhielm, T. Olsson, et al. (2009). "Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis." Neurology **73**(9): 696-701.

Hedstrom, A. K., J. Hillert, T. Olsson, et al. (2013). "Smoking and multiple sclerosis susceptibility." Eur J Epidemiol **28**(11): 867-874.

Hedstrom, A. K., J. Hillert, T. Olsson, et al. (2014). "Alcohol as a modifiable lifestyle factor affecting multiple sclerosis risk." JAMA Neurol **71**(3): 300-305.

Hedstrom, A. K., E. M. Mowry, M. A. Gianfrancesco, et al. (2016). "High consumption of coffee is associated with decreased multiple sclerosis risk; results from two independent studies." J Neurol Neurosurg Psychiatry **87**(5): 454-460.

Heneka, M. T., M. P. Kummer and E. Latz (2014). "Innate immune activation in neurodegenerative disease." Nat Rev Immunol **14**(7): 463-477.

Hernan, M. A., S. S. Jick, G. Logroscino, et al. (2005). "Cigarette smoking and the progression of multiple sclerosis." Brain **128**(Pt 6): 1461-1465.

Hillert, J. (2006). "Multiple sclerosis: Postlinkage genetics." Clin Neurol Neurosurg **108**(3): 220-222.

Hinks, A., A. Barton, S. John, et al. (2005). "Association between the PTPN22 gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that PTPN22 is an autoimmunity gene." Arthritis Rheum **52**(6): 1694-1699.

Hoffman, W., F. G. Lakkis and G. Chalasani (2016). "B Cells, Antibodies, and More." Clin J Am Soc Nephrol **11**(1): 137-154.

Hohlfeld, R. and H. Wekerle (2004). "Autoimmune concepts of multiple sclerosis as a basis for selective immunotherapy: from pipe dreams to (therapeutic) pipelines." Proc Natl Acad Sci U S A **101 Suppl 2**: 14599-14606.

Holick, M. F. (2007). "Vitamin D deficiency." N Engl J Med **357**(3): 266-281.

Holick, M. F. (2009). "Vitamin D status: measurement, interpretation, and clinical application." Ann Epidemiol **19**(2): 73-78.

Holmoy, T. (2007). "Immunopathogenesis of multiple sclerosis: concepts and controversies." Acta Neurol Scand Suppl **187**: 39-45.

Hoppenbrouwers, I. A., Y. S. Aulchenko, A. C. Janssens, et al. (2009). "Replication of CD58 and CLEC16A as genome-wide significant risk genes for multiple sclerosis." J Hum Genet **54**(11): 676-680.

Horrigan, L. A., J. P. Kelly and T. J. Connor (2006). "Immunomodulatory effects of caffeine: friend or foe?" Pharmacol Ther **111**(3): 877-892.

Ingles, S. A., R. W. Haile, B. E. Henderson, et al. (1997). "Strength of linkage disequilibrium between two vitamin D receptor markers in five ethnic groups: implications for association studies." Cancer Epidemiol Biomarkers Prev **6**(2): 93-98.

Jagot, F. and N. Davoust (2016). "Is It worth Considering Circulating microRNAs in Multiple Sclerosis?" Front Immunol **7**: 129.

Jakkula, E., V. Leppa, A. M. Sulonen, et al. (2010). "Genome-wide association study in a high-risk isolate for multiple sclerosis reveals associated variants in STAT3 gene." Am J Hum Genet **86**(2): 285-291.

Jangi, S., R. Gandhi, L. M. Cox, et al. (2016). "Alterations of the human gut microbiome in multiple sclerosis." Nat Commun **7**: 12015.

Jersild, C., A. Svejgaard and T. Fog (1972). "HL-A antigens and multiple sclerosis." Lancet **1**(7762): 1240-1241.

Ji, W., B. Sun and C. Su (2017). "Targeting MicroRNAs in Cancer Gene Therapy." Genes (Basel) **8**(1).

Junker, A., M. Krumbholz, S. Eisele, et al. (2009). "MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47." Brain **132**(Pt 12): 3342-3352.

Jurutka, P. W., L. S. Remus, G. K. Whitfield, et al. (2000). "The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB." Mol Endocrinol **14**(3): 401-420.

Kakalacheva, K. and J. D. Lunemann (2011). "Environmental triggers of multiple sclerosis." FEBS Lett **585**(23): 3724-3729.

Kallberg, H., S. Jacobsen, C. Bengtsson, et al. (2009). "Alcohol consumption is associated with decreased risk of rheumatoid arthritis: results from two Scandinavian case-control studies." Ann Rheum Dis **68**(2): 222-227.

Kamali-Sarvestani, E., A. Nikseresht, E. Aflaki, et al. (2007). "TNF-alpha, TNF-beta and IL-4 gene polymorphisms in Iranian patients with multiple sclerosis." Acta Neurol Scand **115**(3): 161-166.

Kantarci, O. H., D. D. Hebrink, S. J. Achenbach, et al. (2004). "Association of APOE polymorphisms with disease severity in MS is limited to women." Neurology **62**(5): 811-814.

Keller, A., P. Leidinger, J. Lange, et al. (2009). "Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls." PLoS One **4**(10): e7440.

Kingwell, E., J. J. Marriott, N. Jette, et al. (2013). "Incidence and prevalence of multiple sclerosis in Europe: a systematic review." BMC Neurol **13**: 128.

Kleinewietfeld, M. and D. A. Hafler (2013a). "The plasticity of human Treg and Th17 cells and its role in autoimmunity." Semin Immunol **25**(4): 305-312.

Kleinewietfeld, M., A. Manzel, J. Titze, et al. (2013b). "Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells." Nature **496**(7446): 518-522.

Kosmaczewska, A., M. Bilinska, L. Ciszak, et al. (2007). "Different patterns of activation markers expression and CD4+ T-cell responses to ex vivo stimulation in patients with clinically quiescent multiple sclerosis (MS)." J Neuroimmunol **189**(1-2): 137-146.

Kotze, M. J., J. N. de Villiers, L. Warnich, et al. (2006). "Lack of clinical manifestation of hereditary haemochromatosis in South African patients with multiple sclerosis." Metab Brain Dis.

Kragt, J., B. van Amerongen, J. Killestein, et al. (2009). "Higher levels of 25-hydroxyvitamin D are associated with a lower incidence of multiple sclerosis only in women." Mult Scler **15**(1): 9-15.

Kurtzke, J. F. (1980). "Geographic distribution of multiple sclerosis: An update with special reference to Europe and the Mediterranean region." Acta Neurol Scand **62**(2): 65-80.

Kurtzke, J. F. (1983). "Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS)." Neurology **33**(11): 1444-1452.

Langer-Gould, A., S. M. Brara, B. E. Beaber, et al. (2013). "Childhood obesity and risk of pediatric multiple sclerosis and clinically isolated syndrome." Neurology **80**(6): 548-552.

Lassmann, H. (2007). "Multiple sclerosis: is there neurodegeneration independent from inflammation?" J Neurol Sci **259**(1-2): 3-6.

Lassmann, H. and J. van Horssen (2011). "The molecular basis of neurodegeneration in multiple sclerosis." FEBS Lett **585**(23): 3715-3723.

Lassmann, H., J. van Horssen and D. Mahad (2012). "Progressive multiple sclerosis: pathology and pathogenesis." Nat Rev Neurol **8**(11): 647-656.

Lee, Y. H., Y. H. Rho, S. J. Choi, et al. (2007). "The PTPN22 C1858T functional polymorphism and autoimmune diseases--a meta-analysis." Rheumatology (Oxford) **46**(1): 49-56.

LeVine, S. M. (1997). "Iron deposits in multiple sclerosis and Alzheimer's disease brains." Brain Res **760**(1-2): 298-303.

Li, Q. J., J. Chau, P. J. Ebert, et al. (2007). "miR-181a is an intrinsic modulator of T cell sensitivity and selection." Cell **129**(1): 147-161.

Lill, C. M. (2014). "Recent advances and future challenges in the genetics of multiple sclerosis." Front Neurol **5**: 130.

Lin, R., J. Charlesworth, J. Stankovich, et al. (2013). "Identity-by-descent mapping to detect rare variants conferring susceptibility to multiple sclerosis." PLoS One **8**(3): e56379.

Lips, P. (2007). "Relative value of 25(OH)D and 1,25(OH)2D measurements." J Bone Miner Res **22**(11): 1668-1671.

Lublin, F. D. and S. C. Reingold (1996). "Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis." Neurology **46**(4): 907-911.

Lublin, F. D., S. C. Reingold, J. A. Cohen, et al. (2014). "Defining the clinical course of multiple sclerosis: the 2013 revisions." Neurology **83**(3): 278-286.

Lucas, R. M., S. N. Byrne, J. Correale, et al. (2015). "Ultraviolet radiation, vitamin D and multiple sclerosis." Neurodegener Dis Manag **5**(5): 413-424.

Lumeng, C. N., J. L. Bodzin and A. R. Saltiel (2007). "Obesity induces a phenotypic switch in adipose tissue macrophage polarization." J Clin Invest **117**(1): 175-184.

Lundmark, F., K. Duvefelt, E. Iacobaeus, et al. (2007). "Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis." Nat Genet **39**(9): 1108-1113.

Ma, Y., D. L. Trump and C. S. Johnson (2014). "Vitamin D and miRNAs in cancer." Curr Gene Ther **14**(4): 269-275.

Madigand, M., J. J. Oger, R. Fauchet, et al. (1982). "HLA profiles in multiple sclerosis suggest two forms of disease and the existence of protective haplotypes." J Neurol Sci **53**(3): 519-529.

Mahad, D. H., B. D. Trapp and H. Lassmann (2015). "Pathological mechanisms in progressive multiple sclerosis." Lancet Neurol **14**(2): 183-193.

Mamutse, G., J. Woolmore, E. Pye, et al. (2008). "Vitamin D receptor gene polymorphism is associated with reduced disability in multiple sclerosis." Mult Scler **14**(9): 1280-1283.

Manouchehrinia, A., C. R. Tench, J. Maxted, et al. (2013). "Tobacco smoking and disability progression in multiple sclerosis: United Kingdom cohort study." Brain **136**(Pt 7): 2298-2304.

Manouchehrinia, A., M. Weston, C. R. Tench, et al. (2014). "Tobacco smoking and excess mortality in multiple sclerosis: a cohort study." J Neurol Neurosurg Psychiatry **85**(10): 1091-1095.

Martinelli-Boneschi, F., C. Fenoglio, P. Brambilla, et al. (2012). "MicroRNA and mRNA expression profile screening in multiple sclerosis patients to unravel novel pathogenic steps and identify potential biomarkers." Neurosci Lett **508**(1): 4-8.

Martins Silva, B., T. Thorlacius, K. Benediktsson, et al. (2003). "A whole genome association study in multiple sclerosis patients from north Portugal." J Neuroimmunol **143**(1-2): 116-119.

Massa, J., E. J. O'Reilly, K. L. Munger, et al. (2013). "Caffeine and alcohol intakes have no association with risk of multiple sclerosis." Mult Scler **19**(1): 53-58.

Matarese, G., P. B. Carrieri, A. La Cava, et al. (2005). "Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25+ regulatory T cells." Proc Natl Acad Sci U S A **102**(14): 5150-5155.

Matarese, G., P. B. Carrieri, S. Montella, et al. (2010). "Leptin as a metabolic link to multiple sclerosis." Nat Rev Neurol **6**(8): 455-461.

Matesanz, F., B. Rueda, G. Orozco, et al. (2005). "Protein tyrosine phosphatase gene (PTPN22) polymorphism in multiple sclerosis." J Neurol **252**(8): 994-995.

Mattson, M. P. (2005). "NF-kappaB in the survival and plasticity of neurons." Neurochem Res **30**(6-7): 883-893.

Meuth, S. G., K. Gobel and H. Wiendl (2012). "Immune therapy of multiple sclerosis--future strategies." Curr Pharm Des **18**(29): 4489-4497.

Mihailova, S., M. Ivanova, A. Mihaylova, et al. (2005). "Pro- and anti-inflammatory cytokine gene polymorphism profiles in Bulgarian multiple sclerosis patients." J Neuroimmunol **168**(1-2): 138-143.

Mikaeloff, Y., G. Caridade, M. Tardieu, et al. (2007). "Parental smoking at home and the risk of childhood-onset multiple sclerosis in children." Brain **130**(Pt 10): 2589-2595.

Miller, D. H., B. G. Weinshenker, M. Filippi, et al. (2008). "Differential diagnosis of suspected multiple sclerosis: a consensus approach." Mult Scler **14**(9): 1157-1174.

Milo, R. (2016). "Therapeutic strategies targeting B-cells in multiple sclerosis." Autoimmun Rev **15**(7): 714-718.

Milo, R. and A. Miller (2014). "Revised diagnostic criteria of multiple sclerosis." Autoimmun Rev **13**(4-5): 518-524.

Minagar, A., E. G. Toledo, J. S. Alexander, et al. (2004). "Pathogenesis of brain and spinal cord atrophy in multiple sclerosis." J Neuroimaging **14**(3 Suppl): 5S-10S.

Miyake, S., S. Kim, W. Suda, et al. (2015). "Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters." PLoS One **10**(9): e0137429.

Modrego Pardo, P. J., M. A. Latorre, A. Lopez, et al. (1997). "Prevalence of multiple sclerosis in the province of Teruel, Spain." J Neurol **244**(3): 182-185.

Montalban, X., S. L. Hauser, L. Kappos, et al. (2017). "Ocrelizumab versus Placebo in Primary Progressive Multiple Sclerosis." N Engl J Med **376**(3): 209-220.

Muller, K., C. Heilmann, L. K. Poulsen, et al. (1991). "The role of monocytes and T cells in 1,25-dihydroxyvitamin D3 mediated inhibition of B cell function in vitro." Immunopharmacology **21**(2): 121-128.

Mumford, C. J., N. W. Wood, H. Kellar-Wood, et al. (1994). "The British Isles survey of multiple sclerosis in twins." Neurology **44**(1): 11-15.

Munger, K. L., J. Aivo, K. Hongell, et al. (2016). "Vitamin D Status During Pregnancy and Risk of Multiple Sclerosis in Offspring of Women in the Finnish Maternity Cohort." JAMA Neurol **73**(5): 515-519.

Munger, K. L., J. Bentzen, B. Laursen, et al. (2013). "Childhood body mass index and multiple sclerosis risk: a long-term cohort study." Mult Scler **19**(10): 1323-1329.

Munger, K. L., L. I. Levin, B. W. Hollis, et al. (2006). "Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis." Jama **296**(23): 2832-2838.

Munger, K. L., S. M. Zhang, E. O'Reilly, et al. (2004). "Vitamin D intake and incidence of multiple sclerosis." Neurology **62**(1): 60-65.

Munz, C., J. D. Lunemann, M. T. Getts, et al. (2009). "Antiviral immune responses: triggers of or triggered by autoimmunity?" Nat Rev Immunol **9**(4): 246-258.

Naito, S., N. Namerow, M. R. Mickey, et al. (1972). "Multiple sclerosis: association with HL-A3." Tissue Antigens **2**(1): 1-4.

Nesic, D., J. Cheng and L. E. Maquat (1993). "Sequences within the last intron function in RNA 3'-end formation in cultured cells." Mol Cell Biol **13**(6): 3359-3369.

Nguyen, T., P. J. Sherratt and C. B. Pickett (2003). "Regulatory mechanisms controlling gene expression mediated by the antioxidant response element." Annu Rev Pharmacol Toxicol **43**: 233-260.

Nielsen, N. M., K. L. Munger, N. Koch-Henriksen, et al. (2017). "Neonatal vitamin D status and risk of multiple sclerosis: A population-based case-control study." Neurology **88**(1): 44-51.

Nieves, J., F. Cosman, J. Herbert, et al. (1994). "High prevalence of vitamin D deficiency and reduced bone mass in multiple sclerosis." Neurology **44**(9): 1687-1692.

Niino, M., T. Fukazawa, I. Yabe, et al. (2000). "Vitamin D receptor gene polymorphism in multiple sclerosis and the association with HLA class II alleles." J Neurol Sci **177**(1): 65-71.

Noori-Zadeh, A., S. A. Mesbah-Namin, S. Bistoon-Beigloo, et al. (2016). "Regulatory T cell number in multiple sclerosis patients: A meta-analysis." Mult Scler Relat Disord **5**: 73-76.

Noseworthy, J. H., C. Lucchinetti, M. Rodriguez, et al. (2000). "Multiple sclerosis." N Engl J Med **343**(13): 938-952.

O'Connell, R. M., D. S. Rao and D. Baltimore (2012). "microRNA regulation of inflammatory responses." Annu Rev Immunol **30**: 295-312.

O'Connell, R. M., D. S. Rao, A. A. Chaudhuri, et al. (2010). "Physiological and pathological roles for microRNAs in the immune system." Nat Rev Immunol **10**(2): 111-122.

O'Gorman, C., R. Lin, J. Stankovich, et al. (2013). "Modelling genetic susceptibility to multiple sclerosis with family data." Neuroepidemiology **40**(1): 1-12.

Obi, Y., T. Hamano and Y. Isaka (2015). "Prevalence and prognostic implications of vitamin D deficiency in chronic kidney disease." Dis Markers **2015**: 868961.

Ohta, M., T. Okabe, K. Ozawa, et al. (1985). "1 alpha,25-Dihydroxyvitamin D3 (calcitriol) stimulates proliferation of human circulating monocytes in vitro." FEBS Lett **185**(1): 9-13.

Oksenberg, J. R. and S. E. Baranzini (2010). "Multiple sclerosis genetics--is the glass half full, or half empty?" Nat Rev Neurol **6**(8): 429-437.

Oksenberg, J. R. and L. F. Barcellos (2005). "Multiple sclerosis genetics: leaving no stone unturned." Genes Immun **6**(5): 375-387.

Olerup, O. and J. Hillert (1991). "HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation." Tissue Antigens **38**(1): 1-15.

Oliveira, E. M., A. Bar-Or, A. I. Waliszewska, et al. (2003). "CTLA-4 dysregulation in the activation of myelin basic protein reactive T cells may distinguish patients with multiple sclerosis from healthy controls." J Autoimmun **20**(1): 71-81.

Orton, S. M., A. P. Morris, B. M. Herrera, et al. (2008). "Evidence for genetic regulation of vitamin D status in twins with multiple sclerosis." Am J Clin Nutr **88**(2): 441-447.

Orton, S. M., S. V. Ramagopalan, A. E. Para, et al. (2011). "Vitamin D metabolic pathway genes and risk of multiple sclerosis in Canadians." J Neurol Sci **305**(1-2): 116-120.

Otaegui, D., S. E. Baranzini, R. Armananzas, et al. (2009). "Differential micro RNA expression in PBMC from multiple sclerosis patients." PLoS One **4**(7): e6309.

Palacios, N., A. Alonso, H. Bronnum-Hansen, et al. (2011). "Smoking and increased risk of multiple sclerosis: parallel trends in the sex ratio reinforce the evidence." Ann Epidemiol **21**(7): 536-542.

Palmer, E. (2003). "Negative selection--clearing out the bad apples from the T-cell repertoire." Nat Rev Immunol **3**(5): 383-391.

Partridge, J. M., S. J. Weatherby, J. A. Woolmore, et al. (2004). "Susceptibility and outcome in MS: associations with polymorphisms in pigmentation-related genes." Neurology **62**(12): 2323-2325.

Penna, G. and L. Adorini (2000). "1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation." J Immunol **164**(5): 2405-2411.

Penna, G., A. Roncari, S. Amuchastegui, et al. (2005). "Expression of the inhibitory receptor ILT3 on dendritic cells is dispensable for induction of CD4+Foxp3+ regulatory T cells by 1,25-dihydroxyvitamin D3." Blood **106**(10): 3490-3497.

Pereira, C., A. M. Silva, C. Carvalho, et al. (2005). TNFA promoter polymorphisms in Portuguese Multiple Sclerosis patients. 19th European Immunogenetics & Histocompatibility Conference.

Pinholt, M., J. L. Frederiksen, P. S. Andersen, et al. (2005). "Apo E in multiple sclerosis and optic neuritis: the apo E-epsilon4 allele is associated with progression of multiple sclerosis." Mult Scler **11**(5): 511-515.

Pittas, F., A. L. Ponsonby, I. A. van der Mei, et al. (2009). "Smoking is associated with progressive disease course and increased progression in clinical disability in a prospective cohort of people with multiple sclerosis." J Neurol **256**(4): 577-585.

Podbielska, M., N. L. Banik, E. Kurowska, et al. (2013). "Myelin recovery in multiple sclerosis: the challenge of remyelination." Brain Sci **3**(3): 1282-1324.

Polman, C. H., S. C. Reingold, B. Banwell, et al. (2011). "Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria." Ann Neurol **69**(2): 292-302.

Polman, C. H., S. C. Reingold, G. Edan, et al. (2005). "Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria"." Ann Neurol **58**(6): 840-846.

Poorolajal, J., M. Bahrami, M. Karami, et al. (2016). "Effect of smoking on multiple sclerosis: a meta-analysis." J Public Health (Oxf).

Popescu, B. F., I. Pirko and C. F. Lucchinetti (2013). "Pathology of multiple sclerosis: where do we stand?" Continuum (Minneap Minn) **19**(4 Multiple Sclerosis): 901-921.

Provvedini, D. M., C. D. Tsoukas, L. J. Deftos, et al. (1983). "1,25-dihydroxyvitamin D3 receptors in human leukocytes." Science **221**(4616): 1181-1183.

Rajewsky, N. and N. D. Socci (2004). "Computational identification of microRNA targets." Dev Biol **267**(2): 529-535.

Ramagopalan, S. V., M. Cukjati, M. Cernilec, et al. (2008). "Mutations in the hemochromatosis gene and the clinical outcome of multiple sclerosis." J Neuroimmunol **203**(1): 104-107.

Ramagopalan, S. V., G. C. Deluca, K. M. Morrison, et al. (2007). "No effect of APOE and PVRL2 on the clinical outcome of multiple sclerosis." J Neuroimmunol **186**(1-2): 156-160.

Ramagopalan, S. V., J. D. Lee, I. M. Yee, et al. (2013). "Association of smoking with risk of multiple sclerosis: a population-based study." J Neurol **260**(7): 1778-1781.

Ramagopalan, S. V., N. J. Maugeri, L. Handunnetthi, et al. (2009). "Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D." PLoS Genet **5**(2): e1000369.

Rigby, W. F., M. Waugh and R. F. Graziano (1990). "Regulation of human monocyte HLA-DR and CD4 antigen expression, and antigen presentation by 1,25-dihydroxyvitamin D3." Blood **76**(1): 189-197.

Riise, T., M. W. Nortvedt and A. Ascherio (2003). "Smoking is a risk factor for multiple sclerosis." Neurology **61**(8): 1122-1124.

Ristic, S., L. Lovrecic, B. Brajenovic-Milic, et al. (2005). "Mutations in the hemochromatosis gene (HFE) and multiple sclerosis." Neurosci Lett **383**(3): 301-304.

Rodrigues, M. O., A. Fonseca, C. Matias Dias, et al. (2005). "APOE genotypes and dyslipidemias in a sample of the Portuguese population." Clin Chem Lab Med **43**(9): 907-912.

Rojo, A. I., G. McBean, M. Cindric, et al. (2014). "Redox control of microglial function: molecular mechanisms and functional significance." Antioxid Redox Signal **21**(12): 1766-1801.

Roxburgh, R. H., S. R. Seaman, T. Masterman, et al. (2005). "Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity." Neurology **64**(7): 1144-1151.

Rubio, J. P., M. Bahlo, N. Tubridy, et al. (2004). "Extended haplotype analysis in the HLA complex reveals an increased frequency of the HFE-C282Y mutation in individuals with multiple sclerosis." Hum Genet **114**(6): 573-580.

Rubio, J. P., J. Stankovich, J. Field, et al. (2008). "Replication of KIAA0350, IL2RA, RPL5 and CD58 as multiple sclerosis susceptibility genes in Australians." Genes Immun **9**(7): 624-630.

Sadovnick, A. D. (1993). "Familial recurrence risks and inheritance of multiple sclerosis." Curr Opin Neurol Neurosurg **6**(2): 189-194.

Sadovnick, A. D., A. Dircks and G. C. Ebers (1999). "Genetic counselling in multiple sclerosis: risks to sibs and children of affected individuals." Clin Genet **56**(2): 118-122.

Sadovnick, A. D., P. Duquette, B. Herrera, et al. (2007). "A timing-of-birth effect on multiple sclerosis clinical phenotype." Neurology **69**(1): 60-62.

Sadovnick, A. D. and G. C. Ebers (1995). "Genetics of multiple sclerosis." Neurol Clin **13**(1): 99-118.

Salou, M., B. Nicol, A. Garcia, et al. (2015). "Involvement of CD8(+) T Cells in Multiple Sclerosis." Front Immunol **6**: 604.

Santos, M., M. do Carmo Costa, M. Edite Rio, et al. (2004). "Genotypes at the APOE and SCA2 loci do not predict the course of multiple sclerosis in patients of Portuguese origin." Mult Scler **10**(2): 153-157.

Santos, M., J. Pinto-Basto, M. E. Rio, et al. (2003). "A whole genome screen for association with multiple sclerosis in Portuguese patients." J Neuroimmunol **143**(1-2): 112-115.

Sawcer, S. (2008). "The complex genetics of multiple sclerosis: pitfalls and prospects." Brain **131**(Pt 12): 3118-3131.

Sawcer, S., G. Hellenthal, M. Pirinen, et al. (2011). "Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis." Nature **476**(7359): 214-219.

Schmidt, A., N. Oberle and P. H. Krammer (2012). "Molecular mechanisms of treg-mediated T cell suppression." Front Immunol **3**: 51.

Schmidt, H., D. Williamson and A. Ashley-Koch (2007). "HLA-DR15 haplotype and multiple sclerosis: a HuGE review." Am J Epidemiol **165**(10): 1097-1109.

Selmaj, K., C. S. Raine, B. Cannella, et al. (1991). "Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions." J Clin Invest **87**(3): 949-954.

Sena, A., R. Couderc, V. Ferret-Sena, et al. (2009). "Apolipoprotein E polymorphism interacts with cigarette smoking in progression of multiple sclerosis." Eur J Neurol **16**(7): 832-837.

Shimura, M. and T. Miura (1987). "Season of birth in some neurological disorders: multiple sclerosis, ALS, senile dementia." Prog Biometeorol **6**: 163-168.

Shirakawa, A. K., D. Nagakubo, K. Hieshima, et al. (2008). "1,25-dihydroxyvitamin D3 induces CCR10 expression in terminally differentiating human B cells." J Immunol **180**(5): 2786-2795.

Silva, A. M., A. Bettencourt, C. Pereira, et al. (2009). "Protective role of the HLA-A*02 allele in Portuguese patients with multiple sclerosis." Mult Scler **15**(6): 771-774.

Silva, A. M., C. Pereira, A. Bettencourt, et al. (2007a). "The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population." J Neurol Sci **258**(1-2): 69-74.

Silva, A. M., E. Vilhena, A. Bettencourt, et al. (2007b). APOE genotypes in a Portuguese Multiple Sclerosis population: no association with demographic and clinical variables. 23rd Congress of the European Committee for Treatment and Research in Multiple Sclerosis, Prague, Multiple Sclerosis.

Simon, K. C., K. L. Munger, Y. Xing, et al. (2010). "Polymorphisms in vitamin D metabolism related genes and risk of multiple sclerosis." Mult Scler **16**(2): 133-138.

Simpson, L. J. and K. M. Ansel (2015). "MicroRNA regulation of lymphocyte tolerance and autoimmunity." J Clin Invest **125**(6): 2242-2249.

Sinha, S., A. W. Boyden, F. R. Itani, et al. (2015). "CD8(+) T-Cells as Immune Regulators of Multiple Sclerosis." Front Immunol **6**: 619.

Sioka, C., S. Papakonstantinou, S. Markoula, et al. (2011). "Vitamin D receptor gene polymorphisms in multiple sclerosis patients in northwest Greece." J Negat Results Biomed **10**(1): 3.

Smolders, J., J. Damoiseaux, P. Menheere, et al. (2008a). "Vitamin D as an immune modulator in multiple sclerosis, a review." J Neuroimmunol **194**(1-2): 7-17.

Smolders, J., J. Damoiseaux, P. Menheere, et al. (2009a). "Association study on two vitamin D receptor gene polymorphisms and vitamin D metabolites in multiple sclerosis." Ann N Y Acad Sci **1173**: 515-520.

Smolders, J., J. Damoiseaux, P. Menheere, et al. (2009b). "Fok-I vitamin D receptor gene polymorphism (rs10735810) and vitamin D metabolism in multiple sclerosis." J Neuroimmunol **207**(1-2): 117-121.

Smolders, J., P. Menheere, A. Kessels, et al. (2008b). "Association of vitamin D metabolite levels with relapse rate and disability in multiple sclerosis." Mult Scler **14**(9): 1220-1224.

Smolders, J., S. M. Moen, J. Damoiseaux, et al. (2011). "Vitamin D in the healthy and inflamed central nervous system: access and function." J Neurol Sci **311**(1-2): 37-43.

Smolders, J., E. Peelen, M. Thewissen, et al. (2009c). "The relevance of vitamin D receptor gene polymorphisms for vitamin D research in multiple sclerosis." Autoimmun Rev **8**(7): 621-626.

Smolders, J., M. Thewissen, E. Peelen, et al. (2009d). "Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis." PLoS One **4**(8): e6635.

Soilu-Hanninen, M., L. Airas, I. Mononen, et al. (2005). "25-Hydroxyvitamin D levels in serum at the onset of multiple sclerosis." Mult Scler **11**(3): 266-271.

Soilu-Hanninen, M., M. Laaksonen, I. Laitinen, et al. (2008). "A longitudinal study of serum 25-hydroxyvitamin D and intact parathyroid hormone levels indicate the importance of vitamin D and calcium homeostasis regulation in multiple sclerosis." J Neurol Neurosurg Psychiatry **79**(2): 152-157.

Sospedra, M. and R. Martin (2005). "Immunology of multiple sclerosis." Annu Rev Immunol **23**: 683-747.

Sprent, J. and H. Kishimoto (2001a). "The thymus and central tolerance." Philos Trans R Soc Lond B Biol Sci **356**(1409): 609-616.

Sprent, J. and H. Kishimoto (2001b). "The thymus and central tolerance." Transplantation **72**(8 Suppl): S25-28.

Staples, J., A. L. Ponsonby and L. Lim (2010). "Low maternal exposure to ultraviolet radiation in pregnancy, month of birth, and risk of multiple sclerosis in offspring: longitudinal analysis." BMJ **340**: c1640.

Starr, T. K., S. C. Jameson and K. A. Hogquist (2003). "Positive and negative selection of T cells." Annu Rev Immunol **21**: 139-176.

Steckley, J. L., D. A. Dyment, A. D. Sadovnick, et al. (2000). "Genetic analysis of vitamin D related genes in Canadian multiple sclerosis patients. Canadian Collaborative Study Group." Neurology **54**(3): 729-732.

Sundqvist, E., M. Baarnhielm, L. Alfredsson, et al. (2010). "Confirmation of association between multiple sclerosis and CYP27B1." Eur J Hum Genet **18**(12): 1349-1352.

Sundstrom, P. and L. Nystrom (2008). "Smoking worsens the prognosis in multiple sclerosis." Mult Scler **14**(8): 1031-1035.

Suzumura, A. (2013). "Neuron-microglia interaction in neuroinflammation." Curr Protein Pept Sci **14**(1): 16-20.

Tajouri, L., M. Ovcaric, R. Curtain, et al. (2005). "Variation in the vitamin D receptor gene is associated with multiple sclerosis in an Australian population." J Neurogenet **19**(1): 25-38.

Takahashi, K., Y. Nakayama, H. Horiuchi, et al. (2002). "Human neutrophils express messenger RNA of vitamin D receptor and respond to 1alpha,25-dihydroxyvitamin D3." Immunopharmacol Immunotoxicol **24**(3): 335-347.

Templer, D. I., N. H. Trent, D. A. Spencer, et al. (1992). "Season of birth in multiple sclerosis." Acta Neurol Scand **85**(2): 107-109.

Torkildsen, O., J. Aarseth, E. Benjaminsen, et al. (2014). "Month of birth and risk of multiple sclerosis: confounding and adjustments." Ann Clin Transl Neurol **1**(2): 141-144.

Torkildsen, O., N. Grytten, J. Aarseth, et al. (2012). "Month of birth as a risk factor for multiple sclerosis: an update." Acta Neurol Scand Suppl(195): 58-62.

Tremlett, H., D. W. Fadrosh, A. A. Faruqi, et al. (2016). "Gut microbiota in early pediatric multiple sclerosis: a case-control study." Eur J Neurol **23**(8): 1308-1321.

Trowsdale, J. and J. C. Knight (2013). "Major histocompatibility complex genomics and human disease." Annu Rev Genomics Hum Genet **14**: 301-323.

Uria, D. F., P. Abad, M. T. Calatayud, et al. (1997). "Multiple sclerosis in Gijon health district, Asturias, northern Spain." Acta Neurol Scand **96**(6): 375-379.

van Bergen, J., A. Thompson, A. van der Slik, et al. (2004). "Phenotypic and functional characterization of CD4 T cells expressing killer Ig-like receptors." J Immunol **173**(11): 6719-6726.

van der Mei, I. A., A. L. Ponsonby, T. Dwyer, et al. (2007). "Vitamin D levels in people with multiple sclerosis and community controls in Tasmania, Australia." J Neurol **254**(5): 581-590.

van der Walt, A., J. Stankovich, M. Bahlo, et al. (2009). "Apolipoprotein genotype does not influence MS severity, cognition, or brain atrophy." Neurology **73**(13): 1018-1025.

van Etten, E. and C. Mathieu (2005). "Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts." J Steroid Biochem Mol Biol **97**(1-2): 93-101.

van Etten, E., L. Verlinden, A. Giulietti, et al. (2007). "The vitamin D receptor gene FokI polymorphism: functional impact on the immune system." Eur J Immunol **37**(2): 395-405.

Veldman, C. M., M. T. Cantorna and H. F. DeLuca (2000). "Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system." Arch Biochem Biophys **374**(2): 334-338.

Venken, K., N. Hellings, K. Hensen, et al. (2006). "Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression." J Neurosci Res **83**(8): 1432-1446.

Verstuyf, A., G. Carmeliet, R. Bouillon, et al. (2010). "Vitamin D: a pleiotropic hormone." Kidney Int **78**(2): 140-145.

Viglietta, V., C. Baecher-Allan, H. L. Weiner, et al. (2004). "Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis." J Exp Med **199**(7): 971-979.

Vivier, E., D. H. Raulet, A. Moretta, et al. (2011). "Innate or adaptive immunity? The example of natural killer cells." Science **331**(6013): 44-49.

Vivier, E., E. Tomasello, M. Baratin, et al. (2008). "Functions of natural killer cells." Nat Immunol **9**(5): 503-510.

Wagner, C. L., S. N. Taylor, D. D. Johnson, et al. (2012). "The role of vitamin D in pregnancy and lactation: emerging concepts." Womens Health (Lond) **8**(3): 323-340.

Weatherby, S. J., W. Thomson, L. Pepper, et al. (2001). "HLA-DRB1 and disease outcome in multiple sclerosis." J Neurol **248**(4): 304-310.

Weber, M. and D. Schubeler (2007). "Genomic patterns of DNA methylation: targets and function of an epigenetic mark." Curr Opin Cell Biol **19**(3): 273-280.

Wekerle, H. (2017). "B cells in multiple sclerosis." Autoimmunity **50**(1): 57-60.

Westerlind, H., R. Ramanujam, D. Uvehag, et al. (2014). "Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden." Brain **137**(Pt 3): 770-778.

Whitfield, G. K., L. S. Remus, P. W. Jurutka, et al. (2001). "Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene." Mol Cell Endocrinol **177**(1-2): 145-159.

Willer, C. J., D. A. Dyment, A. D. Sadovnick, et al. (2005). "Timing of birth and risk of multiple sclerosis: population based study." BMJ **330**(7483): 120.

Wimalawansa, S. J. (2016). "Non-musculoskeletal benefits of vitamin D." J Steroid Biochem Mol Biol.

Wingerchuk, D. M. and J. L. Carter (2014). "Multiple sclerosis: current and emerging disease-modifying therapies and treatment strategies." Mayo Clin Proc **89**(2): 225-240.

Wion, D., D. MacGrogan, I. Neveu, et al. (1991). "1,25-Dihydroxyvitamin D3 is a potent inducer of nerve growth factor synthesis." J Neurosci Res **28**(1): 110-114.

Wortsman, J., L. Y. Matsuoka, T. C. Chen, et al. (2000). "Decreased bioavailability of vitamin D in obesity." Am J Clin Nutr **72**(3): 690-693.

Wu, C., N. Yosef, T. Thalhamer, et al. (2013). "Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1." Nature **496**(7446): 513-517.

Xu, C., Y. Dai, J. C. Lorentzen, et al. (2001). "Linkage analysis in multiple sclerosis of chromosomal regions syntenic to experimental autoimmune disease loci." Eur J Hum Genet **9**(6): 458-463.

Yeo, T. W., P. L. De Jager, S. G. Gregory, et al. (2007). "A second major histocompatibility complex susceptibility locus for multiple sclerosis." Ann Neurol **61**(3): 228-236.

Yeo, T. W., M. Maranian, S. Singlehurst, et al. (2004). "Four single nucleotide polymorphisms from the vitamin D receptor gene in UK multiple sclerosis." J Neurol **251**(6): 753-754.

Zamboni, P. (2006). "The big idea: iron-dependent inflammation in venous disease and proposed parallels in multiple sclerosis." J R Soc Med **99**(11): 589-593.

Zamboni, P., R. Galeotti, E. Menegatti, et al. (2009). "Chronic cerebrospinal venous insufficiency in patients with multiple sclerosis." J Neurol Neurosurg Psychiatry **80**(4): 392-399.

Zhang, H. L., J. Wu and J. Zhu (2010). "The immune-modulatory role of apolipoprotein E with emphasis on multiple sclerosis and experimental autoimmune encephalomyelitis." Clin Dev Immunol **2010**: 186813.

Zhang, J., Y. Cheng, W. Cui, et al. (2014). "MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with multiple sclerosis and experimental autoimmune encephalomyelitis." J Neuroimmunol **266**(1-2): 56-63.

Zhang, X., J. Reddy, H. Ochi, et al. (2006). "Recovery from experimental allergic encephalomyelitis is TGF-beta dependent and associated with increases in CD4+LAP+ and CD4+CD25+ T cells." Int Immunol **18**(4): 495-503.

Zivadinov, R., B. Weinstock-Guttman, K. Hashmi, et al. (2009). "Smoking is associated with increased lesion volumes and brain atrophy in multiple sclerosis." Neurology **73**(7): 504-510.

Zostawa, J., J. Adamczyk, P. Sowa, et al. (2017). "The influence of sodium on pathophysiology of multiple sclerosis." Neurol Sci **38**(3): 389-398.

CHAPTER 2

Study Design

2.1 - Aim of the study

Despite extensive research, the causal pathway in MS pathogenesis remains unknown. Decades of research have shed some light on the prognostic factors for MS. However, predicting the clinical future of these patients is still a difficult task. With the advent of new genetic technologies, an expansion of known risk genes for MS has emerged. However, their contribution as risk genes was shown to be quite modest.

The overall aim of this study was to investigate genetic and non-genetic factors, involved in immune dysfunction that contributes to disease susceptibility and clinical outcome in Portuguese MS patients.

To evaluate the genetic risk, the study design was based on a hypothesis-driven candidate gene approach. Genes involved in immune system regulation were regarded as of special interest for these studies. Paper 1 explored the role of HLA-DRB1 alleles in MS susceptibility in a large cohort of patients. Papers 2 and 3 investigated other promising candidate genes in MS. In Paper 2 KIR genes that have previously been found of importance to MS and other autoimmune diseases were studied. The Nrf2 pathway is an intrinsic cellular defence system that defends against oxidative, inflammatory, and xenobiotic stress, and was investigated in Paper 3. Epigenetic mechanisms, namely microRNAs, have an effect on MS risk and their role was explored in Paper 4.

Environmental risk factors also contribute to the risk of MS and disease course. This issue is addressed in Chapter 3.2 by focusing on vitamin D. The role of month of birth (Paper 5), vitamin D levels (Paper 6 and 7) and VDR polymorphism FokI (Paper 8) was explored.

2.2 - Subjects and methods

2.2.1 – Analysed Cohorts

2.2.1.1 - Patients

Genetic studies were developed as retrospective case-control studies. A group of unrelated patients with MS was recruited from the neurology outpatient clinic of the Centro Hospitalar do Porto-Hospital de Santo António. In paper 4 a group of unrelated patients with MS from the Centro Hospitalar São João was also included. All patients were from the north of Portugal and the diagnosis of MS was done according to the revised McDonald criteria (Polman et al. 2005). Clinical and demographic information such as sex, age at onset, clinical course, EDSS and MSSS were obtained from the patient database at the time of blood and serum collection.

To investigate the association between the different metabolites, genes and disease aggressiveness, patients with relapsing–remitting (RR) and secondary progressive (SP) disease were subdivided into three groups: (i) benign course (patients with EDSS (Kurtzke 1983) score of ≤3 at least 10 years after disease onset); (ii) non-benign course (EDSS scores >3 after the same period); and (iii) aggressive course (EDSS scores ≥ 6 within 15 years of disease onset) (McDonnell et al. 1999; Silva et al. 2007).

2.2.2.2 - Controls

A group of control subjects, with the same ethnic and geographical origin as the patients, and with no history of neurological diseases, was randomly recruited among healthy blood donors and ICBAS personnel. A questionnaire was performed to exclude family history of neurological and autoimmune diseases.

This study was approved by the Medical Ethical Committee of the hospital and written informed consent was obtained from all participants.

Genetic association studies

The main goal of population-based association studies is to identify genetic differences related to phenotype among individuals in a study population, thus potentially identifying (high) risk or protective alleles. Hardy-Weinberg Equilibrium (HWE) describes and predicts allele and genotype frequencies in ideal, non-evolving populations, in which mutations, gene flow, selection, genetic drift and limited population size do not occur (Ryckman et al. 2008). In such populations the allele and genotype frequencies remain

unchanged over successive generations. Thus, inbreeding, population stratification and selection can lead to divergences of HWE. Nevertheless, deviations from HWE can also be an indicator for disease association and are often underestimated (Wittke-Thompson et al. 2005). In genetic studies HWE has to be tested in order to evaluate possible divergences.

Two approaches for candidate gene analyses can be followed both of which are widely used for genetic association studies. Candidate genes can be chosen under the assumption of functional relevance in the pathogenesis of the disease. The other approach focuses on the replication and further evaluation of association results that have been already described by other groups. Both approaches were used in the genetic studies.

2.2.2 – Methods

2.2.2.1 - DNA/RNA extraction and quantification

Peripheral blood samples (10 ml) from patients and controls were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes using a Salting-Out procedure (Miller et al. 1988). This method involves dehydration and precipitation with a saturated NaCl solution. Buffy coats of nucleated cells were re-suspended in 50 ml polypropylene centrifugation tubes with 40 ml of red cell lysis buffer (1M Tris-HCl, 5M NaCl and 1M MgCl2.6H2O, pH 8.2). The cell lysates were digested overnight at 42°C with 0.2 ml of 10% Sodium lauryl sulphate, 3.5 ml of TE2 buffer (1M Tris-HCL, 5M NaCl and 0.5M EDTA, pH7.2) and 0.1 ml of protease K. After digestion was complete, 1 ml of saturated NaCl (6M) was added to a 15 ml tube and shaken vigorously for 15 seconds, followed by centrifugation at 3000 rpm for 30 minutes at room temperature. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to a 50 ml polypropylene tube. Exactly 2 volumes of absolute ethanol cold (-20ºC) was added and the tubes inverted several times until the DNA precipitated. The precipitated DNA strands were removed with a plastic pipette and transferred to a 1.5 ml microcentrifuge tube containing TE buffer (1M Tris-HCl, 0.5M EDTA, pH 7.2). The DNA was allowed to dissolve 2 hours at 37°C before quantification.

RNA extraction from serum samples was done using the miRNeasy Serum/Plasma Kit. All the procedures were done in a vertical laminar flow chamber with sterilized material. The principle of this kit is that it combines phenol/guanidine-based lysis of samples and silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the

kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis, to denature protein complexes and RNases, and also to remove most of the residual DNA and proteins from the lysate by organic extraction. QIAzol Lysis Reagent is added to serum samples. After addition of chloroform, the lysate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of RNase-free water.

DNA and RNA quantification is an important and necessary step prior to most DNA/RNA analysis methods. The absorbance of a diluted RNA sample was measured at 260 nm for nucleic acid concentration determination. The sample was measured also at 280 nm to detect the presence of other contaminants such as residual proteins and phenol can interfere with absorbance readings. The A260/A280 ratio is used to assess RNA purity. An A260/A280 ratio of 1.8-2.1 is indicative of highly purified RNA.

All the DNA samples were set at a final concentration of 50 ng/µl and all RNA samples were set at a final concentration of 2ng/ µl.

2.2.2.2 - Genotyping

HLA and KIR

For HLA and KIR genotyping, DNA was amplified by polymerase chain reaction with sequence-specific primers (PCR-SSP), based on methods and primer sequences previously described (Olerup et al. 1991; Jones et al. 2006). In order to produce PCR-SSP reactions able to detect and discriminate KIR genes, primers were designed using sequence alignments comprising all KIR allelic variants present in the immunopolymorphism database (IPD) KIR sequence database (http://www.ebi.ac.uk/ipd/kir/). The presence or absence of 14 KIR genes encoding inhibitory (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, KIR3DL3) and activating (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DL4, KIR2DS4, KIR2DS5, KIR3DS1) KIRs was determined. Reactions were also designed for the detection of the HLA-C class I ligands of KIR: epitopes C1 and C2.

Nrf2

Single-labeled probes are a special type of hybridization probe that can detect mutations and single nucleotide polymorphisms. The so-called SimpleProbe format requires only one hybridization probe, labeled with only one fluorophore (Fluorescein), to achieve sequence specificity. Typically such a probe is designed to specifically hybridize to a target sequence that contains the SNP of interest. Once hybridized to its target sequence, the SimpleProbe probe emits more fluorescence than it does when it is not hybridized. A SimpleProbe probe may be labeled at either its 3´- or 5´-end. If a SimpleProbe is free in solution, emission of the reporter dye is quenched by a specific, non-fluorescent quencher. When the probe hybridizes to its target, quenching is reduced and Fluorescein, when excited by the LED of the Real Time Instrument, emits green fluorescence. However, even when the probe is not hybridized, background fluorescence is detectable at 530 nm, so the signal-to-noise ratio is low. By measuring the fluorescence, the instrument can detect melting of the probe-target hybrids as the temperature increases. The more stable the hybridization between SimpleProbe and target sequence, the higher the melting temperature.

FokI

Genotyping of FokI was performed using TaqMan® SNP Genotyping Assay which consists of a predesigned mix of unlabeled polymerase chain reaction primers and the TaqMan® minor groove binding group (MGB) probe (FAM™ and VIC® dye-labeled). The TaqMan® SNP genotyping assay is a rapid fluorophore-based real-time PCR (RT-PCR) method. The TaqMAn® RT-PCR measures fluorescent signals during the exponential stages of the PCR. These assays are performed via PCR amplifying the region containing the polymorphic site. They are based on the usage of two different fluorophore labelled probes, each complementary to the corresponding SNP allele. Allele 1 probe is labelled at the 5’ end with the VIC® fluorescent reporter dye and allele 2 is labelled at the 5’ end with the FAM^{TM} fluorescent reports dye. A quencher (Q) is linked at the 3’ end of the probes. Vicinity of the quencher to the reporter dye like in the intact probe inhibits reporter fluorescence through a Forster Resonance Energy Transfer (FRET). The probes anneal to the complementary sequences during the PCR. Subsequent amplicon generation occurs via the polymerase synthesizing the new DNA strands from the primers and simultaneously degrading the hybridized probes on the target sequence with its 5’-3’ nuclease activity. This separates the reporter dye from the quencher resulting in emission of light. The signals are analysed by the termocycler, which simultaneously generates the genotype data.

All TaqMan SNP Genotyping Assays are designed to work with TaqMan® Universal PCR MasterMix which contains DNA polymerase, dNTPs and optimized mix components and uses the same thermal conditions. These assays were carried out using a Real Time PCR Rotor-Gene RG3000 thermocycler (Corbett). Real Time PCR was performed using 4.0µl TaqMan® Genotyping Master Mix (2×), 0.2 µl TaqMan® SNP Genotyping Assay (TaqMan probes) (20×), 2.8µl DNAse free water and 1µl DNA, to make up the final reaction volume of 8µl. The Real Time PCR thermal conditions were as follows: Initial denaturing at 95°C for 10 min; 40 cycles of 96°C for 15 sec (denaturing) and 60°C for 1 min (annealing/extension). Samples were run with positive controls and blanks.

2.2.2.3 - miRNAs expression

Reverse Transcription (RT) (cDNA synthesis)

The first step of the protocol involves preparing an RT master mix. Each RT requires the following components and volumes, which can be scaled up to suit the required number of reverse transcriptions: 0.15 ul dNTPs, 1 ul reverse transcriptase, 1.5 ul RT buffer, 0.19 ul RNase inhibitor and 4.16 ul nuclease-free water. The master mix was gently mixed, centrifuged to bring to the bottom of the tube and then placed on ice. Each RT reaction should then be prepared at a ratio of 7 ul RT master mix:5 ul total RNA. The final mix then needs to be centrifuged and then incubated on ice for 5 minutes. Finally, the mix is subjected to the following program of heating: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and hold at 4°C. cDNA samples were stored at -20ºC.

TaqMan® microRNA analysis

TaqMan ® MicroRNA Assays use novel stem-loop primers for reverse transcription, followed by real-time TaqMan ® qPCR. The stem-loop RT primer includes 3’ overhang sequence, a stem, and a loop. The 3’ overhang sequence is short, ranging from 5 to 8 nucleotides. The stem-loop structure, which is specific to the 3’ end of the mature miRNA, extends from the very short, mature miRNA molecule and then adds a universal 3’ priming site for follow-up qPCR. This new primer design overcomes a fundamental problem in miRNA quantification because the short length of mature miRNAs prohibits a conventional qPCR assay design. There are several advantages about stem-loop RT primers. First, by annealing a short RT priming sequence to the 3’ miRNA, it gives RT a better specificity for discriminating similar miRNAs. Second, the stem-loop structure prevents hybridization of its RT primer to miRNA precursors, other long RNAs, as well as

genomic DNA. Third, the base stacking of the stem enhances the thermal stability of miRNA and DNA heteroduplex, further improving the RT efficiency for short RT primers. Finally, the stem-loop extends the 3' end of the miRNA by RT. The resulting longer RT product presents a template amenable to real-time TaqMan ® qPCR with high sensitivity and specificity that are largely due to specific PCR primers and the TaqMan ® probe.

MicroRNA Quantification

Methods for relative quantitation of gene expression allow quantifying differences in the expression level of a specific target (gene) between different samples. The data output is expressed as a fold-change or a fold-difference of expression levels.

To obtain accurate relative quantitation of an mRNA target, evaluation of the expression level of an endogenous control (*housekeeping* gene) is recommended. By using an endogenous control as an active reference, we can normalize quantitation of targets for differences in the amount of total nucleic acid added to each reaction. For example, if we determine that a calibrator sample has a two-fold greater amount of endogenous control than a test sample you would expect that the calibrator sample was loaded with two-fold more cDNA than the test sample. Therefore, you would have to normalize the test sample target by two-fold to accurately quantify the fold-differences in target level between calibrator and test samples. In our experiment we used RNU6 (U6 snRNA) as housekeeping gene.

In a real time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).

The Ct values of both controls and the samples of interest are normalized to an appropriate endogenous housekeeping gene.

The comparative Ct method is also known as the $2^{-\Delta\Delta Ct}$. method, where

$$\Delta Ct = Ct, miRNA155 - Ct, RNU6$$

$$\Delta\Delta Ct = \Delta Ct, sample - \Delta Ct, reference$$

Here, ΔCT, sample is the Ct value for any sample (MS patients) normalized to the endogenous housekeeping gene and ΔCt, reference is the Ct value for the controls also normalized to the endogenous housekeeping gene.

For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the reference must be approximately equal. This can be established by looking at how ΔCt varies with template dilution. If the plot of cDNA dilution versus delta Ct is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.

$$2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct,\ MS\ -\ \Delta Ct,\ HI)}$$

2.2.2.4 – 25(OH)D serum levels

Blood was collected in Vacuette® Z Serum Separator Clot Activator tubes for the 25(OH)D serum levels measurements. Serum was obtained by centrifugation and stored in several aliquots at -20°C until analysed. Serum 25(OH)D was chosen as a reliable marker of individual vitamin D status as it reflects vitamin D obtained from food sources and cutaneous synthesis, and is not prone to diurnal variation (Lips 2007).

Serum 25(OH)D was measured using an electro-chemiluminescence binding assay (ECLIA) for the in-vitro determination of total 25-hydroxyvitamin D (Elecsys® Vitamin D total, Cobas, Roche©). First, the sample is incubated with a pre-treatment reagent for 9 minutes. Thereby, the natural VDBP in the sample is denatured to release the bound vitamin D. Second, the sample is further incubated with a recombinant ruthenium-labelled VDBP to form a complex of vitamin D and the ruthenylated-VDBP. Third, with the addition of biotinylated vitamin D a complex consisting of the ruthenium-labelled VDBP and the biotinylated vitamin D is formed. The entire complex becomes bound to the solid phase (by the interaction of biotin and streptavidin-coated microparticles which are captured on the surface of the electrode). Unbound substances are removed. Applying voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via an instrument-specific calibration curve which is generated by 2-point calibration and a calibration master curve provided via the reagent barcode.

2.2.2.5 – Global statistics

Hardy-Weinberg equilibrium was evaluated using Chi-Square (χ2) or Fisher's exact test (for low genotype counts).

Continuous variables were presented as means with standard deviation. The normality of variables distributions was verified using the Kolmogorov-Smirnov test. Differences between two groups were analysed using the parametric student's t test or the nonparametric Mann-Whitney test, as appropriate. In the case of more than two groups, nonparametric Kruskal-Wallis test or one-way ANOVA test were used. In order to evaluate the correlation between variables, Spearman's rank correlation coefficient was applied.

To identify genes contributing to MS susceptibility, a stepwise logistic regression on an allelic level was applied. To select the explanatory variables to be included in the final model, a forward selection method was used, which involves starting with no variables in the model, testing the addition of each variable using likelihood ratio tests as a comparison criterion, adding the variable (if any) that improves the model the most, and repeating this process until none improves the model. It should be noted that Odds Ratios (adjusted ORs) obtained in a multivariable logistic regression model were adjusted for all the other factors included in the model and therefore differ from those obtained when a given gene is compared with all other genes**.**

SNP-phenotype association analysis was undertaken by binary or multinomial logistic regression as appropriate. Unadjusted and adjusted analysis taking into account age, gender and the presence of HLA-DRB1*15 allele in the genotype of HLA-DRB1 locus were performed. The SNP of interest was modelled assuming several related genotypic mechanisms (additive, dominant, recessive, heterozygous advantage and general models) and the minimum p-value from these correlated tests were reported.

Multiple linear regression analysis was used in papers 6 and 7 to study the relationship between a *dependent variable* (response) (vitamin D levels) and *n independent variables* (potential determinants, predictors).

Seasonality was assessed using the Hewitt's test which is a non-parametric test, considered by many authors to be more appropriate for sinusoidal patterns than the Edwards' test (Marrero 1981; Walter 1982), especially when the sample size is not very large (Hewitt et al. 1971; Marrero 1981). In brief, we estimated the expected relative incidence and the Observed Relative Incidence (ORI) of MS cases per month and ranked from 1 to 12 according to the magnitude (12=highest; 1=smallest). Based on the ORI ranks, we determined the rank-sums for successive 6-moth segments and the

statistical significance of the rank-sum values was determined by a table of cumulative probability.

A 5% significance level was used in all analyses. Statistical analyses were performed using Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).

References

Hewitt, D., J. Milner, A. Csima, et al. (1971). "On Edwards' criterion of seasonality and a non-parametric alternative." Br J Prev Soc Med **25**(3): 174-176.

Jones, D. C., R. S. Edgar, T. Ahmad, et al. (2006). "Killer Ig-like receptor (KIR) genotype and HLA ligand combinations in ulcerative colitis susceptibility." Genes Immun **7**(7): 576-582.

Kurtzke, J. F. (1983). "Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS)." Neurology **33**(11): 1444-1452.

Lips, P. (2007). "Relative value of 25(OH)D and 1,25(OH)2D measurements." J Bone Miner Res **22**(11): 1668-1671.

Marrero, O. (1981). "Re: study of seasonality." Am J Epidemiol **113**(4): 481-482.

McDonnell, G. V., H. Mawhinney, C. A. Graham, et al. (1999). "A study of the HLA-DR region in clinical subgroups of multiple sclerosis and its influence on prognosis." J Neurol Sci **165**(1): 77-83.

Miller, S. A., D. D. Dykes and H. F. Polesky (1988). "A simple salting out procedure for extracting DNA from human nucleated cells." Nucleic Acids Res **16**(3): 1215.

Olerup, O. and J. Hillert (1991). "HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation." Tissue Antigens **38**(1): 1-15.

Polman, C. H., S. C. Reingold, G. Edan, et al. (2005). "Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria"." Ann Neurol **58**(6): 840-846.

Ryckman, K. and S. M. Williams (2008). "Calculation and use of the Hardy-Weinberg model in association studies." Curr Protoc Hum Genet **Chapter 1**: Unit 1 18.

Silva, A. M., C. Pereira, A. Bettencourt, et al. (2007). "The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population." J Neurol Sci **258**(1-2): 69-74.

Walter, S. D. (1982). "Study of seasonality." Am J Epidemiol **116**(1): 192-196.

Wittke-Thompson, J. K., A. Pluzhnikov and N. J. Cox (2005). "Rational inferences about departures from Hardy-Weinberg equilibrium." Am J Hum Genet **76**(6): 967-986.

CHAPTER 3

Results

3.1 – Genetic and Epigenetics factors

Paper 1

The protective role of HLA-DRB1*13 in Autoimmune Diseases

The protective role of HLA-DRB1*13 in Autoimmune Diseases

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Abstract

Autoimmune diseases (AIDs) are characterized by a multifactorial aetiology and a complex genetic background, with the MHC region playing a major influence. We genotyped for HLA-DRB1 locus 1228 patients with AIDs-213 with Systemic Lupus Erythematosus-SLE, 166 with Psoriasis or Psoriatic Arthritis-Ps+PsA, 153 with Rheumatoid Arthritis-RA, 67 with Systemic Sclerosis-SSc, 536 with Multiple Sclerosis-MS and 93 with Myasthenia Gravis-MG, and 282 unrelated controls. We confirmed previously established associations of HLA-DRB1*15 (OR=2.17) and HLA-DRB1*03 (OR=1.81) alleles with MS, HLA-DRB1*03 with SLE (OR=2.49), HLA-DRB1*01 (OR=1.79) and HLA-DRB1*04 (OR=2.81) with RA, HLA-DRB1*07 with Ps+PsA (OR=1.79), HLA-DRB1*01 (OR=2.28) and HLA-DRB1*08 (OR=3.01) with SSc and HLA-DRB1*03 with MG (OR=2.98). We further observed a consistent negative association of HLA-DRB1*13 allele with SLE, Ps+PsA, RA and SSc (18.3%, 19.3%, 16.3% and 11.9%, respectively vs. 29.8% in controls). HLA-DRB1*13 frequency in the AIDs group was 20.0% (OR=0.58). Although different alleles were associated with particular AIDs, the same allele, HLA-DRB1*13, was underrepresented in all of the six diseases analysed. This observation suggests that this allele may confer protection for AIDs, particularly for systemic and rheumatic disease. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules, favouring efficient clonal deletion during thymic selection.

Key words: Portugal; 25(OH)D levels; Vitamin D status; healthy adult population.

1. Introduction

Autoimmune diseases (AIDs) are chronic disorders originated by the loss of immunological tolerance to self-antigens. This heterogeneous group of conditions presents common genetic risk factors and share several pathophysiological mechanisms leading to overlapping clinical manifestations targeting specific organs or multiple organ systems [1]. There is evidence that they share similar immunogenetic mechanisms, even though they exhibit varying epidemiological features and clinical manifestations [2, 3]. Underlying these diverse clinical phenotypes is a deregulated immune system with an enriched ability to respond against self. The fact that AIDs share several clinical signs and symptoms (i.e. subphenotypes), and also share physiopathological mechanisms and genetic factors have been called autoimmune tautology and indicates that may have a common origin [4].

The immune system is in charge of the defence against external pathogens. For this purpose, T and B lymphocytes are responsible for the immune response through regulated cell-cell interactions and secretion of cytokines, chemokines, and other inflammatory mediators. This defence against external pathogens must occur without causing unnecessary harm to self. To achieve this delicate balance, the majority of self-reactive T and B lymphocytes are destroyed in the thymus and bone marrow through negative selection [5]. Nevertheless, this process is far from perfect, and self-reactive lymphocytes escape into the periphery. Consequently, peripheral tolerance mechanisms are necessary to keep these self-reactive cells in check [6]. Activated self-reactive T and B cells promote autoimmunity when the effector and regulatory balance of the immune response is disturbed [7].

Major histocompatibility complex (MHC) molecules are widely distributed surface membrane glycoproteins that present antigenic peptides to T-cell receptors (TCR). Developing thymocytes encounter a highly heterogeneous repertoire of self (endogenous) peptide-MHC (pMHC) complexes on thymic epithelial cells, the main thymus antigen presenting cells. The affinity/avidity with which these thymocyte TCRs bind self pMHC determines if it is destined to perish or if it will survive [8]. In this way, a repertoire of peripheral T cells that is essentially self-tolerant is generated [6, 9, 10].

Several hypotheses have been put forward to explain how MHC polymorphisms influence autoimmunity risk or protection. They must do so, somehow, by shaping the central or peripheral T-cell repertoires toward autoimmune resistance or proclivity [8]. A protective MHC profile could achieve this by the selection of a T-cell repertoire with diminished pathogenicity [11]. On the other hand, protective MHC molecules may keep

autoimmunity in check by favouring the negative selection of particular self reactive T-cells [12-14].

The functional basis of the association between specific HLA alleles and development of AIDs can be classically explained by two possible ethiopathogenic models:

The molecular mimicry hypothesis - proposes that certain HLA alleles are more efficient in presenting pathogen epitopes that share structural features with self-peptides to mature T cells. Once the response to the pathogen is initiated the self-antigen is also recognized and disease ensues.

Central selection failure - certain HLA alleles are less efficient at presenting self-peptides to developing T cells in the thymus, so negative selection fails.

A different hypothesis proposes that different alleles can modulate the immunologic profile of an individual, through antigen-independent mechanisms, resulting in either promoting a higher autoimmune predisposition or, in opposition, a more efficient immune regulation. Given the consistent association of HLA-DRB1 alleles to different autoimmune diseases, we explored the idea that the same HLA-DRB1 alleles could be influencing several different autoimmune diseases. To this end we compared the immunogenetic profile in different AIDs. This study includes four autoimmune systemic diseases, namely Systemic Lupus Erythematosus - SLE, Rheumatoid Arthritis - RA, Psoriasis or Psoriatic arthritis - Ps+PsA and Systemic Sclerosis – SSc. Patients with Multiple Sclerosis - MS and Myasthenia Gravis – MG were also included.

2. Patients and Methods

2.1 Patients and Controls

A total of 1228 patients with AIDs - 213 patients with SLE and 153 patients with RA diagnosed according to the American College of Rheumatology (ACR) criteria, 166 patients with Ps+PsA 67 with SSc, 536 with definitive diagnosis of MS according to the revised McDonald criteria and 93 with MG - were recruited from the neurology and medicine outpatient clinic of Centro Hospitalar do Porto - Hospital de Santo António (CHP-HSA). The HLA-DRB1 frequencies of patients were compared with the ones of a control group consisting of 282 unrelated individuals without disease and from the same geographic origin (north of Portugal).

2.2 HLA-DRB1 genotyping

Peripheral blood samples (10 mL) were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes by using a Salting-Out

procedure[15]. DNA was amplified using polymerase chain reaction and sequence-specific primers (PCR-SSP), based on methods previously described [16]. Primers and probes were validated by the Twelfth International Histocompatibility Workshop. PCR products were visualized under ultraviolet light after running in a 1.5% agarose gel containing ethidium bromide.

2.3 Statistical analysis

To identify the HLA-DRB1 genes contributing to the six different AIDs, we applied stepwise logistic regression on an allelic level, using forward selection which involves starting with no variables in the model, testing the addition of each variable using a chosen model comparison criterion, adding the variable (if any) that improves the model the most, and repeating this process until none improves the model. It should be noted that odds ratios (ORs) obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model, and therefore differ from those obtained when a given gene is compared with all other genes. The data were analysed using IBM SPSS 20 statistical software.

3. Results

A total of 1228 cases and 282 controls were analysed and different types of association between alleles and AIDs were found (Table 1). These included three risk alleles for two or more AIDs, two protective alleles for two or more AIDs and three risk alleles for a particular AID. HLA-DRB1*13 was a protective allele for four AIDs: SLE (OR=0.58, p=0.016), Ps+PsA (OR=0.621, p=0.050), RA (OR=0.58, p=0.044) and SSc (OR=0.42, p=0.035). There was a specific risk allele associated with three AIDs. HLA-DRB1*03 was found to be a risk factor for SLE (OR=2.49, $p=4.2x10^{-5}$), MS (OR=1.81, p=0.003) and MG (OR=2.98, $p=6.1x10^{-5}$). There were two risk alleles associated with two AIDs, HLA-DRB1*08 was positively associated with MS (OR=1.73, p=0.033) and SSc (OR=3.01, p=0.004) and HLA-DRB1*01 was found to be a risk factor for RA (OR=1.79, p=0.017) and SSc (OR=2.28, p=0.006).HLA-DRB1*09 was negatively associated with SLE (OR=0.18, p=0.027), MS (OR=0.22, p=0.004) and RA (OR=0.95, p=0.003). Three risk disease-specific alleles were found. HLA-DRB1*04 for RA (OR=2.81, $p=6x10^{-6}$), HLA-DRB1*07 for Ps+PsA (OR=1.79, p=0.006) and HLA-DRB1*15 for MS (OR=2.17, $p=2x10^{-5}$). Considering AIDs as a group, HLA-DRB1*03 frequency was significantly higher (p=0.026, OR=1.49) compared with controls; conversely HLA-DRB1*13 (p=0.003, OR=0.64) and HLA-DRB1*09 (p= 0.001, OR=0.31) frequencies were significantly lower.

Table 1. Associations between HLOA class II and six AIDs: SLE, Ps+PsA, RA, SSc, MS and MG

	Controls (n=282)	**SLE (n=213)**	**Ps+PsA (n=166)**	**RA (n=153)**	**SSc (n=67)**	**MS (n=536)**	**MG (n=93)**	**TOTAL n=1228)**
HLA-DRB1*01	**66 (23.4%)**	40 (18.8%)	39 (23.5%)	**50 (32.7%) OR=1.79 p=0.017**	**28 (41.8%) OR=2.28 p=0.006**	100 (18.7%)	23 (24.7%)	280 (22.8%)
HLA-DRB1*03	**44 (15.6%)**	**73 (34.3%) OR=2.49 p=4.2X10^{-5}**	25 (15.1%)	28 (18.3%)	11 (16.4%)	**123 (22.9%) OR= 1.81 p=0.003**	**33 (35.5%) OR=2.98 p=6.1X10^{-5}**	**293 (23.9%) OR=1.51 p=0.022**
HLA-DRB1*04	**69 (24.5%)**	42 (19.7%)	46 (27.7%)	**73 (47.7%) OR=2.81 p=6X10^{-6}**	13 (19.4%)	128 (23.9%)	23 (24.7%)	325 (26.5%)
HLA-DRB1*07	**72 (25.5%)**	55 (25.8%)	**66 (39.8%) OR=1.79 p=0.006**	38 (24.8%)	14 (20.9%)	147 (27.4%)	23 (24.7%)	343 (27.9%)
HLA-DRB1*08	**24 (8.5%)**	21 (10.0%)	10 (6.0%)	**3 (2.0%) OR=0.24 p=0.026**	**15 (22.4%) OR=3.01 p=0.004**	**65 (12.1%) OR=1.73 p=0.033**	7 (7.5%)	121 (9.9%)
HLA-DRB1*09	**14 (5.0%)**	**2 (1.0%) OR=0.18 p=0.027**	5 (3.0%)	**0 (0.0%)* OR=0.95 p=0.003**	3 (4.5%)	**5 (1.0%) OR=0.22 p=0.004**	2 (2.2%)	**17 (1.4%) OR=0.23 p=1x10^{-4}**
HLA-DRB1*13	**84 (29.8%)**	**39 (18.3%) OR=0.58 p=0.016**	**32 (19.3%) OR=0.62 p=0.050**	**25 (16.3%) OR=0.58 p=0.044**	**8 (11.9%) OR=0.42 p=0.035**	124 (23.1%)	17 (18.3%)	**245 (20.0%) OR=0.58 p=0.004**
HLA-DRB1*15	**56 (19.9%)**	55 (25.8%)	22 (13.3%)	17 (11.1%)	12 (17.9%)	**175 (32.7%) OR=2.17 p=2X10^{-5}**	15 (16.1%)	296 (24.1%)

AIDs: autoimmune diseases; SLE: Systemic Lupus Erythematosus; Ps+PsA: Psoriasis or Psoriatic arthritis; RA: Rheumatoid Arthritis; SSc: Systemic Sclerosis; MS: Multiple Sclerosis; MG: Myasthenia Gravis *Fisher's exact test was used to calculate this value

4. Discussion

Through a systematic review of published works, Cruz-Tapias and collaborators, in 2012, identified some common HLA class II alleles that contribute to susceptibility to AIDs in Latin Americans [3]. The present study is, to date and to the best of our knowledge, the only one that addresses the hypothesis that a HLA-DRB1 allele could influence different autoimmune diseases, using a new cohort, encompassing six different autoimmune diseases.

In this study we observed associations of different HLA-DRB1 alleles with several AIDs. We confirmed positive and negative associations in MS [17, 18], SLE [19-21] , Ps+PsA [22, 23] , RA [24], SSc [25, 26] and MG [27] previously reported in our or other populations.

When AIDs studied were considered as a group, HLA-DRB1*03 allele was significantly overrepresented, as expected [28]. It has been shown that this allele has low affinity for CLIP (class-II-associated invariant chain peptide) and may not require HLA-DM to ensure peptide presentation, preventing efficient peptide selection and allowing the binding of low stability peptides [29]. Concerning the observed negative association with HLA-DRB1*09 we think that this is likely a spurious association, as this is a rare allele and the frequency found in controls is, for some reason, double that the one reported for the Portuguese population [30].

Our observations suggest that the presence of HLA-DRB1*13 allele may confer protection for AIDs. HLA-DRB1*13 is a high frequency allele in the general population both in Portugal [30] and worldwide. Our results confirms that the lower frequency of HLA-DRB1*13 in every individual AIDs groups is not secondary to the deviations granted by the concurrent positive associations.

When the data obtained from previous studies [31-35] are taken into consideration, the HLA-DRB1*13 allele seems to be an universal protective allele for RA. Recently this allele was also described to be protective in SLE in the Japanese population [21] and for ANCA-associated vasculitis in the Dutch population [36]. Subtle structural differences in the HLA molecule have functional implications at the protein level. Specific amino acid patterns at the peptide binding cleft are involved in disease susceptibility, such as the well-known shared epitope first described in the RA susceptibility alleles HLA-DRB1*01 and HLA-DRB1*04 [32, 37]. In a recent study Van Heemst and collaborators identify citrullinated vinculin, present in the joints of $ACPA^+$ RA patients, as an autoantigen targeted by ACPA and $CD4^+$ T cells. These T cells recognize an epitope with the core sequence DERAA, which is also found in many microbes and in protective HLA-

DRB1*13 molecules, presented by predisposing HLA-DQ molecules. Intriguingly, DERAA-directed T cells were not detected in HLA-DRB1*13^{+} donors, indicating that the DERAA epitope from HLA-DRB1*13 could mediate thymic tolerance in these donors, and explain the protective effects associated with HLA-DRB1*13. They suggest that subjects born with HLA-DRB1*13, will present the HLA-DRB1*13-derived DERAA-peptide in the thymus, leading to tolerization of the DERAA-reactive T cell response [38]. The negative association we describe here supports the idea that the HLA-DRB1*13 allele, possibly by its specific structural features, may as well confer resistance to several other AIDs. The protective effect of HLA-DRB1*13 could be explained by a more proficient antigen presentation by these molecules [39, 40], favouring an efficient thymic selection. As a result, negative selection and development of DR-driven autoreactive regulatory T cells are promoted [8].

A different model would relate HLA molecules with the presence of specific endophenotypes indirectly associated with autoimmunity. Other studies of our group suggest that the HLA genotype may primarily influence the general activation state of CD4 T-cells [41]. The protective effect of HLA-DRB1*13 could also be explained by this effect. Curiously, several reports have suggested an association of HLA-DRB1*13 and/or HLA-DQB1*06 with slow disease progression in human immunodeficiency virus (HIV)-infected individuals meaning that among HIV controllers there is an association between the presence of certain class II HLA alleles and strong CD4 T-cell responses [42, 43].

Although different alleles are associated with particular AIDs, the same allele, HLA-DRB1*13, was underrepresented in all six diseases. This difference is statistically significant for the four rheumatic diseases studied. This observation suggests that this allele confers protection to AIDs in general, and particularly to rheumatic diseases.

References

1. Shoenfeld, Y., et al., The mosaic of autoimmunity: genetic factors involved in autoimmune diseases--2008. Isr Med Assoc J, 2008. 10(1): p. 3-7.
2. Anaya, J.M., L. Gomez, and J. Castiblanco, Is there a common genetic basis for autoimmune diseases? Clin Dev Immunol, 2006. 13(2-4): p. 185-95.
3. Cruz-Tapias, P., et al., Shared HLA Class II in Six Autoimmune Diseases in Latin America: A Meta-Analysis. Autoimmune Dis, 2012. 2012: p. 569728.
4. Anaya, J.M., The autoimmune tautology. Arthritis Res Ther, 2010. 12(6): p. 147.
5. Smilek, D.E. and E.W. St Clair, Solving the puzzle of autoimmunity: critical questions. F1000Prime Rep, 2015. 7: p. 17.
6. Sprent, J. and H. Kishimoto, The thymus and central tolerance. Transplantation, 2001. 72(8 Suppl): p. S25-8.
7. Bluestone, J.A., Mechanisms of tolerance. Immunol Rev, 2011. 241(1): p. 5-19.
8. Tsai, S. and P. Santamaria, MHC Class II Polymorphisms, Autoreactive T-Cells, and Autoimmunity. Front Immunol, 2013. 4: p. 321.
9. Starr, T.K., S.C. Jameson, and K.A. Hogquist, Positive and negative selection of T cells. Annu Rev Immunol, 2003. 21: p. 139-76.
10. Palmer, E., Negative selection--clearing out the bad apples from the T-cell repertoire. Nat Rev Immunol, 2003. 3(5): p. 383-91.
11. Luhder, F., et al., Major histocompatibility complex class II molecules can protect from diabetes by positively selecting T cells with additional specificities. J Exp Med, 1998. 187(3): p. 379-87.
12. Schmidt, D., et al., A mechanism for the major histocompatibility complex-linked resistance to autoimmunity. J Exp Med, 1997. 186(7): p. 1059-75.
13. Schmidt, D., et al., Autoantigen-independent deletion of diabetogenic CD4+ thymocytes by protective MHC class II molecules. J Immunol, 1999. 162(8): p. 4627-36.
14. Tsai, S., et al., Antidiabetogenic MHC class II promotes the differentiation of MHC-promiscuous autoreactive T cells into FOXP3+ regulatory T cells. Proc Natl Acad Sci U S A, 2013. 110(9): p. 3471-6.
15. Miller, S.A., D.D. Dykes, and H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res, 1988. 16(3): p. 1215.
16. Olerup, O. and H. Zetterquist, HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens, 1992. 39(5): p. 225-35.
17. Cree, B.A., Multiple sclerosis genetics. Handb Clin Neurol, 2014. 122: p. 193-209.
18. Silva, A.M., et al., The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population. J Neurol Sci, 2007. 258(1-2): p. 69-74.
19. Morris, D.L., et al., Unraveling multiple MHC gene associations with systemic lupus erythematosus: model choice indicates a role for HLA alleles and non-HLA genes in Europeans. Am J Hum Genet, 2012. 91(5): p. 778-93.
20. Vasconcelos, C., et al., HLA in Portuguese systemic lupus erythematosus patients and their relation to clinical features. Ann N Y Acad Sci, 2009. 1173: p. 575-80.
21. Furukawa, H., et al., Human leukocyte antigens and systemic lupus erythematosus: a protective role for the HLA-DR6 alleles DRB1*13:02 and *14:03. PLoS One, 2014. 9(2): p. e87792.
22. Ho, P.Y., et al., Investigating the role of the HLA-Cw*06 and HLA-DRB1 genes in susceptibility to psoriatic arthritis: comparison with psoriasis and undifferentiated inflammatory arthritis. Ann Rheum Dis, 2008. 67(5): p. 677-82.
23. Shawkatova, I., et al., HLA-C, DRB1 and DQB1 alleles involved in genetic predisposition to psoriasis vulgaris in the Slovak population. Folia Microbiol (Praha), 2013. 58(4): p. 319-24.
24. Furukawa, H., et al., Human leukocyte antigen polymorphisms and personalized medicine for rheumatoid arthritis. J Hum Genet, 2015.
25. Flam, S.T., et al., The HLA profiles of mixed connective tissue disease differ distinctly from the profiles of clinically related connective tissue diseases. Rheumatology (Oxford), 2015. 54(3): p. 528-35.
26. Gladman, D.D., et al., HLA markers for susceptibility and expression in scleroderma. J Rheumatol, 2005. 32(8): p. 1481-7.
27. Avidan, N., et al., Genetic basis of myasthenia gravis - a comprehensive review. J Autoimmun, 2014. 52: p. 146-53.

28. Bilbao, J.R., et al., HLA-DRB1 and MICA in autoimmunity: common associated alleles in autoimmune disorders. Ann N Y Acad Sci, 2003. 1005: p. 314-8.
29. Collado, J.A., et al., The Repertoires of Peptides Presented by MHC-II in the Thymus and in Peripheral Tissue: A Clue for Autoimmunity? Front Immunol, 2013. 4: p. 442.
30. Bruno A. Lima, H.A., HLA-A, -C, -B, and -DRB1 allelic and haplotypic diversity in bone marrow volunteer donors from northern Portugal. Organs, Tissues & Cells, 2013. 16: p. 19-26.
31. Klareskog, L., et al., A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. Arthritis Rheum, 2006. 54(1): p. 38-46.
32. van der Helm-van Mil, A.H., et al., An independent role of protective HLA class II alleles in rheumatoid arthritis severity and susceptibility. Arthritis Rheum, 2005. 52(9): p. 2637-44.
33. Jun, K.R., et al., Meta-analysis of the association between HLA-DRB1 allele and rheumatoid arthritis susceptibility in Asian populations. J Korean Med Sci, 2007. 22(6): p. 973-80.
34. Tuokko, J., et al., HLA haplotype analysis in Finnish patients with rheumatoid arthritis. Arthritis Rheum, 2001. 44(2): p. 315-22.
35. van der Woude, D., et al., Protection against anti-citrullinated protein antibody-positive rheumatoid arthritis is predominantly associated with HLA-DRB1*1301: a meta-analysis of HLA-DRB1 associations with anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis in four European populations. Arthritis Rheum, 2010. 62(5): p. 1236-45.
36. Stassen, P.M., et al., HLA-DR4, DR13(6) and the ancestral haplotype A1B8DR3 are associated with ANCA-associated vasculitis and Wegener's granulomatosis. Rheumatology (Oxford), 2009. 48(6): p. 622-5.
37. Michou, L., et al., Validation of the reshaped shared epitope HLA-DRB1 classification in rheumatoid arthritis. Arthritis Res Ther, 2006. 8(3): p. R79.
38. van Heemst, J., et al., Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis. Nat Commun, 2015. 6: p. 6681.
39. Diepolder, H.M., et al., A vigorous virus-specific CD4+ T cell response may contribute to the association of HLA-DR13 with viral clearance in hepatitis B. Clin Exp Immunol, 1998. 113(2): p. 244-51.
40. Ramezani, A., et al., Association of human leukocyte antigen polymorphism with outcomes of hepatitis B virus infection. J Gastroenterol Hepatol, 2008. 23(11): p. 1716-21.
41. Fesel, C., et al., Compensatory T-cell regulation in unaffected relatives of SLE patients, and opposite IL-2/CD25-mediated effects suggested by coreferentiality modeling. PLoS One, 2012. 7(3): p. e33992.
42. Ferre, A.L., et al., HIV controllers with HLA-DRB1*13 and HLA-DQB1*06 alleles have strong, polyfunctional mucosal CD4+ T-cell responses. J Virol, 2010. 84(21): p. 11020-9.
43. Malhotra, U., et al., Role for HLA class II molecules in HIV-1 suppression and cellular immunity following antiretroviral treatment. J Clin Invest, 2001. 107(4): p. 505-17.

Paper 2

The role of KIR2DS1 in Multiple Sclerosis

The role of KIR2DS1 in Multiple Sclerosis

KIR in Portuguese MS patients

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Abstract

Killer Immunoglobulin-like Receptor (KIR) genes may influence both resistance and susceptibility to different autoimmune diseases, but their role in the pathogenesis of Multiple Sclerosis (MS) is still unclear. We investigated the influence of KIR genes on MS susceptibility in 447 MS Portuguese patients, and also whether genetic interactions between specific KIR genes and their HLA class I ligands could contribute to the pathogenesis of MS. We observed a negative association between the activating KIR2DS1 gene and MS (adjusted OR=0.450, p=0.030) independently from the presence of HLA-DRB1*15 allele. The activating KIR2DS1 receptor seems to confer protection against MS most probably through modulation of autoreactive T cells by Natural Killer cells.

Key words: Multiple Sclerosis; KIR; Natural killer cells; Portugal

1. Introduction

Multiple Sclerosis (MS) is the most common inflammatory demyelinating disease of the central nervous system (CNS) in young adults. The cause of MS is unknown, but it is widely believed to be an autoimmune disease occurring in genetically susceptible individuals after exposure to as-yet undefined environmental factors(Dyment et al., 2004). Although the nature of the environmental triggers remains undetermined, progress has been made in characterizing the genetic factors of MS susceptibility. The HLA class II allele DRB1*1501 is a well-established susceptibility factor for this disease (Hafler et al., 2007, Silva et al., 2007), though recent studies reported a protective effect of some HLA class I alleles, such as the HLA-A*02 and Cw*05, independently of HLA-DRB1*1501 allele (Brynedal et al., 2007, Silva et al., 2009, Yeo et al., 2007) and there are now 110 established Multiple Sclerosis risk variants at 103 discrete loci outside of the major histocompatibility complex (Beecham et al., 2013).

Natural killer (NK) cells contribute to both effector and regulatory functions of the innate immune system via their cytotoxic activity, mainly against viral infected cells or tumor cells, and also through their ability to secrete different cytokines (Moretta et al., 2008). These cells may play a regulatory role in MS by modulating the activation and the survival of autoreactive T cells, microglial cells and astrocytes that would otherwise cause inflammatory responses in CNS. Several experimental systems have provided evidence that NK cells have a suppressive role in autoimmunity. NK cells might kill dendritic cells (DC), or they might lyse T cells and silence antigen-specific response. In addition, NK cells might negatively affect T cell response by producing regulatory cytokines such as TGF-β or IL-10 (Morandi et al., 2008, Shi and Van Kaer, 2006).

NK cells are normally restrained by inhibitory receptors that recognize target-cell-expressed MHC class I molecules, they recognize and interact with HLA class I antigens through killer cell immunoglobulin like receptors (KIR). These receptors are present on the cell membrane; it is the cytoplasmatic tail that defines their activating (short [S]) or inhibiting (long [L]) properties. The KIR gene cluster is located on chromosome 19q13 and consists of several genes and pseudogenes, exhibiting considerable structural variation, resulting in all genes rarely being concomitantly present in one given individual. Known ligands include HLA-C, HLA-A, HLA-G and Bw04 molecules. HLA-C alleles can be defined as either ‘group 1’ or ‘group 2’. In group 1 [C1 epitope (Serine at pos 77, Asparagine at pos 80)] are the 2DL2, 2DL3, and 2DS2 binders, Cw*01, Cw*03, Cw*07, Cw*08, Cw*13, Cw*14 and Cw*16. In group 2 [C2 epitope (Asparagine at pos 77, Lysine at pos 80)] are the 2DL1 and 2DS1 binders, Cw*02, Cw*04, Cw*05, Cw*06, Cw*17 and Cw*18. In addition, it was reported ligand for 2DL4 as HLA-G, for 3DL1 (and 3DS1) as

HLA-Bw4 motif, for3DL2 as HLA-A3 and -A11, and for 2DS4 as HLA-Cw*04; ligands for the other KIRs are still unknown (Boyton and Altmann, 2007).

The interaction KIR/HLA results in either activation or inhibitory signals (Biassoni et al., 2009). Triggering of NK cells occurs only when activating signals overcome inhibitory signals. Genetic association studies suggest that KIR receptors may play beneficial roles in viral infections, such as in HIV or HCV and may also modulate susceptibility in autoimmune diseases, for example in type-I diabetes or psoriatic arthritis (Boyton and Altmann, 2007).

Concerning KIR and susceptibility to MS, a recent study showed that HLA-Bw04, a ligand of the inhibitory KIR3DL1 and activatory KIR3DS1 receptors, seems to protect against MS in Norwegians patients and that KIR2DS1 carriers had a lower risk of MS than non-carriers (Lorentzen et al., 2009). In a study from Spain, the authors describe an apparent underrepresentation of KIR3DS1 in MS patients, which might indicate a minor and independent protective role of KIR haplotypes carrying this gene (Ordonez et al., 2009). In a more recent study from Italy, a possible protective role of the activating KIR2DS1 gene was described that was enhanced in the presence of its ligand group HLA-C2 (Fusco et al., 2010). In the last year Garcia-Leon and colleagues (Garcia-Leon et al., 2011) found significantly higher frequencies of KIR2DL5 and KIR3DS1 genes in MS patients and also a protective role of the Bw04 HLA motif. In this context, we investigated the influence of KIR genes on MS susceptibility and disease severity in a Portuguese population, and also assessed whether genetic interactions between specific KIR genes and their HLA class I ligands could contribute to the pathogenesis of MS.

2. Subjects and Methods

2.1 Patients and controls

A group of 447 unrelated patients with a definitive diagnosis of MS, according to the revised McDonald criteria, were recruited from the outpatient neurological clinic of the Centro Hospitalar do Porto – Santo António Hospital (HSA). The Expanded Disability Status Scale (EDSS) and Multiple Sclerosis Severity Scale (MSSS) were used to measure, respectively, physical disability and disease severity. MS patients were randomly included in the study at disease onset. The control group comprised 177 healthy controls (HC). This study was approved by the Medical Ethical Committee of the

hospital and written informed consent was obtained from all participants. Clinical characteristics of the 447 MS patients are shown in Table 1.

Table 1. Clinical features of MS patients.

Clinical variables (n=447)	**Values**
Gender (F:M)	1.9:1
Median age at onset, yr (range)	28 (6-60)
Median disease duration, yr (range)	10 (1-50)
Median EDSS	3.0 (0.0-8.5)
Median MSSS	3.3 (0.0-8.5)
Disease course	
Primary Progressive (PP)	43 (9.6%)
Secondary Progressive (SP)	49 (11%)
Relapsing-remitting (RR)	355 (79.4%)

EDSS, extended disability status scale;
MSSS, Multiple Sclerosis severity score.

2.2 KIR genotyping

Genomic DNA was obtained from proteinase-K treated peripheral blood leukocytes with a salting-out procedure. DNA was amplified by polymerase chain reaction with sequence-specific primers (PCR-SSP), based on methods and primer sequences previously described (Jones et al., 2006). In order to produce PCR-SSP reactions able to detect and discriminate each of the known KIR genes, primers were designed using sequence alignments comprising all KIR allelic variants present in the immunopolymorphism database (IPD) KIR sequence database (http://www.ebi.ac.uk/ipd/kir/). The presence or absence of 14 KIR genes encoding inhibitory (KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL5, KIR3DL1, KIR3DL2, KIR3DL3) and activating (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DL4, KIR2DS4, KIR2DS5, KIR3DS1) KIRs was determined. Reactions were also designed for the detection of the HLA-C class I ligands of KIR: epitopes C1 and C2. Consequently, reactions were included to detect the presence of these alleles to improve overall specificity of the reaction set.

2.3 Statistical analysis

To further identify the KIR genes contributing to MS susceptibility, we applied stepwise logistic regression on an allelic level, using backward selection. Starting from a model with all KIR genes, the least significant gene was removed one at the time until all remaining genes were significant, based on the likelihood-ratio test. It should be noted that odds ratios (ORs) obtained in a multivariable logistic regression analysis are adjusted for all the other genes included in the model, and therefore differ from those obtained when a given gene is compared with all other genes. The possible relation between the presence of specific KIR genes and clinical variables (disease course, age at onset, MSSS) was also performed using logistic regression. All analyses were undertaken with the PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

3. Results

The genotype distribution fell within Hardy–Weinberg distribution in both cohorts. KIR genotype frequencies of patients and HC are reported in table 2. To identify the alleles that confer an effect on MS susceptibility, a stepwise binary logistic regression analysis was performed on all KIR genes and also included HLA-DRB1*15. The alleles that remained significantly associated with susceptibility to MS in the final model are reported in table 3.

Table 2. KIR genes and HLA ligand carrier frequencies in patients and controls.

KIR Gene	KIR Gene Carrier Frequency	
	MS patients (n=447)	Controls (n=177)
Inhibitory genes		
2DL1	98.0	96.6
2DL2	55.5	53.7
2DL3	88.1	89.3
2DL5	50.6	52.5
3DL1	95.1	93.2
3DL2	100	100
3DL3	100	100
Activating genes		
2DS1	35.6	41.8
2DS2	54.8	55.4

2DS3	32.7	29.4
2DL4	99.1	99.4
2DS4	95.3	93.2
2DS5	29.3	34.5
3DS1	36.2	37.9
KIR ligands		
C1C1	29.5	30.5
C1C2	21.5	22.0
C2C2	48.8	47.5
Bw4-	37.7	34.9
Bw4+	62.3	65.1

As expected in the final model HLA-DRB1*15 allele increased the risk of developing the disease (adjusted OR=1.753, p=0.008). The presence of activating KIR2DS1 gene decreased the risk of MS (adjusted OR = 0.450, p=0.030) independently from the presence of HLA-DRB1*15. No significant associations were found for age at onset or disease course.

The frequency of the combination KIR2DS1/C2 group was lower in patients group when compared with controls but the difference was not statistical significant (26.2% vs. 32.2%, p=0.133).

Table 3. Alleles associated with Multiple Sclerosis using logistic regression

	Adjusted Odds ratio	**95% Confidence Interval**	**p**
HLA–DRB1*15	1.753	1.158-2.654	0.008
KIR2DS1	0.450	0.219-0.924	0.030

4. Discussion

The observed associations of KIR variants with several human diseases indicate the functional relevance of KIR polymorphism in immune response. The genes encoding inhibitory KIR are nearly always present in populations at frequencies greater than 90%. The exceptions are those on the so called B haplotypes: KIR2DL2, KIR2DL5A and

KIR2DL5B. Activating KIRs show much greater variation in their presence/absence in different populations. Such extensive diversity between modern populations may indicate that geographically distinct diseases have exerted recent, or perhaps ongoing, selection on KIR repertoires. The differences in frequencies therefore make the choice of appropriate population control for disease studies crucial (Middleton and Gonzelez, 2010).

The primary aim of this study was to examine the possible involvement of KIRs in MS susceptibility, in patients from the north of Portugal. Interactions between KIRs and their HLA class I ligands were also analysed. HLA-DRB1*15 frequencies were considered, to exclude any possible bias due to the known association of this allele with disease susceptibility. We found a negative association of the KIR2DS1 gene with MS, suggesting a possible protective role of this activating gene. The protective effect of KIR2DS1 has been already described in two genetically distinct populations (Norwegians and Italians)(Fusco et al., 2010, Lorentzen et al., 2009). A protective role of the HLA-Cw*05 allele has been described in MS (Yeo et al., 2007). Interestingly this allele belongs to the C2 ligand group, and consequently is a ligand for the KIR2DS1 gene. This is in agreement with the observed protection against MS conferred by the presence of KIR2DS1 and its ligand HLA-Cw*05, independently of HLA-DRB1*15. The decreased risk for the development of MS, associated with KIR2DS1 may result from activation of NK cells and consequently inhibition of autoreactive T cells by lysing autologous DC or lysing autologous T cells, in both cases silencing antigen specific responses (either indirectly or directly, respectively) (see fig 1). Additionally, neurotrophic growth factors secreted by these activated NK cells may contribute to this protection (Graber and Dhib-Jalbut, 2009).

Variation in gene content is a major feature of the KIR complex. Given the extent of KIR locus variability and its effect on a variety of diseases, it seems likely that they may serve as potential therapeutic targets in diseases that directly or indirectly involve the immune response. As stated by Trachtenberg EA, further investigation into the relationship between KIR and HLA polymorphism and susceptibility to MS is critical to better understanding of the innate and adaptative immune response in this disease (Trachtenberg, 2009), for this reason we believe that this study is important.

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Conflict of interest: Authors declare that there aren't any competing financial interests in relation to the work described.

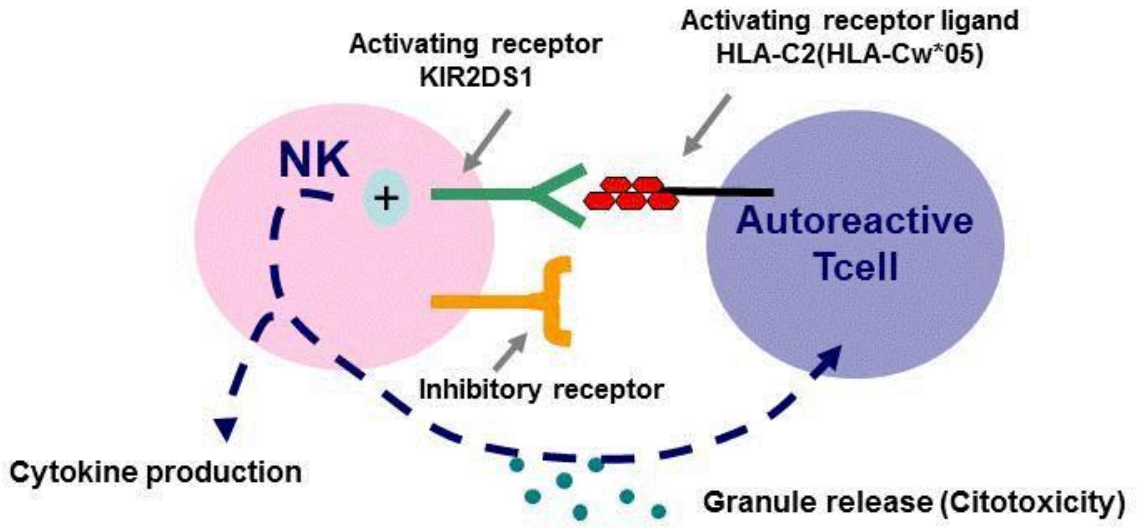

Figure. 1. Schematic figure illustrating how killer immunoglobulin-like receptor (KIR) KIR2DS1 may influence natural killer (NK) cell activation. Activating KIR2DS1, which is associated with protection against Multiple Sclerosis in our study, in the presence of its ligand HLA-C2 (HLA-Cw*05), will lead to an activating signal, and consequently inhibition of autoreactive T cells by lysing autologous DC or lysing autologous T cells, in both cases silencing antigen specific responses (either indirectly or directly, respectively).

References:

Beecham, A. H., Patsopoulos, N. A., Xifara, et al. 2013. Analysis of immune-related loci identifies 48 new susceptibility variants for Multiple Sclerosis. Nat Genet, 45, 1353-60.

Biassoni, R., Ugolotti, E. & De maria, A. 2009. NK cell receptors and their interactions with MHC. Curr Pharm Des, 15, 3301-10.

Boyton, R. J. & Altmann, D. M. 2007. Natural killer cells, killer immunoglobulin-like receptors and human leucocyte antigen class I in disease. Clin Exp Immunol, 149, 1-8.

BrynedaL, B., Duvefelt, K., Jonasdottir, G., Roos, I. M., Akesson, E., Palmgren, J. & Hillert, J. 2007. HLA-A confers an HLA-DRB1 independent influence on the risk of Multiple Sclerosis. PLoS ONE, 2, e664.

Dyment, D. A., Ebers, G. C. & Sadovnick, A. D. 2004. Genetics of Multiple Sclerosis. Lancet Neurol, 3, 104-10.

Fusco, C., Guerini, F. R., Nocera, G., et al. 2010. KIRs and their HLA ligands in Remitting-Relapsing Multiple Sclerosis. J Neuroimmunol.

Garcia-leon, J. A., Pinto-medel, M. J., Garcia-Trujillo, L., et al 2011. Killer cell immunoglobulin-like receptor genes in Spanish Multiple Sclerosis patients. Mol Immunol, 48, 1896-902.

Graber, J. J. & Dhib-Jalbut, S. 2009. Protective autoimmunity in the nervous system. Pharmacol Ther, 121, 147-59.

Hafler, D. A., Compston, A., Sawcer, S., et al 2007. Risk alleles for Multiple Sclerosis identified by a genomewide study. N Engl J Med, 357, 851-62.

Jones, D. C., Edgar, R. S., Ahmad, T., et al 2006. Killer Ig-like receptor (KIR) genotype and HLA ligand combinations in ulcerative colitis susceptibility. Genes Immun, 7, 576-82.

Lorentzen, A. R., Karlsen, T. H., Olsson, M., et al 2009. Killer immunoglobulin-like receptor ligand HLA-Bw4 protects against Multiple Sclerosis. Ann Neurol, 65, 658-66.

Middleton, D. & Gonzelez, F. 2010. The extensive polymorphism of KIR genes. Immunology, 129, 8-19.

Morandi, B., Bramanti, P., Bonaccorsi, I., et al 2008. Role of natural killer cells in the pathogenesis and progression of Multiple Sclerosis. Pharmacol Res, 57, 1-5.

Moretta, A., Marcenaro, E., Parolini, S., Ferlazzo, G. & Moretta, L. 2008. NK cells at the interface between innate and adaptive immunity. Cell Death Differ, 15, 226-33.

Ordonez, D., Sanchez, A. J., Martinez-Rodriguez, J. E., et al 2009. Multiple sclerosis associates with LILRA3 deletion in Spanish patients. Genes Immun, 10, 579-85.

Shi, F. D. & Van Kaer, L. 2006. Reciprocal regulation between natural killer cells and autoreactive T cells. Nat Rev Immunol, 6, 751-60.

Silva, A. M., Bettencourt, A., Pereira, C. et al 2009. Protective role of the HLA-A*02 allele in Portuguese patients with Multiple Sclerosis. Mult Scler, 15, 771-4.

Silva, A. M., Pereira, C., Bettencourt, A., et al 2007. The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population. J Neurol Sci, 258, 69-74.

Trachtenberg, E. A. 2009. Understanding the role of natural killer cell receptors and their human leukocyte antigen ligands in Multiple Sclerosis. Ann Neurol, 65, 626-8.

Yeo, T. W., DE Jager, P. L., Gregory, S. G., et al 2007. A second major histocompatibility complex susceptibility locus for Multiple Sclerosis. Ann Neurol, 61, 228-36.

Paper 3

Nrf2 polymorphisms and Multiple Sclerosis

Nrf2 polymorphisms and Multiple Sclerosis

In preparation

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Abstract

Background: The classic description of MS involves a T-cell mediated process with demyelination and inflammation of the Central Nervous System (CNS). In addition, the release of free radicals (oxygen and nitrogen) by infiltrating monocytes promotes neurodegeneration owing to the fact that cells of the CNS are highly sensitive to excessive oxidative stress. Nuclear factor [erythroid-derived 2]-like 2 (Nrf2) is a basic leucine zipper transcription factor that binds to the promoter sequence of "antioxidant responsive element" (ARE) leading to coordinated up-regulation of ARE driven detoxification and antioxidant genes.

Aims: To investigate the association of two Single Nucleotide Polymorphisms (SNPs) in Nrf2 gene - rs35652124 (-653A/G) and rs6721961 (-617C/A) - with MS susceptibility, disease forms and progression.

Methods: The study included 493 MS patients (387 Relapsing-Remitting (RR), 54 Secondary Progressive (SP) and 52 Primary Progressive (PP)) from the outpatient Neuroimmunology Clinic of CHP–HSA - and 287 healthy controls. SNP genotyping was performed by SimpleProbe allelic discrimination assay.

Results: No significant differences in SNP genotype frequencies were observed between patients and controls. However, the -653GG genotype frequency was statistically higher in RRMS compared to progressive group (10% RRMS vs. 3.8% SPMS+PPMS, p=0.0015). No significant differences were found for the other SNP in relation to disease forms and progression.

Conclusions: Both SNPs reduce the transcription activity of Nrf2 resulting in a decreased detoxification and antioxidant genes transcription. MS patients with the -653GG genotype, and consequently with low Nrf2 expression, appear to be more prone to develop a relapse-remitting course. This is in agreement with experimental data using animal models, where knocking Nrf2 gene out exacerbates experimental autoimmune encephalomyelitis severity.

Introduction

Multiple sclerosis (MS) is the most prevalent chronic inflammatory demyelinating disease of the central nervous system (CNS) affecting around 1 in 1000 people in Europe and US (1). It is thought to be caused by myelin-specific autoreactive $CD4^+$ T (T_H) cells that escape central tolerance, get activated in the periphery, cross the blood brain barrier (BBB) and are reactivated by CNS-resident antigen presenting cells (2). This establishes an inflammatory environment in the CNS causing focal lesions that are characterized by the presence of inflammatory cells, demyelination and axonal damage, leading to the heterogeneity of neurological symptoms (3).

It is generally accepted that MS arises from complex interactions between genetic susceptibility and environmental factors. In the past few years, the number of genes that have been confirmed as important for MS susceptibility has increased substantially. The HLA class II allele DRB1*1501 is a well-established susceptibility factor for this disease (4, 5) but there are now 110 established Multiple Sclerosis risk variants at 103 discrete loci outside of the HLA locus (6). Whether these genes are associated with the clinical phenotype of the disease is less clear (7-9).

There is significant evidence that the pathogenesis of MS involve the generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) associated with mitochondrial dysfunction (10). Nuclear factor [erythroid-derived 2]-like 2 (Nrf2) is a central transcription factor for the antioxidant response (11, 12). In response to alterations in cellular redox status Nrf2 binds to antioxidant response elements (ARE) in the promoters of oxidative-stress regulated genes and induces expression of a battery of antioxidant and detoxifying genes, molecular chaperones and proteasome subunits (11) that aim at restoring redox homeostasis. This is the key cellular mechanism for defense against oxidative damage (11). It has also become apparent that Nrf2 can negatively regulate many pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules, cyclooxygenase-2 and inducible nitric oxide synthase (13). This is thought to be a mechanism of preventing tissue injury associated with inflammatory responses. Presumably, Nrf2 mediates inhibition of NF-kB activity, downregulating the expression of pro-inflammatory genes in innate immune cells (13, 14).

Nrf2 is encoded by the NFE2L2 gene located at chromosome 2q31. Several single nucleotide polymorphisms (SNPs) have been identified in the Nrf2 gene (15). Of special relevance are the rs35652124 (-653A/G) and rs6721961 (-617C/A) polymorphisms, which are located in the promoter region. The rs35652124 and rs6721961 SNPs are predicted to affect Nrf2 myeloid zinc finger 1 (MZF1) and ARE-like promoter binding

sites, respectively (16). These SNPs prevent efficient binding of transcription factors, including Nrf2 itself, to the MZF1 and ARE-like binding sites. Thus, Nrf2 autoregulates its own transcription. Rs35652124 polymorphism has been associated with nephritis in childhood-onset Systemic Lupus Erythematosus (SLE), and that it could be a risk factor for developing kidney dysfunction in SLE patients (17). Taken all of this into account the aim of the present study was to investigate the association of two SNPs in Nrf2 gene -653A/G and -617C/A - with MS susceptibility, disease forms and progression.

Materials and methods

Patients and controls

A total of 493 unrelated patients with a definitive diagnosis of MS, according to the revised McDonald criteria (18), were recruited from the outpatient neurological clinic of the Centro Hospitalar do Porto — Santo António Hospital (HSA). The Expanded Disability Status Scale (EDSS) (19) and Multiple Sclerosis Severity Score (MSSS) (20) were used to measure, respectively, physical disability and disease severity. Clinical features of the 493 MS patients are summarized in Table 1.

Table 1. Clinical features of MS patients.

Clinical variables	Values
Sex (F/M)	323/170
Median age at onset, yr (range)	28 (6-60)
Median disease duration, yr (range)	9 (1-50)
Median EDSS (range)	3.0 (0.0-9.5)
Median MSSS (range)	3.4 (0.0-9.98)
Disease course	
Primary Progressive (PPMS)	52 (10.5%)
Secondary Progressive (SPMS)	54 (11%)
Relapsing-remitting (RRMS)	387 (78.5%)

MS patients were randomly included in the study at disease onset. The control group comprised a total of 287 healthy controls (HC) from the same geographical region. This study was approved by the Medical Ethical Committee of the hospital and written informed consent was obtained from all participants.

SNP's genotyping

Peripheral blood samples (10 mL) were collected in EDTA. Genomic DNA was obtained from proteinase-K–treated peripheral blood leukocytes by using a Salting-Out procedure. SNP's were genotyped with Simple probe assays. PCR products were detected by real-time PCR (RotorGene 6000; Corbett Life Science). The HLA class II data of these patient and control cohorts have been reported previously (5, 21).

Statistics

SNP-phenotype association analysis was undertaken by binary or polytomous logistic regression where appropriate. Unadjusted and adjusted analysis taking into account age, gender and the presence of HLA-DRB1*15 allele was performed. The SNP of interest was modelled assuming several related genotypic mechanisms (additive, dominant, recessive, heterozygous advantage and general models) and the minimum p-value from these correlated tests were reported. The level of significance was setup at 5%. All analyses were undertaken with the IBM SPSS Statistics 23 software (IBM Corporation, Somers, NY, USA).

Results

To investigate if Nrf2 influence the development and course of MS two SNP´s of this gene (rs6721961 and rs35652124) were analysed in a case-control study. For rs6721961 424 patients with MS and 202 controls were genotyped, for rs35652124 493 patients with MS and 287 controls were genotyped.

The frequencies of the two SNP's where very similar to the ones described in others populations. All SNP's were in Hardy-Weinberg equilibrium in controls. When the allele and genotype frequencies of the three SNPs were compared between cases and controls, no significant association was observed with MS susceptibility (data not shown).

Multiple Sclerosis patients can be clinically classified as having a relapsing (RRMS) or progressive disease (SPMS+PPMS) (22). A significantly higher frequency of the GG haplotype of -653A/G SNP was observed among patients with RRMS (10% RRMS vs. 3.8% SPMS+PPMS, p=0.0015). The -653A/G SNP was significantly associated with disease course even when adjusted for age, gender, presence of HLA-DRB1*15 allele,

disease duration (p=0.0010) and EDSS (p=0.0026) and when adjusted for age, gender, presence of HLA-DRB1*15 allele and MSSS (p=0.0088) (Table 2).

Table 2. Binary logistic regression association analysis between SNPs and disease phenotype

Phenotype	SNP	Unadjusted	Adjusted				
			Model 1[a]	Model 2[b]	Model 3[c]	Model 4[d]	Model 5[e]
Disease status (MS vs Controls)	-617C/A	0.599	0.472	0.155	-	-	-
	-653A/G	0.995	0.739	0.295	-	-	-
Disease Course (RR vs SP + PP)	-617C/A	0.385	0.395	0.313	0.372	0.368	0.162
	-653A/G	**0.0015**	**0.0008**	**0.0007**	**0.0010**	**0.0026**	**0.0088**

[a] Adjusted for age and gender.
[b] Adjusted for age, gender and HLA-DRB1*15
[c] Adjusted for age, gender, HLA-DRB1*15 and disease duration.
[d] Adjusted for age, gender, HLA-DRB1*15, disease duration and EDSS.
[e] Adjusted for age, gender, HLA-DRB1*15 and MSSS

Discussion

Because oxidative stress is implicated in the pathogenesis of MS (23) and Nrf2 is a central protein in the cellular defense against oxidative stress (24), genetic variation affecting the efficiency of Nrf2 may contribute to the disease etiopathogenesis. Marzec et al. reported that Nrf2 activity was low in G allele carriers of -653A/G and A allele carriers of -617C/A (16). Consequently the G allele of -653A/G reduce the transcription activity of Nrf2 resulting in a decreased detoxification and antioxidant genes transcription. The results of the present study indicate that MS patients with the -653GG genotype, and consequently with low Nrf2 expression, appear to be more prone to develop a relapse remitting course. Also, the absence of association of Nrf2 SNP´s with disease susceptibility and a positive association with a relapsing onset (severity) are in accordance with the results obtained in animal models - Nrf2-/- mice developed a more severe form of EAE with statistically significant increased mean disease scores and mortality but no difference in incidence (25).

Patients can be clinically classified as having a relapsing or progressive Multiple Sclerosis (22). In RRMS, deterioration results from acute intermittent inflammation, demyelination and axonal injury following the transmigration of inflammatory cells into the CNS (26, 27). Conversely, disability accumulation in SPMS and PPMS results mostly from chronic persistent demyelination, continued widespread axonal injury, and a compartmentalized inflammatory process behind the BBB (28). During the transition from RRMS to SPMS, both peripherally driven and compartmentalized inflammatory processes may coexist.

Infiltrated monocyte-derived macrophages, which form the major cell type in perivascular infiltrates, produce a variety of inflammatory mediators like ROS, nitric oxide, and pro-inflammatory cytokines, which all contribute to neuroinflammation, demyelination, axonal damage, and disease progression. The ROS enhance both monocyte adhesion and migration across brain endothelial cells. Thus, ROS are generally thought to be derived from activated inflammatory cells and to play a role in demyelination and axonal damage in Multiple Sclerosis. Furthermore, free radicals can activate certain transcription factors, such as nuclear transcription NF-KB, which up regulate the expression of many genes such as TNF- α, inducible nitric oxide synthase, intracellular adhesion molecule 1, and vascular-cell adhesion molecule 1. Also, redox reactions are involved in the activity of matrix metallo proteinases, which are important to T cell trafficking into the CNS.

Our results show that patients with the -653GG genotype are more prone to develop a RRMS course. This observation can be explained by the fact that this genotype is associated with low Nrf2 expression, and Nrf2 is a fundamental player in the control of inflammation.

References

1. Petermann F, Korn T. Cytokines and effector T cell subsets causing autoimmune CNS disease. FEBS letters. 2011. Epub 2011/04/12.
2. Goverman J. Autoimmune T cell responses in the central nervous system. Nat Rev Immunol. 2009;9(6):393-407. Epub 2009/05/16.
3. Lassmann H, Bruck W, Lucchinetti CF. The immunopathology of Multiple Sclerosis: an overview. Brain Pathol. 2007;17(2):210-8. Epub 2007/03/29.
4. Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, et al. Risk alleles for Multiple Sclerosis identified by a genomewide study. N Engl J Med. 2007;357(9):851-62. Epub 2007/07/31.
5. Silva AM, Pereira C, Bettencourt A, Carvalho C, Couto AR, Leite MI, et al. The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population. J Neurol Sci. 2007;258(1-2):69-74.
6. Beecham AH, Patsopoulos NA, Xifara DK, Davis MF, Kemppinen A, Cotsapas C, et al. Analysis of immune-related loci identifies 48 new susceptibility variants for Multiple Sclerosis. Nat Genet. 2013;45(11):1353-60. Epub 2013/10/01.
7. Jensen CJ, Stankovich J, Van der Walt A, Bahlo M, Taylor BV, van der Mei IA, et al. Multiple sclerosis susceptibility-associated SNPs do not influence disease severity measures in a cohort of Australian MS patients. PloS one. 2010;5(4):e10003. Epub 2010/04/07.
8. Cree BA. Genetics of primary progressive Multiple Sclerosis. Handbook of clinical neurology. 2014;122:211-30. Epub 2014/02/11.
9. DeLuca GC, Ramagopalan SV, Herrera BM, Dyment DA, Lincoln MR, Montpetit A, et al. An extremes of outcome strategy provides evidence that Multiple Sclerosis severity is determined by alleles at the HLA-DRB1 locus. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(52):20896-901. Epub 2007/12/19.
10. Witherick J, Wilkins A, Scolding N, Kemp K. Mechanisms of oxidative damage in Multiple Sclerosis and a cell therapy approach to treatment. Autoimmune diseases. 2010;2011:164608. Epub 2011/01/05.
11. Sykiotis GP, Bohmann D. Stress-activated cap'n'collar transcription factors in aging and human disease. Science signaling. 2010;3(112):re3. Epub 2010/03/11.
12. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochemical and biophysical research communications. 1997;236(2):313-22. Epub 1997/07/18.
13. Kim J, Cha YN, Surh YJ. A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. Mutation research. 2010;690(1-2):12-23. Epub 2009/10/06.
14. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M, Kensler TW, et al. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. The Journal of clinical investigation. 2006;116(4):984-95. Epub 2006/04/06.
15. Cho HY, Marzec J, Kleeberger SR. Functional polymorphisms in Nrf2: implications for human disease. Free radical biology & medicine. 2015;88(Pt B):362-72. Epub 2015/06/29.
16. Marzec JM, Christie JD, Reddy SP, Jedlicka AE, Vuong H, Lanken PN, et al. Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2007;21(9):2237-46. Epub 2007/03/27.
17. Cordova EJ, Velazquez-Cruz R, Centeno F, Baca V, Orozco L. The NRF2 gene variant, -653G/A, is associated with nephritis in childhood-onset systemic lupus erythematosus. Lupus. 2010;19(10):1237-42. Epub 2010/05/29.
18. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for Multiple Sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. 2011;69(2):292-302. Epub 2011/03/10.
19. Kurtzke JF. Rating neurologic impairment in Multiple Sclerosis: an expanded disability status scale (EDSS). Neurology. 1983;33(11):1444-52. Epub 1983/11/01.
20. Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, et al. Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. Neurology. 2005;64(7):1144-51.
21. Bettencourt A, Carvalho C, Leal B, Bras S, Lopes D, Martins da Silva A, et al. The Protective Role of HLA-DRB1(*)13 in Autoimmune Diseases. Journal of immunology research. 2015;2015:948723. Epub 2015/11/26.

22. Lublin FD, Reingold SC, Cohen JA, Cutter GR, Sorensen PS, Thompson AJ, et al. Defining the clinical course of Multiple Sclerosis: the 2013 revisions. Neurology. 2014;83(3):278-86. Epub 2014/05/30.
23. Ohl K, Tenbrock K, Kipp M. Oxidative stress in Multiple Sclerosis: Central and peripheral mode of action. Experimental neurology. 2016;277:58-67. Epub 2015/12/03.
24. Sandberg M, Patil J, D'Angelo B, Weber SG, Mallard C. NRF2-regulation in brain health and disease: implication of cerebral inflammation. Neuropharmacology. 2014;79:298-306. Epub 2013/11/23.
25. Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA. The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. Toxicological sciences : an official journal of the Society of Toxicology. 2010;114(2):237-46. Epub 2009/11/17.
26. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in Multiple Sclerosis brains. Brain. 2009;132(Pt 5):1175-89. Epub 2009/04/03.
27. Lassmann H. Multiple sclerosis: is there neurodegeneration independent from inflammation? J Neurol Sci. 2007;259(1-2):3-6. Epub 2007/03/21.
28. Lassmann H, van Horssen J, Mahad D. Progressive Multiple Sclerosis: pathology and pathogenesis. Nat Rev Neurol. 2012;8(11):647-56. Epub 2012/09/26.

Paper 4

Serum microRNA-155 in Multiple Sclerosis patients

Serum microRNA-155 in Multiple Sclerosis patients

In preparation

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Introduction: Multiple Sclerosis (MS) is an autoimmune, inflammatory neurodegenerative disorder of the central nervous system. MicroRNAs (miRNAs) are a class of small noncoding RNAs which have recently been described to be regulatory modulators of gene expression, controlling different biological processes, including immune responses. MicroRNA-155 (miRNA-155) is a multifunctional molecule that plays a crucial role in inflammation. Its expression is up-regulated in a variety of tissues including whole blood, CD4+and CD8+ T cells, serum and brain lesions of MS patients. Also, it promotes Th1 and Th17 responses and increases the permeability of the blood brain barrier promoting sustained inflammation, contributing to MS pathogenesis.

Aim: To analyse the expression of circulating miRNA-155 in the serum of MS patients.

Methods: The study included 60 MS patients (38 female and 22 male) and 55 healthy controls (HC). RNA extraction from serum samples was done using the miRNeasy Serum/Plasma Kit. MiRNA-155 gene expression was detected with a TaqMan® miRNA assay. Relative expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Differences in ΔCt were evaluated using a two-tailed Student's t-test.

Results: The serum concentration of miRNA-155 was significantly higher in MS patients compared with HC (fold change 4.60; $p<0.0001$).

Discussion: Circulating miRNAs have emerged as potential biomarkers for several human diseases including MS. As far as is known, miRNA-155 is the only miRNA consistently increased in MS brain and spinal cord lesions and in peripheral blood mononuclear cells. In this study miRNA-155 levels were increased in patient's serum which supports the findings of Zhang et al, 2014. These observations can have important implications for the development of new therapeutic strategies.

Introduction

MicroRNAs (miRNAs) are short (about 22 nucleotides in length) single-stranded regulatory RNAs that modulate gene expression, at the posttranscriptional level, by repressing translation of specific messenger RNA (mRNA) targets, resulting in downregulation of protein expression (Krol et al. 2010; Pasquinelli 2012). These molecules regulate approximately 90% of protein-coding genes, and play a central role in various biological processes including immune call lineage commitment, differentiation, proliferation, apoptosis and maintenance of immune homeostasis (O'Connell et al. 2010). miRNAs have been detected in several body fluids, including plasma, serum, cerebrospinal fluid (CSF), breast milk, urine, tears, semen, and saliva (Cortez et al. 2011). They are highly stable in the blood, being resistant to circulating ribonucleases and severe conditions such as extended storage, freeze-thaw, and extreme pH due to their packaging in lipid vesicles such as exosomes, binding to RNA-binding protein, and association with high-density lipoprotein.

One of the best-characterized miRNAs is miR-155, which has pleiotropic functions in inflammation, autoimmunity, and cell plasticity (Baltimore et al. 2008; Kong et al. 2008; Faraoni et al. 2009; O'Connell et al. 2010; Vigorito et al. 2013). Thus far, miR-155 has been shown to induce a decrease in the expression levels of multiple identified transcripts, but the effect is modest, characteristic of fine-tuning regulation (Selbach et al. 2008; Guo et al. 2010). In the context of neuroinflammation, miR-155 has been shown to be one of the most highly elevated miRNAs in acute MS lesions (Junker et al. 2009). miR-155 expression was highly correlated with disease severity in patients with Multiple Sclerosis, it was significantly increased (fold change = 3.65; $P < 0.001$) in sera samples from a cohort of 31 MS patients (Zhang et al. 2014). Also, brain endothelial miR-155 is a negative regulator of blood-brain barrier function during neuroinflammation (Lopez-Ramirez et al. 2014) and also contributes to the regulation of leukocyte adhesion at the inflamed BBB (Cerutti et al. 2016). The aim of this study was to analyse the expression of miR-155 in the sera of a large group of Portuguese MS patients.

Materials and methods

Patients and controls

A total of 60 unrelated patients with a definitive diagnosis of MS, according to the revised McDonald criteria (Polman et al. 2011), were recruited from the outpatient neurological clinic of the Centro Hospitalar do Porto — Santo António Hospital (HSA) and Centro Hospitalar de São João. The Expanded Disability Status Scale (EDSS) (Kurtzke 1983)

and Multiple Sclerosis Severity Scale (MSSS) (Roxburgh et al. 2005) were used to measure, respectively, physical disability and disease severity. Clinical and demographic information such as sex, age at onset, clinical course, EDSS and MSSS at the time of serum sampling were collected from the patient database. Clinical features of the 60 MS patients are summarized in Table 1.

Table 1. Clinical features of MS patients.

Clinical variables	Values
Sex (F/M)	38/22
Median age at onset, yr (range)	28 (16-53)
Median disease duration, yr (range)	15 (1-30)
Median EDSS (range)	3.0 (0.0-8.0)
Median MSSS (range)	3.4 (0-9.33)
Disease course	
Relapse-Remitting (RRMS)	46 (76.7%)
Secondary Progressive (SPMS)	14 (23.3%)

MS patients were randomly included in the study at disease onset. The control group comprised a total of 75 healthy controls (HC) from the same geographical region. This study was approved by the Medical Ethical Committee of the hospital and written informed consent was obtained from all participants.

miRNAs expression levels quantification

Peripheral blood was collected in tubes without anticoagulant (Vacuette, GBO, Germany), centrifuge and serum aliquots were stored at -20ºC. RNA was extracted using the miRNeasy® Serum/Plasma Kit (Qiagen, Germany), according to the manufacturer's protocol. The synthesis of cDNA was performed with the Taqman®MicroRNA reverse Transcription-Applied Biosystems Kit (Applied Biosystems, USA) and specific primers for miR-155 (Taqman® MicroRNA Assays – Applied Biosystems, USA). The quantitative RT-PCR amplification was run with specific primers and probes for the studied miRNAs

(Taqman® MicroRNA Assays – Applied Biosystems, USA) and a NzySpeedy qPCR mastermix (Nzytech, Portugal). Each reaction was performed in triplicate and the average Ct value was used in analysis. The relative expression was calculated using the 2 -ΔΔCT method. MiRNAs level was evaluated in serum, a cell-free body fluid that is not known to have constant levels of a particular RNA species, hindering expression normalization by an endogenous control or housekeeping gene. To overcome this problem, the same serum volume was used for each subject and the same threshold was used for each target so that expression levels could be comparable between samples. Therefore, micro-RNAs levels are expressed as 50-Ct (Wang et al. 2010).

Statistical analysis

Continuous variables were given as means with standard deviation. The normality of distribution was verified using the Kolmogorov-Smirnov test. Differences between two groups were analysed using the parametric student's t test or the nonparametric Mann-Whitney test, as appropriate. In the case of more than two groups, the nonparametric Kruskal-Wallis 1-way ANOVA test or the One way ANOVA test was used. In order to evaluate the correlation between variables, Spearman's rank correlation coefficient was applied. All the analyses were carried out using the Statistical Package for the Social Sciences (SPSS), v.23 software. The level of significance for comparisons was set at $p < 0.05$.

Results

The normality of the distribution of examined microRNA was assessed using the Kolmogorov-Smirnov test. The resulting *p* value ($p>0.05$) allowed for the maintenance of the null hypothesis (the distribution in these groups is normal) and parametric tests were used for subsequent analysis.

Means of the normalized expression (ΔCt) obtained for miRNA155 was performed in patients and in healthy controls. For MS patient the ΔCt mean ± SD was 25.02±0.99 and for HI controls was 21.3±2.36 (Figure 1). This difference was statistically significant ($p<0.0001$).

Multiple Sclerosis patients presented a higher expression of miR-155 gene. For this gene the relative quantification in MS was 3.72, and by definition 1 for HI. These values mean that the expression of miR-155 gene for MS patients is about 13 times higher in MS patients than in the HI.

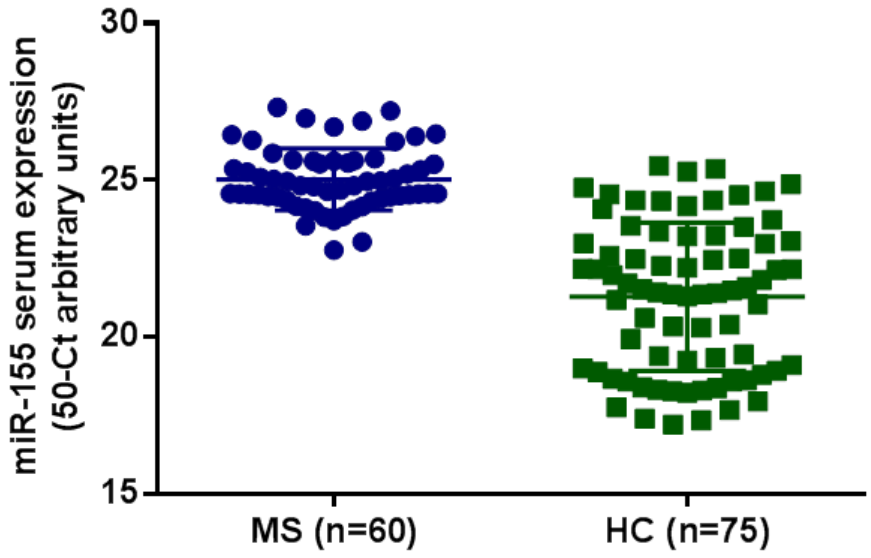

Figure 1. Circulating miR-155 expression levels in healthy controls and MS patients.

Discussion

Circulating miRNAs have emerged as potential biomarkers for several human diseases including Multiple Sclerosis (Jagot et al. 2016).

Serum concentration of circulating miRNA-155 was significantly higher in MS patients compared with HC which is in accordance with previous studies performed in serum (Zhang, Cheng et al. 2014) and in PBMC (Paraboschi et al. 2011; Waschbisch et al. 2011) but not with Fenoglio C et al. (Fenoglio et al. 2011) that found no differences in the expression levels of miR-155 in patients as compared with controls ($P>0.05$).

miRNA-155 is the only miRNA consistently increased in MS brain and spinal cord lesions, in peripheral blood mononuclear cells and in serum (Xinting Ma et al., 2014). Several data reveal that miR-155 is involved in immune responses, including B and T cell differentiation and development and its overexpression results in human chronic inflammatory condition (O'Connell et al. 2012).

Deregulated miRNA levels in biological fluids could represent a new source of biomarkers in MS that could be helpful for disease prognosis and for discrimination of clinical subtype (Keller et al. 2009), thereby helping therapeutic decisions or monitoring of therapeutic effects. These observations can have important implications for the development of new therapeutic strategies (Li et al. 2012; Zare-Shahabadi et al. 2013; Aslani et al. 2017).

References

Aslani, S., N. Jafari, et al. (2017). "Epigenetic Modifications and Therapy in Multiple Sclerosis." Neuromolecular Med 19(1): 11-23.

Baltimore, D., M. P. Boldin, et al. (2008). "MicroRNAs: new regulators of immune cell development and function." Nat Immunol 9(8): 839-845.

Cerutti, C., P. Soblechero-Martin, et al. (2016). "MicroRNA-155 contributes to shear-resistant leukocyte adhesion to human brain endothelium in vitro." Fluids Barriers CNS 13(1): 8.

Cortez, M. A., C. Bueso-Ramos, et al. (2011). "MicroRNAs in body fluids--the mix of hormones and biomarkers." Nat Rev Clin Oncol 8(8): 467-477.

Faraoni, I., F. R. Antonetti, et al. (2009). "miR-155 gene: a typical multifunctional microRNA." Biochim Biophys Acta 1792(6): 497-505.

Fenoglio, C., C. Cantoni, et al. (2011). "Expression and genetic analysis of miRNAs involved in CD4+ cell activation in patients with Multiple Sclerosis." Neurosci Lett 504(1): 9-12.

Guo, H., N. T. Ingolia, et al. (2010). "Mammalian microRNAs predominantly act to decrease target mRNA levels." Nature 466(7308): 835-840.

Jagot, F. and N. Davoust (2016). "Is It worth Considering Circulating microRNAs in Multiple Sclerosis?" Front Immunol 7: 129.

Junker, A., M. Krumbholz, et al. (2009). "MicroRNA profiling of Multiple Sclerosis lesions identifies modulators of the regulatory protein CD47." Brain 132(Pt 12): 3342-3352.

Keller, A., P. Leidinger, et al. (2009). "Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls." PLoS One 4(10)

Kong, W., H. Yang, et al. (2008). "MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA." Mol Cell Biol 28(22): 6773-6784.

Krol, J., I. Loedige, et al. (2010). "The widespread regulation of microRNA biogenesis, function and decay." Nat Rev Genet 11(9): 597-610.

Kurtzke, J. F. (1983). "Rating neurologic impairment in Multiple Sclerosis: an expanded disability status scale (EDSS)." Neurology 33(11): 1444-1452.

Li, J. S. and Z. X. Yao (2012). "MicroRNAs: novel regulators of oligodendrocyte differentiation and potential therapeutic targets in demyelination-related diseases." Mol Neurobiol 45(1): 200-212.

Lopez-Ramirez, M. A., D. Wu, et al. (2014). "MicroRNA-155 negatively affects blood-brain barrier function during neuroinflammation." FASEB J 28(6): 2551-2565.

O'Connell, R. M., D. Kahn, et al. (2010). "MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development." Immunity 33(4): 607-619.

O'Connell, R. M., D. S. Rao, et al. (2012). "microRNA regulation of inflammatory responses." Annu Rev Immunol 30: 295-312.

O'Connell, R. M., D. S. Rao, et al. (2010). "Physiological and pathological roles for microRNAs in the immune system." Nat Rev Immunol 10(2): 111-122.

Paraboschi, E. M., G. Solda, et al. (2011). "Genetic association and altered gene expression of mir-155 in Multiple Sclerosis patients." Int J Mol Sci 12(12): 8695-8712.

Pasquinelli, A. E. (2012). "MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship." Nat Rev Genet 13(4): 271-282.

Polman, C. H., S. C. Reingold, et al. (2011). "Diagnostic criteria for Multiple Sclerosis: 2010 revisions to the McDonald criteria." Ann Neurol 69(2): 292-302.

Roxburgh, R. H., S. R. Seaman, et al. (2005). "Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity." Neurology 64(7): 1144-1151.

Selbach, M., B. Schwanhausser, et al. (2008). "Widespread changes in protein synthesis induced by microRNAs." Nature 455(7209): 58-63.

Vigorito, E., S. Kohlhaas, et al. (2013). "miR-155: an ancient regulator of the immune system." Immunol Rev 253(1): 146-157.

Wang, G., L. S. Tam, et al. (2010). "Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus." J Rheumatol 37(12): 2516-2522.

Waschbisch, A., M. Atiya, et al. (2011). "Glatiramer acetate treatment normalizes deregulated microRNA expression in relapsing remitting Multiple Sclerosis." PLoS One 6(9): e24604.

Zare-Shahabadi, A., Y. Renaudineau, et al. (2013). "MicroRNAs and Multiple Sclerosis: from physiopathology toward therapy." Expert Opin Ther Targets 17(12): 1497-1507.

Zhang, J., Y. Cheng, et al. (2014). "MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with Multiple Sclerosis and experimental autoimmune encephalomyelitis." J Neuroimmunol 266(1-2): 56-63.

3.2 – Vitamin D

Paper 5

Multiple Sclerosis and birth timing in Northern Portugal

Multiple Sclerosis and birth timing in Northern Portugal

In preparation

Andreia Bettencourt, Maria José Sá, Berta Silva, Paulo P Costa, Ana Martins Silva

Abstract

Background: Month of birth has been described as a risk factor for Multiple Sclerosis (MS) susceptibility and disease phenotype in different studies.

Purpose: To assess whether month of birth is associated with MS susceptibility and/or with disease progression in Northern Portuguese MS patients.

Methods: The month of birth of MS patients from the North of Portugal, born between 1929 and 1993 was compared with the month of births in the general population during the same period and from the same geographical region.

Results: Significantly less patients with MS (51.1%) were born in October compared with controls (Bonferroni corrected p=0.005). The January-June period is a period of higher incidence (p=0.0325). No significant differences were found concerning disease progression.

Conclusions: Multiple Sclerosis seems to be more frequent in individuals born in January-June but birth timing does not seem to influence disease progression.

Keywords: Multiple Sclerosis; month of birth; season of birth; Disease progression; Portugal.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. It has a complex etiopathogenesis and it is widely accepted to be an autoimmune disorder, occurring in genetically susceptible individuals after exposure to infectious, nutritional, and climatic or other undefined environmental factors [1]. Even though genes are important for MS development, epidemiological studies clearly show that environment has also a prominent role in contributing to MS risk. Environmental factors with the strongest evidence for involvement in MS are Epstein-Barr virus (EBV), smoking, and latitude/vitamin D.

The level of ultraviolet radiation, influencing directly (non-vitamin D pathways) or through the generation of vitamin D is a strong candidate [2]. Within regions of temperate climate, MS incidence and prevalence increases with latitude [3]. Higher exposure to ultraviolet radiation [4], higher vitamin D intake [5], and also higher serum vitamin D concentrations [6] seem to be associated with a reduced risk of onset of Multiple Sclerosis. This evidence comes from both case-control and cohort studies. Seasonal deficiency in maternal vitamin D concentrations may be linked to excess risk of Multiple Sclerosis at birth. Because pregnant women have a reduced outdoor activity and increased physiological needs, they are vulnerable for the development of vitamin D deficiency during pregnancy [7]. Low concentrations of neonatal vitamin D are associated with an increased risk of MS [8, 9].

Month of birth has been described as a risk factor for MS susceptibility. Several studies have suggested that a springtime birth substantially increase the risk of developing MS. The risk of developing MS in countries located in the northern hemisphere is greater for those individuals born in April/May and less for those born in October/November [10-16]. A meta-analysis of 2013 revealed an excessive MS risk in individuals born in spring and a reduced risk in individuals born in autumn, imputing this observation to UV light exposure and maternal Vitamin D levels [17]. Nevertheless, this has not been confirmed in other studies [18-23]. Also, a study from Barros et al. [24] does not support the hypothesis of month of birth as risk factor for Multiple Sclerosis in Portugal.

Concerning disease progression, there are also inconsistent findings on how month of birth might influence the prognosis of MS. Two independent studies have described that, patients born in the winter months have an earlier disease onset than those born in the high-risk months [25, 26]. In 2006 Tremlett and Devonshire [27] reported that there was some evidence to suggest that the gestational period had a small effect on later disease progression in British Columbia, Canada. In a follow-up study with 2837 patients also

from British Columbia, Canada, and 810 patients from Groningen, the Netherlands, Koch et al. found no association between the season or month of birth and disease progression [28]. More recently, Lucenti et al [29], studied 1782 patients from Italy and found that birth timing is not associated with MS progression.

Taking into account all the studies described previously we may assume that the month of birth effect seems to be most noticeable in high-risk areas for MS, and especially in areas with low sunlight exposure (Northern hemisphere). In areas with a high sunlight exposure, there seems to be little or no month of birth effects. Portugal was considered to be a low-medium prevalence zone for MS [3], but following epidemiological studies suggest that it should be considered a medium prevalence zone [30]. Data on MS prevalence in Portugal points for a prevalence of 47 per 100 000 in Santarem, Centre of Portugal [30, 31]. In 2015, a study from the District of Braga, North of Portugal, found a prevalence of 39.82/100 000 inhabitants [32]. Portugal is one of the European countries with more hours of sunshine consequently the study of month of birth in MS may be of particular interest.

2. Materials and Methods

2.1 Patients and controls

A total of 502 MS patients, followed at the outpatient Neuroimmunology Clinic of Centro Hospitalar do Porto – Hospital de Santo António, a tertiary center in the North of Portugal were consecutively enrolled. MS patients were diagnosed according to Poser and McDonald criteria. The study was approved by the Ethical Committee of the hospital. Samples and clinical information were collected after written informed consent was obtained. The control sample was obtained from Statistics Portugal, the entity responsible for ensuring the production and dissemination of official statistical information in Portugal. It comprised the live births records in the same geographical area and for the same time period of birth as patients.

2.2 Demographic and clinical variables

Demographic and clinical data were collected at enrolment. The following variables were recorded for each patient: sex, ethnicity, clinical course, age at disease onset and EDSS score at the last visit (at least 1 month after the last relapse). The Multiple Sclerosis severity score (MSSS) was used as a measure of disease severity.

2.3 Statistical analysis

The distribution of month of birth in MS patients was compared with the distribution in the corresponding Portuguese population (birth years 1929-1993 as reported by Statistics Portugal) by Chi square test. Each month was analysed separately and compared with the other 11 months, in terms of odds ratios (ORs) and 95% confidence intervals (CI) using 2x2 table for the Chi square test. Bonferroni correction was used to correct for the 12 comparisons. Seasonality was assessed using the Hewitt's test [33]. The Hewitt's test is a non-parametric test, considered by many authors to be more appropriate for sinusoidal patterns then the Edwards' test [34, 35], especially when the sample size is not very large [33, 34]. In brief, we estimated the expected relative incidence (ERI) and the observed relative incidence (ORI) of MS cases per month and ranked from 1 to 12 according to the magnitude (12=highest; 1=smallest) (Table 2). Based on the ORI ranks shown in the last column of Table 2, we determined the rank-sums for successive 6-moth segments (Table 3) and the statistical significance of the rank-sum values was determined by a table of cumulative probability [33].

MSSS scores of each month and season of birth were compared with all the others combined, using Bonferroni's correction to account for multiple comparisons. 95% confidence intervals (CI) for medians were computed. Differences were considered significant at p>0.005. Statistical analyses were performed using the IBM SPSS Statistics 23.0 software (IBM Corp, Armonk, NY).

3. Results

Five hundred and two patients with a mean age at onset of 29.8 years (SD: 9.4, range 6-60) and mean disease duration of 11 years (SD: 8.9, range 1-50) were included in this study. The frequency of females was 65.3%. The most frequent form of presentation was relapsing-remitting (78.2%), 115 patients had progressive MS.

Age at onset was not statistically different in patients born in hotter six-months period from April to September (mean: 29.6 years) from patients born between October and March (mean: 30.1 years, p=0.558). The number of patients with MS born in each month versus the other 11 months was compared with the general population (Table 1). In this study 51.1% fewer people with MS were born in October, this was significant even after Bonferroni correction (21 observed vs. 42 expected, χ^2=11.87, p=0.0006, p_c=0.007).The peak birth month was February, the risk of MS was 43.5% higher for people born in this month (51 observed vs. 37 expected, χ^2=5.87, p=0.015) (Table 1).

The rank-sums for successive 6 month segments (Table 3) indicate the January-June period as of higher incidence and if we assume a prior hypothesis of seasonality, the

corresponding rank-sum (51) is statistically significant according to the Hewitt test (p=0.0325). On the other hand, if we do not assume a prior hypothesis of seasonality we have to look to the probability of the *maximum* rank-sum (p=0.2908).

Figure 1 shows the median score by month of birth. The median of the individual months was not statistically different from the overall median score. The peak MSSS was May.

Table 1. Observed number of patients with Multiple Sclerosis compared with the expected number, according to month of birth.

	MS patients (n=502)		Observed/expected births (95% CI)	χ^2	p value	p_c value*
Month	**Observed nº of births**	**Expected nº of births**				
January	50	40	1.28 (0.95-1.71)	2.72	0.099	-
February	51	**37**	**1.43 (1.07-1.91)**	**5.87**	**0.015**	**-**
March	43	43	1.01 (0.74-1.38)	0.00	0.956	-
April	41	45	0.91 (0.66-1.25)	0.36	0.548	-
May	48	44	1.10 (0.82-1.48)	0.40	0.526	-
June	43	43	1.01 (0.74-1.38)	0.00	0.959	-
July	36	44	0.80 (0.57-1.13)	1.59	0.208	-
August	53	42	1.28 (0.96-1.70)	2.84	0.092	-
September	41	43	0.95 (0.69-1.31)	0.10	0.750	-
October	21	**42**	**0.47 (0.30-0.73)**	**11.87**	**0.0006**	**0.007**
November	37	39	0.94 (0.67-1.31)	0.14	0.706	-
December	38	40	0.95 (0.68-1.32)	0.09	0.769	-

*pc=Bonferroni correction

Table 2. Total number of births in MS and control groups, estimates of expected relative incidence (ERI), observed relative incidence (ORI) and ORI ranks.

Month	**Births (n=1,150,362)**	**MS (n=502)**	**days in month**	**ERI (x10^3)**	**ERI (502)**	**ORI (x10^3)**	**Rank**
Jan	91645	50	31	0.0371	42.6	0.0463	10
Feb	84416	51	28	0.0335	38.5	0.0463	11
Mar	97744	43	31	0.0371	42.6	0.0374	7.5
Apr	102762	41	30	0.0359	41.3	0.0328	5.5
May	100797	48	31	0.0371	42.6	0.0404	9
Jun	97802	43	30	0.0359	41.3	0.0361	7.5
Jul	100788	36	31	0.0371	42.6	0.0303	2
Aug	97354	53	31	0.0371	42.6	0.0462	12
Sep	98538	41	30	0.0359	41.3	0.0342	5.5
Oct	97370	21	31	0.0371	42.6	0.0183	1
Nov	89986	37	30	0.0359	413	0.0338	3
Dec	91160	38	31	0.0371	42.6	0.0354	4

Table 3. Hewitt test rank-sums for successive 6-month segments with the corresponding p values presented in Hewitt et al. [33].

Period	Rank-sum	Cumulative Probability	Estimated Cumulative Probability
Jan to Jun	**51**	**0.0325**	0.2908
Feb to Jul	49	0.0660	0.4958
Mar to Aug	48	0.0898	0.6086
Apr to Sep	50	0.0465	0.3826
May to Oct	43	0.2944	0.9904
Jun to Nov	38	1.0000	1.0000
Jul to Dec	27	1.0000	1.0000
Aug to Jan	29	1.0000	1.0000
Sep to Feb	30	1.0000	1.0000
Oct to Mar	28	1.0000	1.0000
Nov to Apr	35	1.0000	1.0000
Dec to May	40	0.4686	1.0000

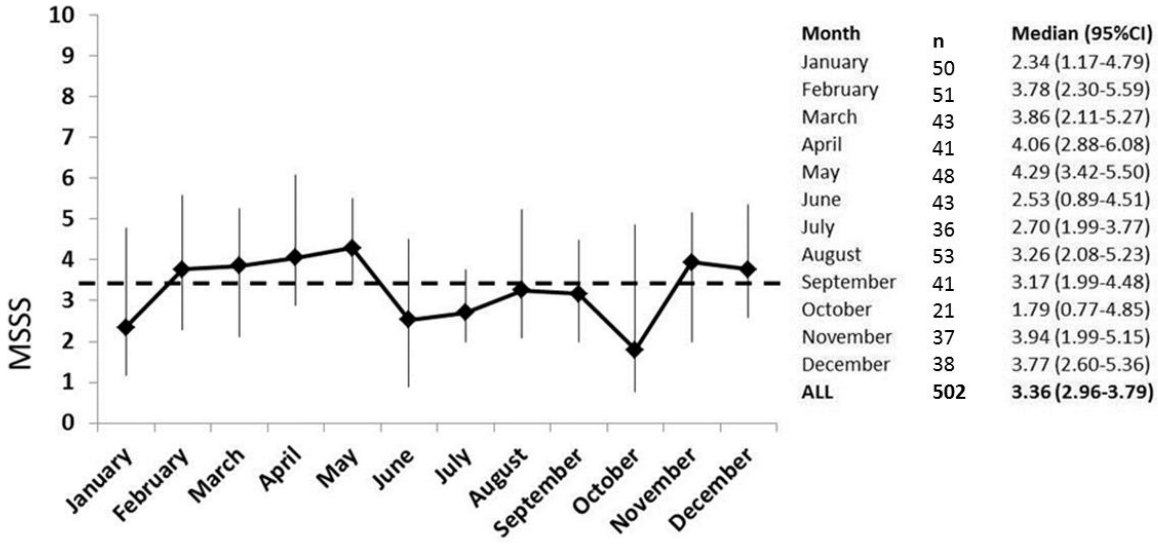

Figure 1. MSSS median scores with corresponding 95%CI, by patients month of birth. The dashed line represents the MSSS overall median.

4. Discussion

A consistent finding that month or season of birth has an effect on MS susceptibility is described in studies from the Northern hemisphere [17]. In the Southern hemisphere, a reverse pattern was detected, with an excess in November–December and a decrease in May–June [36]. Like in most studies we also found less MS births than expected in the autumn, with lowest point being in October. However, when we analysed the data with the Hewitt's test, considered by many authors to be more appropriate for sinusoidal patterns, we found an excess of births of MS patients in the period of January-June, with a peak in February, if a prior hypothesis of seasonality is assumed. Is there sufficient evidence for us to assume a prior hypothesis of seasonality taking into account the previous studies?

Our results are not consistent with a previous study from the Portuguese MS population [24], where a group of 421 MS patients, also from the North of Portugal, was studied. Some of the discordant results between the positive and negative studies in the literature can be explained by the fact that, while the month of birth effect is more prominent in high-risk areas for MS, especially in areas with low sunlight exposure, this effect seems to be negligible or non-existent in areas with high sunlight exposure [14, 37]. Also, in 2013, Fiddes et al. [38] stated that, in the absence of adequate control for confounding factors, such as year of birth and place of birth, the reported associations of MS with month of birth are probably false positives. In our study we used a control population matched by year of birth and place of birth (we only included statistics from the same districts of our patients).

Low concentrations of neonatal vitamin D are associated with an increased risk of MS [8, 9]. The seasonal fluctuations in vitamin D levels might result in decreased vitamin D concentrations in utero, which could explain the month-of-birth effect in MS. This observation suggests a role for the intrauterine environment. Winter levels of circulating vitamin D in pregnant women and newborns are low [39]. Vitamin D helps tune the fetal immune system by suppressing inflammatory cytokines and promoting self-tolerance [40]. Vitamin D is also considered a neuroactive steroid affecting brain development and function. It plays an essential role in myelination, which is important for connectivity in the brain. Experimental data on animal fetal development suggest that low maternal vitamin D has important implications for the developing brain [41]. Also, cerebral white matter is responsive to vitamin D and neurons and glial cells have vitamin D receptors [42]. A genetic study in humans has further implicated vitamin D as a strong environmental candidate by showing direct functional interaction with the major locus that determines susceptibility to Multiple Sclerosis [43, 44]. Although human evidence

concerning fetal development has been difficult to obtain, the body of related evidence to date has led some to recommend antenatal supplementation with vitamin D to prevent Multiple Sclerosis [45, 46].

The association of the period of birth with the risk of developing MS is relatively consistent; however a correlation with disease phenotype is more controversial. In this study we didn't find any association between MS birth and age at onset. It is interesting to observe that, even though that, like in the risk analysis, the median MSSS also has a peak in spring months and the lowest point was in October, the difference was not statistically significant. This is in agreement with Koch et al [28] and with a more recent study from Italy that also found no association between birth timing and MS progression [29].

As a conclusion we can say that MS seems to be more frequent in individuals born in January-June but birth timing does not seem to influence disease progression.

Conflict of interest

No conflict of interest exists regarding the present paper.

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References

[1] Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. Lancet Neurol. 2010;9:727-39.
[2] Koch MW, Metz LM, Agrawal SM, Yong VW. Environmental factors and their regulation of immunity in Multiple Sclerosis. J Neurol Sci. 2013;324:10-6.
[3] Kurtzke JF. Geographic distribution of Multiple Sclerosis: An update with special reference to Europe and the Mediterranean region. Acta Neurol Scand. 1980;62:65-80.
[4] van der Mei IA, Ponsonby AL, Dwyer T, Blizzard L, Simmons R, Taylor BV, et al. Past exposure to sun, skin phenotype, and risk of Multiple Sclerosis: case-control study. BMJ. 2003;327:316.
[5] Munger KL, Zhang SM, O'Reilly E, Hernan MA, Olek MJ, Willett WC, et al. Vitamin D intake and incidence of Multiple Sclerosis. Neurology. 2004;62:60-5.
[6] Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of Multiple Sclerosis. Jama. 2006;296:2832-8.
[7] Chaudhuri A. Why we should offer routine vitamin D supplementation in pregnancy and childhood to prevent Multiple Sclerosis. Med Hypotheses. 2005;64:608-18.
[8] Nielsen NM, Munger KL, Koch-Henriksen N, Hougaard DM, Magyari M, Jorgensen KT, et al. Neonatal vitamin D status and risk of Multiple Sclerosis: A population-based case-control study. Neurology. 2017;88:44-51.
[9] Munger KL, Aivo J, Hongell K, Soilu-Hanninen M, Surcel HM, Ascherio A. Vitamin D Status During Pregnancy and Risk of Multiple Sclerosis in Offspring of Women in the Finnish Maternity Cohort. JAMA neurology. 2016;73:515-9.
[10] Disanto G, Chaplin G, Morahan JM, Giovannoni G, Hypponen E, Ebers GC, et al. Month of birth, vitamin D and risk of immune-mediated disease: a case control study. BMC medicine. 2012;10:69.
[11] Saastamoinen KP, Auvinen MK, Tienari PJ. Month of birth is associated with Multiple Sclerosis but not with HLA-DR15 in Finland. Mult Scler. 2012;18:563-8.
[12] Bayes HK, Weir CJ, O'Leary C. Timing of birth and risk of Multiple Sclerosis in the Scottish population. European neurology. 2010;63:36-40.
[13] Willer CJ, Dyment DA, Sadovnick AD, Rothwell PM, Murray TJ, Ebers GC. Timing of birth and risk of Multiple Sclerosis: population based study. BMJ. 2005;330:120.
[14] Grytten N, Torkildsen O, Aarseth JH, Benjaminsen E, Celius EG, Dahl OP, et al. Month of birth as a latitude-dependent risk factor for Multiple Sclerosis in Norway. Mult Scler. 2013;19:1028-34.
[15] Torkildsen O, Aarseth J, Benjaminsen E, Celius E, Holmoy T, Kampman MT, et al. Month of birth and risk of Multiple Sclerosis: confounding and adjustments. Annals of clinical and translational neurology. 2014;1:141-4.
[16] Verheul F, Smolders J, Trojano M, Lepore V, Zwanikken C, Amato MP, et al. Fluctuations of MS births and UV-light exposure. Acta Neurol Scand. 2013;127:301-8.
[17] Dobson R, Giovannoni G, Ramagopalan S. The month of birth effect in Multiple Sclerosis: systematic review, meta-analysis and effect of latitude. J Neurol Neurosurg Psychiatry. 2013;84:427-32.
[18] Akhtar S, Alroughani R, Al-Shammari A, Al-Abkal J, Ayad Y. Non-parametric analysis of seasonality in birth and Multiple Sclerosis risk in second generation of migrants in Kuwait. BMC neurology. 2014;14:170.
[19] Sadovnick AD, Yee IM. Season of birth in Multiple Sclerosis. Acta Neurol Scand. 1994;89:190-1.
[20] Givon U, Zeilig G, Dolev M, Achiron A. The month of birth and the incidence of Multiple Sclerosis in the Israeli population. Neuroepidemiology. 2012;38:64-8.
[21] Fragoso YD, Shearer KD, Adoni T, Alves-Leon SV, Bidin Brooks JB, Comini-Frota ER, et al. Month of birth does not seem to interfere with the development of Multiple Sclerosis later in life in Brazilian patients. Neuroepidemiology. 2012;39:70-1.
[22] Fragoso YD, Adoni T, Almeida SM, Alves-Leon SV, Arruda WO, Barbagelata-Aguero F, et al. Multiple sclerosis in South America: month of birth in different latitudes does not seem to interfere with the prevalence or progression of the disease. Arquivos de neuro-psiquiatria. 2013;71:573-9.
[23] Villar-Quiles RN, Matias-Guiu JA, Ortega G, Gonzalez-Suarez I, Oreja-Guevara C, Matias-Guiu J. Analysis of the Relationship between the Month of Birth and Risk of Multiple Sclerosis in a Spanish Population. European neurology. 2016;76:202-9.

[24] Barros P, de Sa JM, Sa MJ. Month of birth and risk of Multiple Sclerosis in a Portuguese population. Clin Neurol Neurosurg. 2013;115:1762-5.
[25] Sotgiu S, Pugliatti M, Sotgiu MA, Fois ML, Arru G, Sanna A, et al. Seasonal fluctuation of Multiple Sclerosis births in Sardinia. J Neurol. 2006;253:38-44.
[26] McDowell TY, Amr S, Langenberg P, Royal W, Bever C, Culpepper WJ, et al. Time of birth, residential solar radiation and age at onset of Multiple Sclerosis. Neuroepidemiology. 2010;34:238-44.
[27] Tremlett HL, Devonshire VA. Does the season or month of birth influence disease progression in Multiple Sclerosis? Neuroepidemiology. 2006;26:195-8.
[28] Koch M, De Keyser J, Tremlett H. Timing of birth and disease progression in Multiple Sclerosis. Mult Scler. 2008;14:793-8.
[29] Lucenti A, Galimberti S, Barizzone N, Naldi P, Comi G, Martinelli Boneschi F, et al. Multiple sclerosis progression is not associated with birth timing in Italy. J Neurol Sci. 2014;346:194-6.
[30] De Sa J, Paulos A, Mendes H, Becho J, Marques J, Roxo J. The prevalence of Multiple Sclerosis in the District of Santarem, Portugal. J Neurol. 2006;253:914-8.
[31] de Sa J. [Epidemiology of Multiple Sclerosis in Portugal and Spain]. Revista de neurologia. 2010;51:387-92.
[32] Figueiredo J, Silva A, Cerqueira JJ, Fonseca J, Pereira PA. MS Prevalence and Patients' Characteristics in the District of Braga, Portugal. Neurology research international. 2015;2015:895163.
[33] Hewitt D, Milner J, Csima A, Pakula A. On Edwards' criterion of seasonality and a non-parametric alternative. British journal of preventive & social medicine. 1971;25:174-6.
[34] Walter SD. Study of seasonality. Am J Epidemiol. 1982;116:192-6.
[35] Marrero O. Re: study of seasonality. Am J Epidemiol. 1981;113:481-2.
[36] Staples J, Ponsonby AL, Lim L. Low maternal exposure to ultraviolet radiation in pregnancy, month of birth, and risk of Multiple Sclerosis in offspring: longitudinal analysis. BMJ. 2010;340:c1640.
[37] Torkildsen O, Grytten N, Aarseth J, Myhr KM, Kampman MT. Month of birth as a risk factor for Multiple Sclerosis: an update. Acta Neurol Scand Suppl. 2012:58-62.
[38] Fiddes B, Wason J, Kemppinen A, Ban M, Compston A, Sawcer S. Confounding underlies the apparent month of birth effect in Multiple Sclerosis. Ann Neurol. 2013;73:714-20.
[39] Newhook LA, Sloka S, Grant M, Randell E, Kovacs CS, Twells LK. Vitamin D insufficiency common in newborns, children and pregnant women living in Newfoundland and Labrador, Canada. Maternal & child nutrition. 2009;5:186-91.
[40] Smolders J, Damoiseaux J, Menheere P, Hupperts R. Vitamin D as an immune modulator in Multiple Sclerosis, a review. J Neuroimmunol. 2008;194:7-17.
[41] Harms LR, Burne TH, Eyles DW, McGrath JJ. Vitamin D and the brain. Best practice & research Clinical endocrinology & metabolism. 2011;25:657-69.
[42] Smolders J, Moen SM, Damoiseaux J, Huitinga I, Holmoy T. Vitamin D in the healthy and inflamed central nervous system: access and function. J Neurol Sci. 2011;311:37-43.
[43] Ramagopalan SV, Maugeri NJ, Handunnetthi L, Lincoln MR, Orton SM, Dyment DA, et al. Expression of the Multiple Sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D. PLoS genetics. 2009;5:e1000369.
[44] Berlanga-Taylor AJ, Disanto G, Ebers GC, Ramagopalan SV. Vitamin D-gene interactions in Multiple Sclerosis. J Neurol Sci. 2011;311:32-6.
[45] Chaudhuri A. Why we should offer routine vitamin D supplementation in pregnancy and childhood to prevent Multiple Sclerosis. Medical Hypotheses. 2005;64:608-18.
[46] Fernandes de Abreu DA, Landel V, Feron F. Seasonal, gestational and postnatal influences on Multiple Sclerosis: the beneficial role of a vitamin D supplementation during early life. J Neurol Sci. 2011;311:64-8.

Paper 6

Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal

Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal

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Abstract

Vitamin D *status* in human populations has become a matter of great concern, in the wake of a multitude of published works that document widespread vitamin D deficiency across Europe, even in countries with abundant sunlight. In Portugal there are no measures of 25-hydroxyvitamin D – 25(OH)D – levels in the general adult population. The purpose of this study was to measure 25(OH)D levels in a healthy population cohort and investigate the possible association with season and selected demographic and laboratory measurements.

A cohort of 198 participants (18-67 years) living in the north of Portugal, Porto, conducted in July and August 2015 (summer time) and April 2016 (winter time) was studied to evaluate serum 25(OH)D levels. Sociodemographic characteristics (age, sex and body mass index) and season of the year were taken into account as possible 25(OH)D levels codeterminants.

In the whole group, the mean level of serum 25(OH)D was 55.4±23.4 nmol/L, with 48% of the population presenting levels compatible with vitamin D deficiency (below 50 nmol/L). In the winter period, this value reaches 74%. No statistically significant differences were observed between genders (57.4±23.9 vs. 53.3±22.8 nmol/L, p=0.219) as well as no statistically significant correlation was found between age and 25(OH)D levels (p=0.349). As expected higher levels of 25(OH)D were observed in summer than in winter (68.2±21.5 *vs*. 42.2±16.9 nmol/L; p<0.0001). Serum 25(OH)D levels were significantly lower in obese compared to non-obese subjects (46.6±17.6 vs. 57.7±24.2 nmol/L, p=0.012).

Vitamin D deficiency is prevalent in this area, affecting almost half of the population. Body mass index and season are predictors for lower 25-hydroxyvitamin D levels and vitamin D *status*. An effective strategy to prevent vitamin D deficiency and insufficiency should be envisaged and implemented in our population.

Key words: Portugal; 25(OH)D levels; Vitamin D status; healthy adult population.

1. Introduction

Vitamin D is unique among vitamins, since it works as a hormone and can be synthesized on the skin as a result of exposure to sunlight. It is acquired both through nutrition (10-20%) and by cutaneous synthesis under the action of sunlight [1]. Dietary sources of vitamin D include fish oils and, in some countries (USA and Northern Europe) fortified food products (dairy and bread products). In Portugal, vitamin supplements containing vitamin D exist in the market. However, the main source of vitamin D results from cutaneous synthesis on sun exposure and is dependent on various factors such as the geographical area latitude, altitude, season, time of day, the exposed body surface and exposure duration, use of sunscreens, skin pigmentation, obesity and age [2].

Vitamin D_3 or cholecalciferol, after formation in the skin, and vitamin D_2 or D_3, from dietary sources, are hydroxylated in the liver, resulting in the formation of 25-hydroxyvitamin D [25(OH)D], the main circulating form. This form subsequently undergoes hydroxylation in the kidney and other organs to generate the biologically active, dihydroxylated form of vitamin D, calcitriol or $1,25(OH)_2D$, which acts through specific vitamin D receptors [1]. The vitamin D role on the maintenance of calcium serum levels, by promoting calcium and phosphorus absorption from the intestine and calcium bone reabsorption, is well known [3]. Recent evidences correlate insufficient vitamin D levels with an increased risk of developing other non-bone-related disorders: cardiovascular diseases, hypertension, malignant neoplasia, type I diabetes mellitus, Multiple Sclerosis, dementia, rheumatoid arthritis, and infectious disease [2-4].The identification of vitamin D receptors in immune system cells and the discovery that dendritic cells can produce the metabolically active form of vitamin D have led to the suggestion that vitamin D is also an immune modulator [5].

The high prevalence of inadequate vitamin D is nowadays seen as a public health problem affecting several countries in Europe and the USA, particularly in those people at risk for osteoporosis and its consequences [2]. Vitamin D deficiency screening is accomplished through measurement of 25(OH)D, which is the best index for assessing vitamin D reserve in the body [2], due to its greater half-life comparing with the metabolically active form. Only at-risk populations are routinely tracked for vitamin D deficiency, including the elderly, the institutionalized, pregnant women and post-menopausal women (increased risk of fractures) [3]. Much debate has taken place over the definition of vitamin D deficiency. Most agree that a 25(OH)D concentration <50nmol/L, or 20 ng/mL, is an indication of vitamin D deficiency, whereas a 25(OH)D concentration of 51–74 nmol/L, or 21–29 ng/mL, is considered to indicate insufficiency; concentrations >75 nmol/L, or 30 ng/mL, are considered to be adequate [6-9]. The

optimal serum 25(OH)D levels are those for which calcium absorption is optimized, parathyroid hormone (PTH) levels reduced and the greatest benefit to the bone and muscle function are obtained; currently levels above 75 nmol/L (30 ng/mL) are recommended.

Several studies have described inadequacy of 25-hydroxyvitamin D all over the Europe, although vitamin D *status* within different European countries shows a high variation [10-12]. In Portugal, the prevalence of vitamin D deficiency is unknown because there are no epidemiological studies in adult healthy individuals; however, several studies in healthy pediatric populations and in specific hospital populations have been published [3, 13-22]. In 2009 a study in a healthy pediatric population from Porto was published. A group of 45 children (33F, 12M; 2.5-16 years) were evaluated in winter and spring. None of them was supplemented after the first year of life. Values above 100 nmol/L were considered optimal, 75-100 nmol/L sufficient, 50-74 nmol/L relative insufficient and <50 nmol/L deficient. Vitamin D deficiency was found in 26% of the studied population during the months with less sunlight. According to these cut off values, 80% of the children did not achieve optimal levels [23]. In another pediatric study, 73 children (37F, 36M), aged 12 months to 17 years, from the outpatient clinic of Centro Hospitalar do Porto, were studied. The study occurred between March 2008 and July 2010. The children were divided in to pre-school age (12 months to 5 years; 23.3% (17/73)) and school age (6 to 17 years; 76.7% (56/73)). Normal 25(OH)D levels (>75 nmol/L) were observed in 17.8% of the children (11% with optimal values, >100nmol/L; and 6.8% with sufficient values, 75-100 nmol/L). On the other hand, 82.2% had low 25(OH)D levels (42.5% with relative insufficiency, 50-74 nmol/L; and 39.7% with deficiency, <50 nmol/L). Gender, residential area, BMI and season were not related to 25(OH)D levels. It was observed that school age children had higher vitamin D deficiency (p=0.013), thus establishing a relation with age [24]. Another cohort of 122 healthy children and adolescents (5-18 years) from Porto was studied. They were observed in the pediatric outpatient clinic during the winter and spring of 2011/2012. Vitamin D *status* was observed to be insufficiency (≥20 and <30 ng/mL) in 92.5% of the cases, from which 47.8% presented deficiency (≥10 and <20 ng/mL) and 6% severe deficiency (<10 ng/mL). Only 7.5% of the sample had an adequate vitamin D *status* (≥30 ng/mL) [25].

As already stated, vitamin D *status* is often studied in specific groups that have increased risk of vitamin D deficiency or osteoporosis, such as hospitalized or elderly people. In such groups, confounding of variables makes it difficult to translate findings to the general population [26]. Thus, the aim of the current study is to evaluate vitamin D *status* in non-supplemented healthy adults living in Porto, north of Portugal.

2. Subjects and Methods

2.1 Subjects

The study was conducted in Porto (~41° N; elevation: 104 m), in July and August 2015 (summer time) and April 2016 (winter time). Two hundred healthy blood donors voluntarily participated in this study. Two men were excluded, because they were taking multivitamin supplementation. The average age of these individuals was 43.1±12.1 years. Subjects were stratified in three groups according to age [27].

A questionnaire about age, gender, weight, height, ethnicity, nationality, place of birth, occupation, sun exposure, sunscreens use, eating habits, smoking habits, physical activity, diagnosed pathologies, use of medicines and food supplements, was answered during blood donation. In order to analyse the influence of BMI on the 25(OH)D levels, the subjects were divided into two groups based on BMI values: BMI<30 kg/m^2 (non-obese) or BMI≥30 kg/m^2 (obese). Written informed consent was obtained for each volunteer, and the study was approved by the Ethics Committee of Centro Hospitalar do Porto, according to Declaration of Helsinki.

2.2 Laboratory Measurements

Blood was collected in Vacuette® Z Serum Clot Activator tubes for the measurement of PTH and in Vacuette® Z Serum Separator Clot Activator tubes for the other measurements. Serum was obtained by centrifugation and stored in several aliquots at -20°C until analysed. Serum 25(OH)D was chosen as a reliable marker of individual vitamin D *status* as it reflects vitamin D obtained from food sources and cutaneous synthesis, and it is not prone to diurnal variation.

Serum 25(OH)D was measured using an electro-chemiluminescence binding assay (ECLIA) for the in-vitro determination of total 25-hydroxyvitamin D (Elecsys® Vitamin D total, Cobas, Roche©). The reference range for 25(OH)D was >75 nmol/L (measurement range: 7.50-175 nmol/L). The serum PTH concentration was assessed using an electro-chemiluminescence assay with 15-65 pg/mL as a reference range. Serum total calcium, phosphate and creatinine concentrations were measured by routine laboratory methods in a Cobas Integra 800.

2.3 Statistical Analyses

Continuous data were checked for normality using the Kolmogorov-Smirnov test and natural logarithm (ln) transformations were used for skewed variables previous to the

statistical analysis. Differences between groups were tested using the Student's t-test or one-way ANOVA (continuous variables) and χ^2 test (dichotomous variables). Pearson's or Spearman's correlation coefficients were calculated to test relationships between continuous variables.

Multiple linear regression analysis was used to consider potential determinants of 25(OH)D levels (dependent variable). The following independent variables: age and BMI (as continuous variables), season and gender (as categorical variables) were included in the model.

A *p*-value below 0.05 was considered to be statistically significant. Statistical analyses were performed using *Statistical Package for the Social Sciences* software (version 23, IBM SPSS Statistics, NY, USA).

3. Results

The characteristics of the study population are shown in Table1. Approximately 48% were women, and the mean age (±SD) of the study population was 43.1±12.1 years. No statistically significant differences were observed between genders. The frequency of obesity was significantly higher in this population compared with the general Portuguese population (22.7% vs. 14.2%, p=0.001, OR=1.77, 95%CI=1.26-2.50) [28].

The mean serum 25(OH)D concentration was 55.4±23.4 nmol/L in all participants (median 50.9 nmol/L) with no significant differences between men and women (57.4±23.9 vs. 53.3±22.8 nmol/L; p=0.219). Fifty women (52.6%) and 45 men (43.7%) were deficient in 25(OH)D but the gender difference was not statistically significant (Table 1).

No statistically significant correlation was found between age and 25(OH)D levels (p=0.349). When subjects were categorized in groups according to age (Figure 1), no differences in 25(OH)D levels between the 3 groups (p=0.311) were found.

Table 1 - Characteristics of the study population.

Characteristics	Total (n=198)	Women (n=95)	Men (n=103)
Sociodemographics			
Age, years, mean ± SD	43.1±12.1	41.9±12.5	44.2±11.7
Season			
Summer, n (%)	101 (51.1)	44 (46.3)	57 (55.3)
Winter, n (%)	97 (49.0)	51 (53.7)	46 (44.7)
BMI, mean ± SD	27.0±4.3	26.9±4.4	27.2±4.2
Laboratory measurements			
PTH levels (pg/mL), mean ± SD	44.9±14.7	45.6±12.6	44.3±16.5
Creatinine levels (mg/dL), mean ± SD	0.8±0.2	0.8±0.1	0.8±0.2
Total calcium levels (mmol/L), mean ± SD	2.4±0.1	2.4±0.1	2.4±0.1
Phosphorus levels (mmol/L), mean ± SD	1.0±0.2	1.0±0.2	1.0±0.2
25(OH)D levels (nmol/L)			
Mean ± SD	55.4±23.4	53.3±22.8	57.4±23.9
<50 nmol/L (deficiency), n (%)	95 (48.0)	50 (52.6)	45 (43.7)
50-75 nmol/L (insufficiency), n (%)	60 (30.3)	27 (28.4)	33 (32.0)
>75 nmol/L (optimal), n (%)	43 (21.7)	18 (18.9)	25 (24.3)

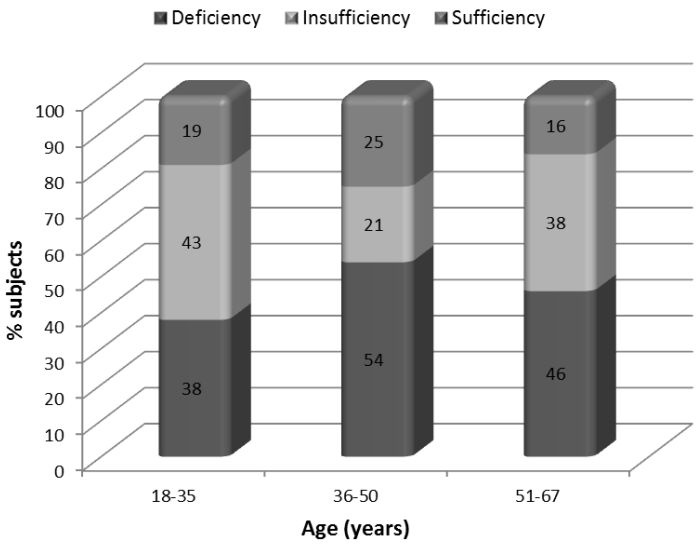

Figure 1 – Global prevalence of vitamin D deficiency and insuficiency by age.

Body mass index was negatively correlated with 25(OH)D levels (p=0.001, r=-0.237) (Figure 2). In conformity, 25(OH)D levels were significantly lower in obese compared to non-obese subjects (46.6±17.6 vs. 57.7±24.2 nmol/L, p=0.012) (Figure 3).

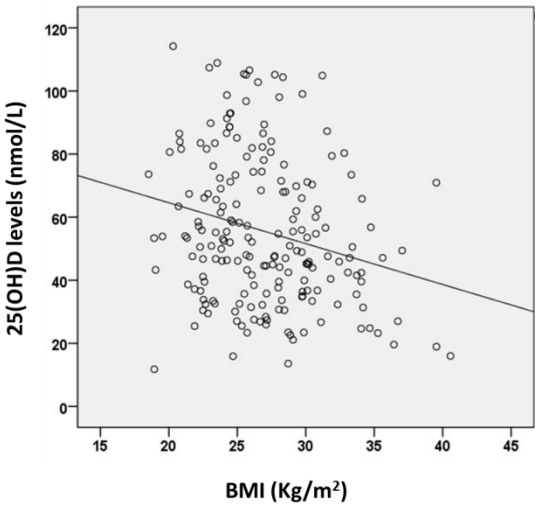

Figure 2 –Correlation between 25(OH)D and BMI.

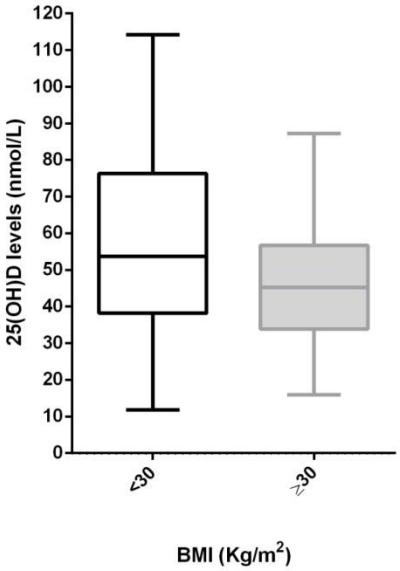

Figure 3- Comparison of serum 25 (OH)D levels between obese (BMI≥30) and non-obese (BMI<30) individuals.

In the winter period, 74.2% of the studied population had a 25(OH)D concentration below 50.0 nmol/L compared with 22.8% in the summer period (p<0.0001). Only 5 individuals (5.2%) presented optimal levels of 25(OH)D in winter, and 38 (37.6%) in summer (Table 2).

Table 2 – Differences in 25(OH)D concentration according to season.

25(OH)D levels (nmol/L)	Winter (n=97)	Summer (n=101)	p
Mean ± SD	42.2±16.9	68.2±21.5	<0.0001
<50 nmol/L (deficiency), n (%)	72 (74.2)	23 (22.8)	
50-75 nmol/L (insufficiency), n (%)	20 (20.6)	40 (39.6)	<0.0001
>75 nmol/L (optimal), n (%)	5 (5.2)	38 (37.6)	

In multiple linear regression analysis, controlling for age and gender, significant associations between 25(OH)D levels and season and BMI were found. Winter and higher BMI were significantly associated with lower serum 25(OH)D levels (Table 3).

Table 3 – Results of a multiple linear regression analysis on determinants of 25(OH)D levels

Variable	B	SE	p
Intercept	4.224	0.189	<0.0001
Age	-0.002	0.002	0.499
Season	0.482	0.054	<0.0001
Gender	-0.40	0.054	0.456
Body Mass Index (kg/m^2)	-0.17	0.007	0.010
Corr .r^2=0.341			

4. Discussion

There are many studies on vitamin D *status* of the general population in the USA, Canada, Asia Pacific, Middle East, Africa and across Europe [29], but to the best of our knowledge this is the first conducted in a Portuguese healthy adult population.

Globally, vitamin D deficiency is more prevalent in winter, women, older age groups, individuals with darker skin, and higher latitudes [7, 30, 31]. In the present study, the frequency of 25(OH)D deficiency was significantly higher in winter, confirming the well-known seasonal fluctuation in 25(OH)D concentration. No association between 25(OH)D levels and gender was observed in our study, although women presented slightly lower levels of 25(OH)D but the difference was not statistically significant. It has been established that the ageing skin produces less vitamin D [32]. However, in our study, we did not find any association between vitamin D *status* and age.

The negative association of vitamin D *status* with obesity is well documented in different studies [33]. This is probably due to the decreased bioavailability of vitamin D from cutaneous and dietary sources because of its sequestration in body fat compartments [34]. Our observations confirm the association of vitamin D *status* with BMI. Furthermore, an inverse correlation between vitamin D *status* and BMI was found.

There is ongoing debate related to the optimal levels of 25(OH)D. All available evidence suggest that children and adults should maintain a blood level of 25(OH)D above 50

nmol/L to prevent rickets and osteomalacia, respectively. However, to maximize vitamin D effect on calcium, bone, and muscle metabolism, the 25(OH)D blood level should be above 75 nmol/L. Numerous epidemiological studies have suggested that a 25(OH)D blood level above 75 nmol/L may have additional health benefits in reducing the risk of common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases [35].

In this study we observed that almost half of the studied population presented serum 25(OH)D values suggestive of vitamin D deficiency, reaching 74% in winter. This observation is in line with a recent study that suggests that vitamin D deficiency is widespread across Europe, even in countries with abundant sunlight, and at prevalence rates that meet the criteria of a pandemic [10]. An effective strategy to prevent vitamin D deficiency and insufficiency should be envisaged. For specific high-risk groups use of vitamin D supplements would be an effective measure. For the general population fortification of widely used foods could be considered, especially in winter.

5. Conclusions

The present study analysed serum 25-hydroxyvitamin D levels in healthy adults between 18-67 years of age. BMI and season are predictors for lower 25(OH)D levels and vitamin D *status* in this population. The strengths of this work comprise a detailed questionnaire documenting demographic data and blood sampling taking place through summer as well as wintertime. On the other hand, although the questionnaire included data about sun exposure, sunscreens use, eating habits, smoking habits, physical activity, diagnosed pathologies, and use of medicines and/or food supplements, these parameters were only used to exclude confounding factors that could bias our results, and were not used in the analysis of the results, as that was beyond the aim of the present study.

Conflict of interest

The authors have declared no conflict of interests.

Funding source

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References

1. Mithal, A., et al., Global vitamin D status and determinants of hypovitaminosis D. Osteoporos Int, 2009. 20(11): p. 1807-20.
2. Declaração Portuguesa da Vitamina D, 2009, Sociedade Portuguesa de Medicina Interna.
3. Alves, M., et al., Vitamina D–importância da avaliação laboratorial. Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo, 2013. 8(1): p. 32-39.
4. Cutolo, M., Vitamin D and autoimmune rheumatic diseases. Rheumatology (Oxford), 2009. 48(3): p. 210-2.
5. Carvalho, C., et al., Association between vitamin D receptor (VDR) gene polymorphisms and systemic lupus erythematosus in Portuguese patients. Lupus, 2015. 24(8): p. 846-53.
6. Dawson-Hughes, B., et al., Estimates of optimal vitamin D status. Osteoporos Int, 2005. 16(7): p. 713-6.
7. Holick, M.F., Vitamin D deficiency. N Engl J Med, 2007. 357(3): p. 266-81.
8. Holick, M.F., Vitamin D status: measurement, interpretation, and clinical application. Ann Epidemiol, 2009. 19(2): p. 73-8.
9. Souberbielle, J.C., et al., Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: Recommendations for clinical practice. Autoimmun Rev, 2010. 9(11): p. 709-15.
10. Cashman, K.D., et al., Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr, 2016. 103(4): p. 1033-44.
11. Pludowski, P., et al., Vitamin d status in central europe. Int J Endocrinol, 2014. 2014: p. 589587.
12. Quraishi, S.A., C.A. Camargo, Jr., and J.E. Manson, Low vitamin D status in Europe: moving from evidence to sound public health policies. Am J Clin Nutr, 2016. 103(4): p. 957-8.
13. Boura, M., et al., Hypovitaminosis D in HIV-infected patients in Lisbon: a link with antiretroviral treatment. J Int AIDS Soc, 2014. 17(4 Suppl 3): p. 19826.
14. Castro, F.D., et al., Lower Levels of Vitamin D Correlate with Clinical Disease Activity and Quality of Life in Inflammatory Bowel Disease. Arq Gastroenterol, 2015. 52(4): p. 260-265.
15. Lucas, R., L. Costa, and H. Barros, Ingestão de Cálcio e Vitamina D numa Amostra Urbana de Mulheres Portuguesas. Arquivos de Medicina (online), 2005. 19(1-2): p. 7-14.
16. Matias, P.J., et al., 25-Hydroxyvitamin D3, arterial calcifications and cardiovascular risk markers in haemodialysis patients. Nephrol Dial Transplant, 2009. 24(2): p. 611-8.
17. Matias, P.J., et al., Cholecalciferol supplementation in hemodialysis patients: effects on mineral metabolism, inflammation, and cardiac dimension parameters. Clin J Am Soc Nephrol, 2010. 5(5): p. 905-11.
18. Nunes, J.P. and C.S. Martins, Myocardial infarction, hypovitaminosis D and vitiligo. Rev Port Cardiol, 2010. 29(5): p. 839-40.
19. Peixoto, D., et al., Avaliação dos níveis de vitamina D na artrite idiopática juvenil. Acta Pediátrica Portuguesa, 2013. 44(4): p. 183-184.
20. Santiago, T., et al., [Hypovitaminosis D in patients admitted to an internal medicine ward]. Acta Med Port, 2012. 25(2): p. 68-76.
21. Santos, M.J., V. Fernandes, and F.M. Garcia, [Vitamin D Insufficiency in a Hospital Population: A Photograph from the Laboratory Perspective]. Acta Med Port, 2015. 28(6): p. 726-34.
22. Silva, L., et al., [Vitamin D measurement in Portuguese patients with fragility fractures]. Acta Reumatol Port, 2010. 35(3): p. 352-7.
23. Monteiro, T., Carência de vitamina D: um problema de saúde pública não reconhecido e frequente no Grande Porto? Acta Pediátrica Portuguesa, 2009. 40(2): p. 49-52.
24. Rocha, A.M.R., Avaliação do estado de Vitamina D numa população pediátrica do grande Porto, in Instituto de Ciências Biomédicas Abel Salazar (ICBAS) 2012, University of Porto: Porto.
25. Ferreira, S., et al., Status de vitamina D e de mineralização óssea em crianças e adolescentes residentes na cidade do Porto, in Sociedade Portuguesa de Ciências da Nutrição e Alimentação (SPCNA)2012, Revista SPCNA. p. 54.
26. van Grootheest, G., et al., Determinants of plasma 25-hydroxyvitamin D levels in healthy adults in the Netherlands. Neth J Med, 2014. 72(10): p. 533-40.
27. Cashman, K.D., et al., Vitamin D status of Irish adults: findings from the National Adult Nutrition Survey. Br J Nutr, 2013. 109(7): p. 1248-56.
28. do Carmo, I., et al., Overweight and obesity in Portugal: national prevalence in 2003-2005. Obes Rev, 2008. 9(1): p. 11-9.

29. Hilger, J., et al., A systematic review of vitamin D status in populations worldwide. Br J Nutr, 2014. 111(1): p. 23-45.
30. Lips, P., Worldwide status of vitamin D nutrition. J Steroid Biochem Mol Biol, 2010. 121(1-2): p. 297-300.
31. Gozdzik, A., et al., Low wintertime vitamin D levels in a sample of healthy young adults of diverse ancestry living in the Toronto area: associations with vitamin D intake and skin pigmentation. BMC Public Health, 2008. 8: p. 336.
32. MacLaughlin, J. and M.F. Holick, Aging decreases the capacity of human skin to produce vitamin D3. The Journal of Clinical Investigation. 76(4): p. 1536-1538.
33. Lagunova, Z., et al., The dependency of vitamin D status on body mass index, gender, age and season. Anticancer Res, 2009. 29(9): p. 3713-20.
34. Wortsman, J., et al., Decreased bioavailability of vitamin D in obesity. Am J Clin Nutr, 2000. 72(3): p. 690-3.
35. Holick, M.F., et al., Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab, 2011. 96(7): p. 1911-30.

Paper 7

Serum 25-hydroxyvitamin D levels in Multiple Sclerosis patients from the North of Portugal

Serum 25-hydroxyvitamin D levels in Multiple Sclerosis patients from the North of Portugal

Submitted

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Abstract

BACKGROUND AND PURPOSE: Increasing evidence has shown that individuals with Multiple Sclerosis (MS) have lower 25-hydroxyvitamin D [25(OH)D] levels compared to healthy controls. There is no information regarding 25(OH)D levels and MS in Portugal. Therefore the aim of the current study was to examine the levels of 25(OH)D in a group of patients with MS and in healthy matched controls, as well as the association of 25(OH)D levels with disease course, disability and severity.

METHODS: A group of 244 unrelated Portuguese patients, with a definitive diagnosis of MS, and 198 ethnically matched healthy controls were included in the study. A sub-group of patients with recent disease onset was included. Serum 25(OH)D was measured using an electrochemiluminescence binding assay.

RESULTS: The mean serum level of 25(OH)D in patients with MS was 39.9±22.0 nmol/L, which was significantly lower ($p<0.0001$) than those in healthy controls, 55.4±23.4 nmol/L. There was a negative correlation between 25(OH)D levels and EDSS ($r=-0.293$, $p<0.0001$) and MSSS scores ($r=-0.293$, $p<0.0001$). In multiple logistic regression analysis adjusted for age, gender, disease form, EDSS, disease duration and MSSS, 25(OH)D levels were independently associated with EDSS ($p=0.004$) and disease duration ($p=0.016$), and with MSSS ($p=0.001$).

CONCLUSION: In accordance with the majority of the literature, low serum 25(OH)D levels were associated with susceptibility and disability in MS patients from Portugal. Lower serum 25(OH)D levels were also found in patients with a recent disease onset, supporting vitamin D levels as a risk factor for MS.

Key-words: Vitamin D; 25(OH)D; Multiple sclerosis; disability; susceptibility; Portugal

1. Introduction

Multiple Sclerosis (MS) is the most common inflammatory demyelinating disease of the central nervous system (CNS) in young adults. The cause of MS remains poorly understood, but it is widely believed to be an autoimmune disease occurring in genetically susceptible individuals after exposure to as-yet undefined environmental factors [1].

Among non-infectious environmental factors, there is a recent increase in studies investigating vitamin D levels in MS pathogenesis. Vitamin D is the main regulator of calcium and phosphorus levels in the body, and deficiency is associated with rickets in children, and with osteomalacia and osteoporosis in adults. Several reports indicate that vitamin D has pleiotropic effects, significantly affecting the regulation of immune responses, restoring beneficial proportions of the populations of Th2 and Th1 lymphocytes, with the overall effect of attenuating inflammatory reactions [2, 3].

Increasing evidence has shown that individuals with MS have lower 25-hydroxyvitamin D [25(OH)D] levels compared to healthy controls. Since 2010 several studies have addressed the influence of vitamin D levels in disease susceptibility [4-18] (Table 1). In 2014, a meta-analysis of previous studies concluded that low vitamin D levels are associated with an increased risk of MS [19]. In a large prospective study, published in 2006, Munger et al. found that the risk of MS decreased with increasing of serum levels of 25-hydroxyvitamin D [20]. Also, two other studies showed evidence for possible neuroprotection of vitamin D in clinically isolated syndrome [21, 22]. Nevertheless it is less clear whether vitamin D has a role in MS progression.

Regarding disease course, lower levels of 25(OH)D were found in secondary-progressive (SP) MS when compared to relapsing-remitting (RR) MS [23]. In a retrospective longitudinal study, Muris et al. [24] assessed whether the vitamin D status in RRMS patients is associated with the time of conversion to SPMS and found an association between low vitamin D status at the start of RRMS and the early conversion to SPMS. Lower 25(OH)D levels in patients with RRMS have been associated with higher clinical and radiographic disease activity [16, 25-27] and with the degree of disability in fully ambulatory RR patients [28].

There is no data regarding vitamin D levels in MS patients in Portugal, therefore the aim of the current study was to examine the levels of 25(OH)D in a population-based group of patients with MS and in healthy matched controls, as well as the association of 25(OH)D levels with disease course, disability and severity.

Table 1. Summary of the case-control studies that studied the influence of vitamin D levels in MS susceptibility since 2010

Study	**Country**	**Study design**	**Sample size (MS/Control)**	**Season**	**25(OH)D levels (nmol/L)**		
					MS	**Control**	**p**
Shaygannejad (2010) [4]	Iran	Case-control	(50/50)	NA	48.0	62.0	0.036
Lonergan (2011) [5]	Ireland	Case-control	(329/226)	Winter	38.6	36.4	n.s.
Gelfand (2011) [6]	USA	Case-control	(339/342)	NA	29.7	36.6	0.0001
Hatamian (2013)[7]	Iran	Case-control	(52/52)	NA	66.1	92.6	0.003
Kirbas (2013)[8]	Turkey	Case-control	(30/30)	NA	67.9	106.3	0.001
Mazdeh (2013)[9]	Iran	Case-control	(75/100)	Winter Summer	NA 29.4	NA 58.5	 0.003
Shahbeigi (2013)[10]	Iran	Case-control	(98/17)	Summer	79.0	89.3	0.047
Hejazi (2014)[11]	Iran	Case-control	(37/37)	Winter	20.7	15.8	n.s.
Niino (2015)[12]	Japan	Case-control	(70/40)	Winter	42.7	49.9	<0.05
Behrens (2016)[13]	Germany	Case-control	(76/76)	All year	NA	NA	0.002
Karampoor (2016)[14]	Iran	Case-control	(1000/700)	Winter	36.2	64.4	NA
Becker (2016)[15]	Brazil	Case-control	(67/61)	Winter Summer	58.0 74.8	67.7 77.3	0.957 0.115
Brola (2016)[16]	Poland	Case-control	(184/78)	Winter Summer	33.4 60.6	36.4 62.6	0.012 0.256
Yamout (2016)[17]	Lebanon	Case-control	(50/99)	All year	53.9	36.2	0.002
Zhang (2016)[18]	China	Case-control	(141/282)	Winter Summer	39.7	51.4	<0.0001
Bettencourt (2017)	Portugal	Case-control	(244/198)	Winter Summer	34.5 48.7	42.2 68.2	0.0003 <0.0001

2. Subjects and methods

2.1 - Patients and controls

From a total of 632 unrelated Portuguese patients with a definitive diagnosis of MS, according to the revised McDonald criteria [29], recruited from the neurology outpatient clinic of Centro Hospitalar do Porto – Hospital de Santo António (HSA) a subgroup of 244 patients, that had 25(OH)D levels measured before supplementation, were selected. The Expanded Disability Status Scale (EDSS) [30] and Multiple Sclerosis Severity Scale (MSSS) [31] were used to measure, respectively, physical disability and disease severity. The control group comprised 198 ethnically matched healthy controls (HC) and their evaluation was described previously [32]. A sub-group of recently diagnosed (2012-2015) patients (n=74), in which vitamin D levels were measured at diagnosis, was studied independently. Individuals were excluded if they had disorders related to vitamin D deficiency such as rickets or parathyroid pathologies and also if they consumed drugs or supplements containing vitamin D or calcium. This study was approved by the hospital Medical Ethical Committee and written informed consent was obtained from all participants.

2.2 - 25(OH)D measurement

Blood was collected in Vacuette® Z Serum Separator Clot Activator tubes. Serum was obtained by centrifugation and stored in several aliquots at -20°C until analysed. Serum total 25(OH)D was chosen as a reliable marker of individual vitamin D status as it reflects vitamin D obtained from food sources and cutaneous synthesis, and it is not prone to diurnal variation. Serum 25(OH)D was measured using an electrochemiluminescence binding assay (ECLIA) for the *in vitro* determination of total 25-hydroxyvitamin D (Elecsys® Vitamin D total, Cobas, Roche©), measurement range: 7.50-175 nmol/L.

Much debate has taken place over the definition of vitamin D deficiency, nevertheless a 25(OH)D concentration <50nmol/L (20 ng/ml) is currently considered an indication of vitamin D deficiency, whereas a 25(OH)D concentration of 50–75 nmol/L (20–30 ng/ml), is considered to indicate insufficiency; concentrations >75 nmol/L (30 ng/ml), are considered to be adequate [33-36].

2.3 - Statistical analysis

Continuous data were checked for normality using the Kolmogorov-Smirnov test. Differences between groups were tested using the Mann-Whitney U test and Kruskal-

Wallis test. Spearman's correlation coefficients were calculated to test interactions between continuous variables.

Multivariate linear regression was used to test the association of 25(OH)D levels with EDSS and MSSS, adjusting for age, gender and disease course; and multivariate logistic regression was used to test the association of 25 (OHD levels with MS status adjusting for age and gender.

A p-value below 0.05 was considered to be statistically significant. Statistical analyses were performed using Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).

3. Results

A total of 244 MS patients (93 male and 151 female; mean age: 41.1±11.3 years) and 198 healthy controls (103 male and 95 female; mean age: 43.1±12.1 years) were studied. Demographic and clinical characteristics of all participants are presented in Table 2.

Table 2. Characteristics of Multiple Sclerosis patients and healthy controls

	Total group of MS patients (n=244)	**Patients with recent disease onset (n=74)**	**Healthy controls (n=198)**
Females/males (% F)	151/93 (62%)	48/26 (65%)	95/103 (48%)
Mean age, years (SD)	41.1±11.3	34.0±8.3	43.1±12.1
Mean disease duration, years (SD)	10.7±8.7	2.2±1.1	-
Disease Course Relapse-remitting (RRMS) Secondary Progressive (SPMS) Primary Progressive (PPMS)	 191 (78.3%) 26 (10.7%) 27 (11%)	 69 (93%) - 5 (7%)	 - - -
EDSS, median (minimum – maximum)	2.5 (0.0-9.0)	1.0 (0.0-6.5)	-
MSSS, median (minimum – maximum)	2.9 (0.11-9.79)	2.2 (0.40-9.79)	-
25(OH)D (nmol/L) Mean ± SD <50 nmol/L (deficiency), n (%) 50-75 nmol/L (insufficiency), n (%) >75 nmol/L (optimal), n (%)	 39.9±22.1 162 (66.4) 62 (25.4) 20 (8.2)	 39.6±21.1 53 (71.6) 14 (18.9) 7 (9.5)	 55.4±23.4 95 (48.0) 60 (30.3) 43 (21.7)

The vitamin D serum levels were significantly lower (p<0.0001) in patients compared to healthy individuals (39.9±22.0 nmol/L vs. 55.4±23.4 nmol/L, respectively). Vitamin D levels of recently diagnosed MS patients were also significantly lower (p<0.0001) compared to healthy individuals (39.6±20.9 nmol/L vs. 55.4±23.4 nmol/L, respectively). About 66% of the patients presented 25(OH)D deficiency compared with 48% of the healthy controls (p<0.0001) (Table 2).

As expected the levels of 25(OH)D were higher in summer than in winter either in patients and in controls (Figure 1). The 25(OH)D mean concentration in winter was 34.5±20.2 in MS vs. 42.2±16.9 in controls, p=0.0003. In summer the 25(OH)D mean concentration was 48.7±22.1 in patients vs. 68.2±21.5 in healthy controls, p<0.0001. Logistic regression analysis indicated that serum 25(OH)D levels, age and gender were significantly associated with MS susceptibility (Table 3). These results showed that for every 1 nmol/L increase in 25(OH)D levels, the odds for MS decreased (OR=0.97; 95%CI=0.96-0.98; p<0.0001).

Table 3. Variables that significantly affect Multiple Sclerosis risk.

Variable	**B**	**SE**	**p**	**OR (95%CI)**
25(OH)D levels (nmol/L)	-0.031	0.005	<0.0001	0.970 (0.961-0.979)
Age (years)	-0.022	0.009	0.015	0.979 (0.962-0.996)
Gender	0.525	0.206	0.011	1.691 (1.129-2.534)

Dependent variable: controls = 0 and patients = 1.
Independent variables: age, gender (male=0 and female=1) and 25(OH)D levels.
25(OH)D: 25-hydroxyvitamin

In what regards disease course (RRMS vs. SPMS+PPMS groups), patients with a progressive course presented significantly lower 25(OH)D levels (42.7±21.7 vs. 29.9±20.4, p<0.0001).

Spearman rank correlation analyses revealed a significant inverse correlation between 25(OH)D levels and EDSS (r=-0.293, p<0.0001) and also with MSSS (r=-0.323, p<0.0001). To test for potential confounding factors, 25(OH)D levels were assessed in multiple logistic regression analysis adjusted for age, gender, disease form, EDSS, disease duration and MSSS. Vitamin D levels were independently associated with EDSS (p=0.004) and disease duration (p=0.016), and with MSSS (p=0.001) (Table 4).

Table 4. Results of a multiple logistic regression analysis on determinants of 25(OH)D levels

Covariable	**Bivariate model**		**Multivariate model with disease duration and EDSS**		**Multivariate model with MSSS**	
	p value	**OR (CI 95%)**	**p value**	**OR (CI 95%)**	**p value**	**OR (CI 95%)**
Age	0.087	0.98 (0.96-1.00)	0.570	0.99 (0.96-1.02)	0.986	1.00 (0.97-1.03)
Gender	0.835	0.94 (0.55-1.63)	0.633	0.87 (0.49-1.55)	0.606	0.86 (0.48-1.53)
Disease course	**0.002**	**3.56 (1.59-7.97)**	0.654	1.28 (0.43-3.82)	0.570	1.35 (0.48-3.77)
Disease duration	0.874	1.00 (0.97-1.03)	**0.016**	**1.05 (1.01-1.10)**	-	-
EDSS	**<0.0001**	**0.77 (0.68-0.88)**	**0.004**	**0.73 (0.59-0.90)**	-	-
MSSS	**<0.0001**	**0.77 (0.70-0.87)**	**-**	**-**	**0.001**	**0.80 (0.69-0.92)**

Dependent variable: 25(OH)D levels<50 nmol/L = 0 and 25(OH)D levels≥50 nmol/L = 1.
Independent variables: age, gender (male=0 and female=1), disease course (SPMS+PPMS=0 and RRMS =1), disease duration, EDSS and MSSS.
MS: Multiple Sclerosis; EDSS: Expanded Disability Status Scale; MSSS: MSSS: MS Severity Scale; PPMS: primary progressive MS; RRMS: relapsing–remitting MS; SPMS: secondary progressive MS

4. Discussion

There is compelling evidence indicating that lower levels of vitamin D are associated with an increased risk and disease activity in MS. In the present study 25(OH)D serum levels were significantly lower in patients compared to controls. These results are in agreement with several previous studies [6-9, 12-14, 16, 18].

Vitamin D levels are lower in winter [37], as a result of seasonal changes, and this was also observed in this study. As a consequence, the difference in vitamin D levels between patients and controls in winter was reduced, but remained statistically significant.

Vitamin D deficiency is common among patients with MS, in the current study more than half of the patients (66%) had 25(OH)D deficiency compared to 48% in the healthy controls. Restriction of mobility due to advanced MS-related disability, leading to limited sunlight exposure, may confound the efforts of establishing a causal relationship between vitamin D deficiency and disease susceptibility or outcome, as the levels of vitamin D are rarely known before disease onset and diagnosis. In this setting, our study of a sub-group of patients with recent disease onset, demonstrating 25(OH)D deficits already at this early stage of the disease, may be of particular value. This observation is in accordance with results from a prospective study [20] and from Behrens et al. [13] that showed, in the last year, that “clinically hardly affected patients”, in the earliest phases of MS, also present low 25(OH)D levels.

Vitamin D plays a role in adaptive and innate immunity [38]. In MS, the vitamin D mechanism of action is likely to be related to the development of self-tolerance, as vitamin D regulates T helper cell and dendritic cell function, and induces regulatory T cells, thus resulting in a decreased Th1 driven autoimmune response [39]. These immunomodulatory effects give support to the hypothesis that a higher vitamin D status reduces disability progression, which could, for example, prevent relapses in RRMS. Nevertheless, there are contradictory evidences regarding whether vitamin D insufficiency has an adverse effect on MS outcomes. Longitudinal studies show a relationship between low serum vitamin D levels and disease activity as observed on magnetic resonance imaging [27, 40, 41], a higher relapse risk [25, 42] and increased disability progression [28, 43]. Low vitamin D levels were also associated with higher disability, assessed by the EDSS score, in some studies [18, 23]. In the study by van der Mei et al. [44], performed in 136 MS patients and 272 healthy controls in Australia, was observed that patients with higher disability (EDSS>3) had greater vitamin D insufficiency than cases with low disability. Recently, Thouvenot et al. [28], in a cohort of

181 MS patients from France, found a negative correlation between vitamin D levels and EDSS score in the overall patient population, but in relapsing-remitting MS patients, vitamin D levels were only correlated with disability scores for EDSS < 4. However association with higher disability was not confirmed in other studies [15, 24, 27, 45]. Because patients with prolonged disease duration have higher disability, they could be expected to have less outdoor exposure and to be at risk of vitamin D deficiency for this same reason. These facts make it difficult to establish a causal explanation for the association of longer disease duration with worse EDSS score as well as the inverse significant relationship between 25(OH)D levels and disease duration.

Some of the strengths of this study are the inclusion of a relatively large group of patients with MS and controls, and the inclusion of seasonal variation. It should be noted that 25(OH)D levels may not reflect the true biological activity, as downstream events in the vitamin D signaling, or other factors that could modulate vitamin D bioavailability were not taken into account. In conclusion, vitamin D levels in patients with MS were significantly lower than in healthy subjects, also a significant association was found between vitamin D level and disability.

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Conflicts of interest

The authors declare that they have no conflict of interest.

References

1. Goodin DS. Chapter 11-The epidemiology of Multiple Sclerosis: insights to disease pathogenesis. In: Douglas SG, editor. Handbook of clinical neurology: Elsevier; 2014. p. 231-66.
2. Szymczak I, Pawliczak R. The Active Metabolite of Vitamin D3 as a Potential Immunomodulator. Scandinavian journal of immunology. 2016;83(2):83-91.
3. Smolders J, Damoiseaux J, Menheere P, Hupperts R. Vitamin D as an immune modulator in Multiple Sclerosis, a review. J Neuroimmunol. 2008;194(1-2):7-17.
4. Shaygannejad V, Golabchi K, Haghighi S, Dehghan H, Moshayedi A. A Comparative Study of 25 (OH) Vitamin D Serum Levels in Patients with Multiple Sclerosis and Control Group in Isfahan, Iran. International journal of preventive medicine. 2010;1(3):195-201.
5. Lonergan R, Kinsella K, Fitzpatrick P, Brady J, Murray B, Dunne C, et al. Multiple sclerosis prevalence in Ireland: relationship to vitamin D status and HLA genotype. J Neurol Neurosurg Psychiatry. 2011;82(3):317-22.
6. Gelfand JM, Cree BA, McElroy J, Oksenberg J, Green R, Mowry EM, et al. Vitamin D in African Americans with Multiple Sclerosis. Neurology. 2011;76(21):1824-30.
7. Hatamian H, Bidabadi E, Seyed Saadat SM, Saadat NS, Kazemnezhad E, Ramezani H, et al. Is serum vitamin D levels associated with disability in patients with newly diagnosed Multiple Sclerosis? Iranian journal of neurology. 2013;12(2):41-6.
8. Kirbas A, Kirbas S, Anlar O, Turkyilmaz AK, Cure MC, Efe H. Investigation of the relationship between vitamin D and bone mineral density in newly diagnosed Multiple Sclerosis. Acta neurologica Belgica. 2013;113(1):43-7.
9. Mazdeh M, Seifirad S, Kazemi N, Seifrabie MA, Dehghan A, Abbasi H. Comparison of vitamin D3 serum levels in new diagnosed patients with Multiple Sclerosis versus their healthy relatives. Acta medica Iranica. 2013;51(5):289-92.
10. Shahbeigi S, Pakdaman H, Fereshtehnejad SM, Nikravesh E, Mirabi N, Jalilzadeh G. Vitamin d3 concentration correlates with the severity of Multiple Sclerosis. International journal of preventive medicine. 2013;4(5):585-91.
11. Hejazi E, Amani R, SharafodinZadeh N, Cheraghian B. Comparison of Antioxidant Status and Vitamin D Levels between Multiple Sclerosis Patients and Healthy Matched Subjects. Multiple sclerosis international. 2014;2014:539854.
12. Niino M, Sato S, Fukazawa T, Masaki K, Miyazaki Y, Matsuse D, et al. Decreased serum vitamin D levels in Japanese patients with Multiple Sclerosis. J Neuroimmunol. 2015;279:40-5.
13. Behrens JR, Rasche L, Giess RM, Pfuhl C, Wakonig K, Freitag E, et al. Low 25-hydroxyvitamin D, but not the bioavailable fraction of 25-hydroxyvitamin D, is a risk factor for Multiple Sclerosis. Eur J Neurol. 2016;23(1):62-7.
14. Karampoor S, Zahednasab H, Ramagopalan S, Mehrpour M, Safarnejad Tameshkel F, Keyvani H. 25-hydroxyvitamin D levels are associated with Multiple Sclerosis in Iran: A cross-sectional study. J Neuroimmunol. 2016;290:47-8.
15. Becker J, Callegaro D, Lana-Peixoto MA, Talim N, Vidaletti T, de Paula Correa M, et al. Hypovitaminosis D association with disease activity in relapsing remitting Multiple Sclerosis in Brazil. J Neurol Sci. 2016;363:236-9.
16. Brola W, Sobolewski P, Szczuchniak W, Goral A, Fudala M, Przybylski W, et al. Association of seasonal serum 25-hydroxyvitamin D levels with disability and relapses in relapsing-remitting Multiple Sclerosis. European journal of clinical nutrition. 2016;70(9):995-9.
17. Yamout B, Karaky NM, Mahfouz RA, Jaber F, Estaitieh N, Shamaa D, et al. Vitamin D receptor biochemical and genetic profiling and HLA-class II genotyping among Lebanese with Multiple Sclerosis - A pilot study. J Neuroimmunol. 2016;293:59-64.
18. Zhang Y, Liu G, Han X, Dong H, Geng J. The association of serum 25-hydroxyvitamin D levels with Multiple Sclerosis severity and progression in a case-control study from China. J Neuroimmunol. 2016;297:127-31.
19. Duan S, Lv Z, Fan X, Wang L, Han F, Wang H, et al. Vitamin D status and the risk of Multiple Sclerosis: a systematic review and meta-analysis. Neurosci Lett. 2014;570:108-13.
20. Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of Multiple Sclerosis. Jama. 2006;296(23):2832-8.
21. Mowry EM, Pelletier D, Gao Z, Howell MD, Zamvil SS, Waubant E. Vitamin D in clinically isolated syndrome: evidence for possible neuroprotection. Eur J Neurol. 2016;23(2):327-32.
22. Martinelli V, Dalla Costa G, Colombo B, Dalla Libera D, Rubinacci A, Filippi M, et al. Vitamin D levels and risk of Multiple Sclerosis in patients with clinically isolated syndromes. Mult Scler. 2014;20(2):147-55.

23. Smolders J, Menheere P, Kessels A, Damoiseaux J, Hupperts R. Association of vitamin D metabolite levels with relapse rate and disability in Multiple Sclerosis. Mult Scler. 2008;14(9):1220-4.
24. Muris AH, Rolf L, Broen K, Hupperts R, Damoiseaux J, Smolders J. A low vitamin D status at diagnosis is associated with an early conversion to secondary progressive Multiple Sclerosis. J Steroid Biochem Mol Biol. 2015.
25. Runia TF, Hop WC, de Rijke YB, Buljevac D, Hintzen RQ. Lower serum vitamin D levels are associated with a higher relapse risk in Multiple Sclerosis. Neurology. 2012;79(3):261-6.
26. Mesliniene S, Ramrattan L, Giddings S, Sheikh-Ali M. Role of vitamin D in the onset, progression, and severity of Multiple Sclerosis. Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists. 2013;19(1):129-36.
27. Fitzgerald KC, Munger KL, Kochert K, Arnason BG, Comi G, Cook S, et al. Association of Vitamin D Levels With Multiple Sclerosis Activity and Progression in Patients Receiving Interferon Beta-1b. JAMA neurology. 2015;72(12):1458-65.
28. Thouvenot E, Orsini M, Daures JP, Camu W. Vitamin D is associated with degree of disability in patients with fully ambulatory relapsing-remitting Multiple Sclerosis. Eur J Neurol. 2015;22(3):564-9.
29. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for Multiple Sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol. 2011;69(2):292-302.
30. Kurtzke JF. Rating neurologic impairment in Multiple Sclerosis: an expanded disability status scale (EDSS). Neurology. 1983;33(11):1444-52.
31. Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, et al. Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. Neurology. 2005;64(7):1144-51.
32. Bettencourt A, Boleixa D, Reis J, Oliveira JC, Mendonça D, Costa PP, et al. Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal. J Steroid Biochem Mol Biol. 2016.
33. Dawson-Hughes B, Heaney RP, Holick MF, Lips P, Meunier PJ, Vieth R. Estimates of optimal vitamin D status. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2005;16(7):713-6.
34. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-81.
35. Holick MF. Vitamin D status: measurement, interpretation, and clinical application. Annals of epidemiology. 2009;19(2):73-8.
36. Souberbielle JC, Body JJ, Lappe JM, Plebani M, Shoenfeld Y, Wang TJ, et al. Vitamin D and musculoskeletal health, cardiovascular disease, autoimmunity and cancer: Recommendations for clinical practice. Autoimmunity reviews. 2010;9(11):709-15.
37. Maxwell JD. Seasonal variation in vitamin D. Proc Nutr Soc. 1994;53(3):533-43.
38. Gatti D, Idolazzi L, Fassio A. Vitamin D: not just bone, but also immunity. Minerva medica. 2016;107(6):452-60.
39. Royal W, 3rd, Mia Y, Li H, Naunton K. Peripheral blood regulatory T cell measurements correlate with serum vitamin D levels in patients with Multiple Sclerosis. J Neuroimmunol. 2009;213(1-2):135-41.
40. Mowry EM, Waubant E, McCulloch CE, Okuda DT, Evangelista AA, Lincoln RR, et al. Vitamin D status predicts new brain magnetic resonance imaging activity in Multiple Sclerosis. Ann Neurol. 2012;72(2):234-40.
41. Loken-Amsrud KI, Holmoy T, Bakke SJ, Beiske AG, Bjerve KS, Bjornara BT, et al. Vitamin D and disease activity in Multiple Sclerosis before and during interferon-beta treatment. Neurology. 2012;79(3):267-73.
42. Simpson S, Jr., Taylor B, Blizzard L, Ponsonby AL, Pittas F, Tremlett H, et al. Higher 25-hydroxyvitamin D is associated with lower relapse risk in Multiple Sclerosis. Ann Neurol. 2010;68(2):193-203.
43. Ascherio A, Munger KL, White R, Kochert K, Simon KC, Polman CH, et al. Vitamin D as an early predictor of Multiple Sclerosis activity and progression. JAMA neurology. 2014;71(3):306-14.
44. van der Mei IA, Ponsonby AL, Dwyer T, Blizzard L, Taylor BV, Kilpatrick T, et al. Vitamin D levels in people with Multiple Sclerosis and community controls in Tasmania, Australia. J Neurol. 2007;254(5):581-90.
45. Yildiz M, Tettenborn B, Putzki N. Vitamin D levels in Swiss Multiple Sclerosis patients. Swiss medical weekly. 2011;141:w13192.

Paper 8

The vitamin D receptor gene FokI polymorphism and Multiple Sclerosis in a Northern Portuguese population

The vitamin D receptor gene FokI polymorphism and Multiple Sclerosis in a Northern Portuguese population

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Abstract

Background: The cause of Multiple Sclerosis (MS) remains poorly understood, but it is widely believed to be an autoimmune disease occurring in genetically susceptible individuals after exposure to as-yet undefined environmental factors. One of these environmental factors is vitamin D, a well-known immune modulator. The biologically active form of vitamin D, 1,25-dihydroxyvitamin D3, has been shown to exert its immune modulatory properties through its nuclear receptor (VDR) namely by inhibiting the proliferation of Th cells. The purpose of this study was to evaluate the influence of FokI VDR polymorphism in MS development and progression.

Methods: A group of 533 unrelated Portuguese patients with a definitive diagnosis of MS and 446 ethnically matched healthy controls were included in the study. FokI was genotyped using a PCR-based TaqMan Genotyping Assay and serum 25-hydroxyvitamin D [25(OH)D] was also assessed.

Results: A statistically significant higher frequency of the ff genotype was observed in MS patients [15.6% vs. 10.1%, p=0.012, OR(95%CI)=1.69(1.12-2.54)]. No differences were observed in the frequencies of the FokI polymorphism according to disease course or with progression of disability. None of the genotypes was significantly associated with 25(OH)D serum levels.

Conclusions: An association between FokI ff genotype and MS susceptibility was found, but not with disease form or progression. Additional clinical and experimental studies should take the FokI VDR polymorphism into account, and further clarify the role of vitamin D, its metabolites and its receptor in MS.

Key-words: VDR; Multiple Sclerosis; FokI; Portugal

1. Introduction

Multiple sclerosis (MS) is the most common inflammatory demyelinating disease of the central nervous system (CNS) in young adults. The cause of MS remains unknown, but it is widely believed to be an autoimmune disease occurring in genetically susceptible individuals after exposure to as-yet undefined environmental factors [1]. Nevertheless, a number of risk factors are now known to be important for the development of this disease [2].

Combined with Epstein-Barr virus (EBV) infection and smoking, vitamin D deficiency has been also described as an environmental risk factor for MS. Progress has been made in understanding its role in both disease cause and progression [3]. Physiological functions of the biologically active, dihydroxylated form of vitamin D (calcitriol or $1,25(OH)_2D$) were initially thought to be limited to bone metabolism, but the identification of vitamin D receptors (VDR) in immune system cells and the discovery that dendritic cells can produce the metabolically active form of vitamin D have led to the suggestion that vitamin D can also act as an immune modulator [4, 5], and consequently implicated in several immune-mediated diseases [6]. The interaction of vitamin D metabolites with cells of the immune system has been proven in vitro and in animal models. The evidence obtained from these studies strongly supports a model in which vitamin D mediates a shift to a more anti-inflammatory immune response, and in particular to enhanced regulatory T cell functionality [7, 8].

Calcitriol performs its biological activities through VDR-mediated gene regulation [9].It binds to VDR in the cytoplasm of target cells, and then the ligand-receptor complex enters the cell nucleus where it functions as a ligand-activated transcription factor [10]. VDR influences the expression of more than 200 genes and the majority of these genes are involved in immunity, such as the HLA-DRB1*15 allele, a risk factor for MS with a highly efficient vitamin D responsive element in its promoter [11].

In humans, the VDR gene is located on chromosome 12q13.1, extends over 100 kb and includes eight protein-coding exons, six untranslated exons, eight introns and two promoter regions. Four common single-nucleotide polymorphisms (SNPs) in the VDR gene have been extensively investigated: FokI C>T (rs2228570/rs10735810), BsmI A>G (rs1544410), ApaI G>T (rs7975232) and TaqI C>T (rs731236). BsmI and ApaI SNPs are both located in intron 8, and the TaqI is a silent SNP in exon 9. Although these three polymorphisms do not produce any structural change on the VDR protein, they are in strong linkage disequilibrium. The VDR FokI restriction site defines a SNP in the first of two potential translation initiation start sites for the VDR mRNA. Two protein variants can be expressed, corresponding to the two available start sites: the longer VDR, encoded

by the alternative allele form (ATG) (designated f/M1), is three amino acids longer and 1.7 times less efficient than the common allele form (ACG) (designated F/M4). This has functional consequences for the intra-cellular activity namely the amino acid structure created by the FokI-f allele will reduce the transcriptional activity [12-14].

Several studies investigating VDR polymorphisms and their association with MS have been published in the last years but with inconclusive results [15-19]. These discrepancies can be explained by different factors such as small sample size groups, geographical diversity and different genetic population backgrounds. To the best of our knowledge no previous studies have addressed the association of VDR polymorphisms and MS in the Portuguese population; therefore our purpose was to evaluate this association in order to contribute to the ongoing debate.

2. Subjects and methods

2.1 Patients and controls

A group of 533 unrelated Portuguese patients with a definitive diagnosis of MS, according to the revised McDonald criteria, were recruited from neurology outpatient clinic of Centro Hospitalar do Porto – Hospital Santo António. The control group comprised 446 ethnically matched healthy blood donors (HC), from the same geographical area (North of Portugal). Individuals were excluded if they had disorders related to vitamin D deficiency such as rickets or parathyroid pathologies and also if they consumed drugs or supplements containing vitamin D or calcium. This study was approved by the hospital Medical Ethical Committee and written informed consent was obtained from all participants. The demographic and clinical features of the studied groups are presented in Table 1.

Table 1. Characteristics of the MS patients and control population.

	MS Patients (n=533)	Controls (n=446)
Sex		
F	348 (65.3%)	248 (55.6%)
M	185 (34.7%)	198 (44.4%)
Age at onset (years)	30.2±9.3	-
Disease Duration (years)	10.3±8.5	-
Disease Course		-
RR	424 (79.5%)	
SP	59 (11.1%)	
PP	50 (9.4%)	
EDSS (mean±SD)	3.0±2.3	-
MSSS (mean±SD)	3.9±2.8	-

2.2 Clinical parameters

Sex, age, clinical phenotype (RR-Relapsing Remitting, SP-Secondary progressive, PP-Primary progressive) and disease duration were retrieved from patient's database. The Expanded Disability Status Scale (EDSS) [20] and Multiple Sclerosis Severity Scale (MSSS) [21] were used to measure physical disability and disease progression, respectively. Patients were stratified into three groups according to the rate of disease disability progression. Patients with MSSS<3 (n=249) were considered slow progressors. Patients with MSSS>6 (n=145) were classified as fast progressors. Patients with MSSS 3-6 (n=139) were included in the mid-rate progressors group.

2.3 DNA samples and genotyping

Peripheral blood samples from MS patients and controls were collected in EDTA tubes. Genomic DNA was obtained from proteinase-K treated peripheral blood leukocytes with a salting-out procedure [22]. The FokI (rs2228570/rs10735810) was genotyped using a pre-designed TaqMan® allelic discrimination assay from Applied Biosystems (Foster City, CA, USA) in a Rotor Gene 6000 Real-Time PCR machine (Corbett Life Science).

2.4 25(OH)D measurement

Blood was collected in Vacuette® Z Serum Separator Clot Activator tubes. Serum was obtained by centrifugation and stored in several aliquots at -20°C until analysed. Serum samples were available for a limited number of patients (n=269). Serum 25-hydroxyvitamin D [25(OH)D] was chosen as a reliable marker of individual vitamin D status as it reflects vitamin D obtained both from food sources and cutaneous synthesis, and it is not prone to diurnal variation. The serum levels of this metabolite were determined as previously described [23].

2.5 Statistical analysis

Genotypes were examined for deviation from the Hardy-Weinberg equilibrium by using the exact Chi-square test. Logistic regression was used to estimate Odds Ratio (OR) and 95% CIs with correction for sex. The major homozygous genotype was used as the reference group. 25(OH)D levels were checked for normality using the Kolmogorov-Smirnov test and after natural logarithm (ln) transformations were used for the analysis. Differences between groups were tested using the one-way ANOVA test. A p-value<0.05

was considered to be statistically significant. Statistical analyses were performed using Statistical Package for the Social Sciences software (version 23, IBM SPSS Statistics, NY, USA).

3. Results

The genotypic and allelic frequencies of the FokI polymorphism in patients and controls are described in Table 2. Both groups were in Hardy-Weinberg equilibrium (p>0.05).

A statistically significant higher frequency of FokI ff genotype was observed in MS patients (15.6% vs. 10.1%, p=0.012, OR (95%CI)=1.687(1.120-2.541)). Based on the known role of vitamin D in MS etiopathogenesis, we further analysed whether allele variants of the VDR gene polymorphism FokI could have any influence on disease form or disability progression. No differences were observed between frequencies of FokI polymorphism in the patients accordingly to disease form (p=0.996) (Table 3). When genotype and allele frequencies of FokI were compared among patients with different rate of progression no significant differences were found (Table 4). Concerning 25(OH)D serum levels no significant differences were observed in relation to the different FokI genotypes (p=0.256).

Table 2. VDR FokI polymorphism genotype and allelic frequencies in MS patients and in healthy controls.

	MS (n=533)	Controls (n=446)	OR (95%CI)	p
Genotype				
FF (CC)	223 (41.8%)	204 (45.7%)	1	
Ff (CT)	227 (42.6%)	197 (44.2%)	1.054 (0.805-1.380)	0.701
ff (TT)	83 (15.6%)	45 (10.1%)	1.687 (1.120-2.541)	**0.012**
Allele				
F (C)	673 (63.1%)	605 (67.8%)	0.812 (0.673-0.980)	**0.030**
f (T)	393 (36.9%)	287 (32.2%)	1.231 (1.020-1.485)	

Table 3. Distribution of the genotype and allele frequencies of the FokI polymorphism according to clinical MS-phenotype.

	RR (n=424)	SP (n=59)	PP (n=50)	p
Genotypes				
FF (CC)	180 (42.5%)	24 (40.7%)	20 (40%)	
Ff (CT)	179 (42.2%)	25 (42.4%)	22 (44%)	0.996
Ff (TT)	65 (15.3%)	10 (16.9%)	8 (16%)	
Alleles				
F (C)	539 (63.6%)	73 (61.9%)	62 (62%)	0.905
f (T)	309 (36.4%)	45 (38.1%)	38 (38%)	

Table 4. Distribution of the genotype and allele frequencies of the FokI polymorphism in MS patients with different rate of disability progression.

	Slow progressors (n=249)	Mid-rate progressors (n=139)	Fast progressors (n=145)	p
Genotypes				
FF (CC)	99 (40.4%)	60 (43.2%)	63 (43.4%)	
Ff (CT)	102 (41.6%)	58 (41.7%)	64 (44.1%)	0.701
ff (TT)	44 (18%)	21 (15.1%)	18 (12.4%)	
Alleles				
F (C)	300 (61.2%)	178 (64%)	190 (65.5%)	0.468
f (T)	190 (38.8%)	100 (36%)	100 (34.5%)	

4. Discussion

Multiple sclerosis is the most common inflammatory demyelinating disease of the CNS in young adults and its etiology remains poorly understood. Progress has been made in characterizing its genetic susceptibility factors. The HLA class II allele DRB1*1501 is a well-established susceptibility factor for this disease [24], though subsequent studies reported a protective effect of some HLA class I alleles, such as the HLA-A*02 and Cw*05, independently of HLA-DRB1*1501 allele [25]. There are at least 110 established Multiple Sclerosis risk variants at 103 discrete loci outside the major histocompatibility complex [26].

The association of FokI with MS has been previously investigated in patient-control studies but the results have been conflicting. A British study from 2004 described a trend towards an under-expression of FokI ff genotype in MS patients [27], but subsequent studies did not report any associations of this polymorphism with MS susceptibility. The discrepancy found in the different studies might be caused by clinical heterogeneity, ethnicity, geographical factors, and interactions with other genetic or environmental factors and/or small sample size (low statistical power). Sample size issues may be overcome through meta-analyses, which will increase statistical power and resolution by pooling the results of independent analyses, but do not surmount the other possible confounding factors. In 2009 Smolders et al [28] hypothesized that an association of VDR polymorphisms with MS might only be penetrant in a population with a sufficient vitamin D status, meaning that these polymorphisms are likely to influence the response of vitamin D metabolism only on exposure to sufficient amounts of vitamin D, which may also explain some of the reported negative results.

To the best of our knowledge, this is the first study to investigate the association between MS and FokI, in both disease development and progression, in a Portuguese population. An association between FokI ff genotype and MS susceptibility was found, but there were no associations with disease forms or progression, which is in agreement with a study from Cierny and colleagues that also did not find significant association between this SNP and the rate of disease disability progression [29]. Also, no differences were observed between the different FokI genotypes and 25(OH)D serum levels. Nevertheless the main goal of this paper was not the investigation of the role of the FokI genotypes in predicting vitamin D levels; this issue will be addressed in a follow-up analysis.

The FokI F (C) allele leads to the expression of a 3 amino acid shorter protein (424 amino acids) than the f (T) allele (427 amino acids). The shorter length of the VDR protein apparently increases translational activity [13] which may explain why peripheral blood mononuclear cells with the FF genotype were more efficient in exerting the $1,25(OH)_2D_3$ effects than the ff genotype [30]. Calcitriol has been shown to have immune modulatory properties, namely by inhibiting the proliferation of Th cells. Thus, we hypothesize that individuals carrying the FF genotype may benefit from a more balanced T cell response, possibly preventing the development of autoimmune diseases.

A strength of the present study is the relatively large sample size of both populations. It also has some limitations, namely only one polymorphism of the VDR gene was studied, and we cannot exclude that other genetic variants of the VDR gene could be associated with MS susceptibility. As stated by Smolders and colleagues, there are many players in vitamin D metabolism, whose exact roles and significance are not yet fully understood. Additional clinical and experimental studies should take the FokI VDR polymorphism into account, and further clarify the role of vitamin D, its metabolites and its receptor in MS [28]. Also, assessment of SNPs in VDR gene can assist in the identification of individuals at risk of vitamin D insufficiency and may be useful to adjust treatment in individuals with an insufficient response to vitamin D supplementation [31].

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Conflicts of interest

The authors declare that they have no conflict of interest.

References

[1] Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. Lancet Neurol. 2010;9:727-39.
[2] van der Mei I, Lucas RM, Taylor BV, Valery PC, Dwyer T, Kilpatrick TJ, et al. Population attributable fractions and joint effects of key risk factors for Multiple Sclerosis. Mult Scler. 2016;22:461-9.
[3] Pakpoor J, Ramagopalan S. Evidence for an Association Between Vitamin D and Multiple Sclerosis. Current topics in behavioral neurosciences. 2015;26:105-15.
[4] Kamen DL, Tangpricha V. Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. J Mol Med (Berl). 2010;88:441-50.
[5] Wei R, Christakos S. Mechanisms Underlying the Regulation of Innate and Adaptive Immunity by Vitamin D. Nutrients. 2015;7:8251-60.
[6] Rosen Y, Daich J, Soliman I, Brathwaite E, Shoenfeld Y. Vitamin D and autoimmunity. Scandinavian journal of rheumatology. 2016:1-9.
[7] Smolders J, Damoiseaux J, Menheere P, Hupperts R. Vitamin D as an immune modulator in Multiple Sclerosis, a review. J Neuroimmunol. 2008;194:7-17.
[8] Smolders J, Damoiseaux J. Vitamin D as a T-cell modulator in Multiple Sclerosis. Vitamins and hormones. 2011;86:401-28.
[9] Hewer S, Lucas R, van der Mei I, Taylor BV. Vitamin D and Multiple Sclerosis. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia. 2013;20:634-41.
[10] VanAmerongen BM, Dijkstra CD, Lips P, Polman CH. Multiple sclerosis and vitamin D: an update. European journal of clinical nutrition. 2004;58:1095-109.
[11] Ramagopalan SV, Maugeri NJ, Handunnetthi L, Lincoln MR, Orton SM, Dyment DA, et al. Expression of the Multiple Sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D. PLoS genetics. 2009;5:e1000369.
[12] Whitfield GK, Remus LS, Jurutka PW, Zitzer H, Oza AK, Dang HT, et al. Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. Molecular and cellular endocrinology. 2001;177:145-59.
[13] Jurutka PW, Remus LS, Whitfield GK, Thompson PD, Hsieh JC, Zitzer H, et al. The polymorphic N terminus in human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIB. Mol Endocrinol. 2000;14:401-20.
[14] Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA, Van Leeuwen JP. Genetics and biology of vitamin D receptor polymorphisms. Gene. 2004;338:143-56.
[15] Smolders J, Peelen E, Thewissen M, Menheere P, Tervaert JW, Hupperts R, et al. The relevance of vitamin D receptor gene polymorphisms for vitamin D research in Multiple Sclerosis. Autoimmun Rev. 2009;8:621-6.
[16] Tizaoui K, Kaabachi W, Hamzaoui A, Hamzaoui K. Association between vitamin D receptor polymorphisms and Multiple Sclerosis: systematic review and meta-analysis of case-control studies. Cellular & molecular immunology. 2015;12:243-52.
[17] Garcia-Martin E, Agundez JA, Martinez C, Benito-Leon J, Millan-Pascual J, Calleja P, et al. Vitamin D3 receptor (VDR) gene rs2228570 (Fok1) and rs731236 (Taq1) variants are not associated with the risk for Multiple Sclerosis: results of a new study and a meta-analysis. PLoS One. 2013;8:e65487.
[18] Yeo TW, Maranian M, Singlehurst S, Gray J, Compston A, Sawcer S. Four single nucleotide polymorphisms from the vitamin D receptor gene in UK Multiple Sclerosis. J Neurol. 2004;251:753-4.
[19] Simon KC, Munger KL, Kraft P, Hunter DJ, De Jager PL, Ascherio A. Genetic predictors of 25-hydroxyvitamin D levels and risk of Multiple Sclerosis. J Neurol. 2011;258:1676-82.
[20] Kurtzke JF. Rating neurologic impairment in Multiple Sclerosis: an expanded disability status scale (EDSS). Neurology. 1983;33:1444-52.
[21] Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, et al. Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. Neurology. 2005;64:1144-51.
[22] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16:1215.
[23] Bettencourt A, Boleixa D, Reis J, Oliveira JC, Mendonça D, Costa PP, et al. Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal. J Steroid Biochem Mol Biol. 2016.

[24] Silva AM, Pereira C, Bettencourt A, Carvalho C, Couto AR, Leite MI, et al. The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population. J Neurol Sci. 2007;258:69-74.
[25] Silva AM, Bettencourt A, Pereira C, Santos E, Carvalho C, Mendonca D, et al. Protective role of the HLA-A*02 allele in Portuguese patients with Multiple Sclerosis. Mult Scler. 2009;15:771-4.
[26] Beecham AH, Patsopoulos NA, Xifara DK, Davis MF, Kemppinen A, Cotsapas C, et al. Analysis of immune-related loci identifies 48 new susceptibility variants for Multiple Sclerosis. Nat Genet. 2013;45:1353-60.
[27] Partridge JM, Weatherby SJ, Woolmore JA, Highland DJ, Fryer AA, Mann CL, et al. Susceptibility and outcome in MS: associations with polymorphisms in pigmentation-related genes. Neurology. 2004;62:2323-5.
[28] Smolders J, Damoiseaux J, Menheere P, Tervaert JW, Hupperts R. Fok-I vitamin D receptor gene polymorphism (rs10735810) and vitamin D metabolism in Multiple Sclerosis. J Neuroimmunol. 2009;207:117-21.
[29] Cierny D, Michalik J, Kurca E, Dobrota D, Lehotsky J. FokI vitamin D receptor gene polymorphism in association with Multiple Sclerosis risk and disability progression in Slovaks. Neurological research. 2015;37:301-8.
[30] Colin EM, Weel AE, Uitterlinden AG, Buurman CJ, Birkenhager JC, Pols HA, et al. Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1, 25-dihydroxyvitamin D3. Clinical endocrinology. 2000;52:211-6.
[31] Herrmann M, Farrell CL, Pusceddu I, Fabregat-Cabello N, Cavalier E. Assessment of vitamin D status - a changing landscape. Clin Chem Lab Med. 2017;55:3-26.

CHAPTER 4

Discussion and Conclusions

4.1 – General Discussion

Multiple Sclerosis is an autoimmune and neurodegenerative disorder affecting approximately 2.3 million people worldwide (Browne et al. 2014). It is characterized by chronic inflammation and areas of demyelination (lesions or plaques) in the CNS, impairing the electrical conduction of nerve impulses (Schaeffer et al. 2015). MS is considered an autoimmune disease with autoreactive T lymphocytes recognizing components of the myelin sheath, nonetheless neurodegenerative processes also contribute to impairment (Ransohoff et al. 2015; Martin et al. 2016). It displays several characteristics that are common to numerous autoimmune diseases, including moderate polygenic heritability, modulation by environmental factors, clinical and genetic heterogeneity, and higher frequency in women.

The primary aim of this thesis was to demonstrate the value of selected factors (genetic and non-genetic) involved in immune dysfunction in Multiple Sclerosis. This chapter summarizes and discusses the importance of the results obtained in this thesis in an integrated way, and how these findings may direct future research.

4.1.1 – Genetic and epigenetic factors

Genes within the HLA region are the strongest genetic risk factors associated with MS. Some HLA class II and class I genes are particularly relevant modifiers of disease risk: the class II variant HLA-DRB1*15:01 has a strong association with an increased risk of MS (OR≈3), whereas the class I variant HLA‑A*02 is associated with protection (OR≈0.6). The absence of HLA-A*02 and the presence of HLA-DRB1*15:01 has a combined OR of ≈5. (Brynedal et al. 2007; Moutsianas et al. 2015).

Our group has showed, over the last years, that some genetic variants are correlated with increased/decreased risk of MS in the Portuguese population (Bettencourt et al. 2012). In 2007 we described the association of HLA-DRB1*15 and HLA-DRB1*03 alleles and MS susceptibility in a group of 248 patients (Silva et al. 2007). In 2009 we reported the association of HLA-A*02 allele with disease protection in 342 patients, independently of HLA-DRB1*15 allele (Silva et al. 2009). In **paper 1** we confirmed the association of HLA-DRB1*15 and HLA-DRB1*03 alleles and MS in a larger group of patients (n=536) (Bettencourt et al. 2015). The pathways through which HLA molecules determine MS risk or protection remain to be fully revealed. It is generally accepted that they do so by shaping the central and peripheral T cell repertoires of the host. The functional basis of the association between specific HLA alleles and development of MS can be classically

explained by two possible ethiopathogenic models: *The molecular mimicry hypothesis* - certain HLA alleles are more efficient at presenting pathogen epitopes that share structural features with self-peptides to mature T cells. Once the response to the pathogen is initiated the self-antigen is also recognized and disease ensues. *Central selection failure* - certain HLA alleles are less efficient at presenting self-peptides to developing T cells in the thymus, so negative selection fails. HLA class II molecules associated with MS susceptibility (HLA-DRB1*15 and HLA-DRB1*03) would promote positive selection of pathogenic autoreactive thymocytes in the cortex, while failing to trigger their subsequent deletion in the medulla. On the other hand, protective alleles (HLA-A*02) would select a repertoire of T cells that would lead to the deletion of the majority of the autoreactive T cells through negative selection (Fugger et al. 2009).

As already stated, central selection is a process far from perfect and self-reactive lymphocytes can escape into the periphery. Consequently, peripheral tolerance mechanisms are necessary to control these self-reactive T cells. One of these mechanisms involves innate immunity, namely natural killer cells (Gross et al. 2016). NK cells have been shown to suppress activated T cells through secretion of anti-inflammatory cytokines (Morandi et al. 2008; Moretta et al. 2008) and/or through their cytolytic function (Shi et al. 2006). Killer cell immunoglobulin-like receptors represent one of the human natural killer cell receptor families that recognize MHC class I molecules as their ligands. Inhibitory and activating receptors, and their HLA ligands, determine NK responsiveness (Bashirova et al. 2006; Parham et al. 2013). **In paper 2** we investigated the influence of KIR genes, and their HLA class I ligands, on MS susceptibility. A negative association between the activating KIR2DS1 gene and MS, independently from the presence of the HLA-DRB1*15 allele, was observed. This result is in accordance with the literature (Shahsavar et al. 2016). The decreased risk for the development of MS associated with KIR2DS1 may be due to activation of NK cells and consequent inhibition of autoreactive T cells, either by lysing autologous DC or by lysing autologous T cells, in both cases silencing antigen specific responses. Additionally, neurotrophic growth factors secreted by activated NK cells may contribute to disease protection. Interestingly, this immunological mechanism of protection was observed in the treatment of MS with Daclizumab (anti-CD25 depleting monoclonal antibodies). The originally presumed mechanism of action of Daclizumab was that the blockade of CD25 (IL-2Rα) would inhibit the proliferation of the recently activated T cells (Martin 2012). However, anti-CD25 treatment reduced only moderately the number of CD4 and CD8 T cells. Nonetheless, a marked increase in NK cells, especially $CD56^{bright}$ (regulatory NK cells), was unexpectedly observed (Bielekova et al. 2009). These $CD56^{bright}$ NK cells are able to

inhibit CD4 T cell responses, although the exact mechanism is currently unknown (Bielekova et al. 2006).

The failure of central and peripheral tolerance, responsible for the immune deregulation observed in MS, may play a role in the pathogenesis of the disease; it courses with an inflammatory process and with oxidative stress, which also promotes tissue damage. Recent data point to an important role of anti-oxidative pathways for tissue protection in chronic MS, particularly involving the transcription factor Nrf2. As already stressed, Nrf2 is a central transcription factor for the antioxidant response (Itoh et al. 1997; Sykiotis et al. 2010). In response to alterations in cellular redox status Nrf2 binds to antioxidant response elements in the promoters of oxidative-stress regulated genes, and induces the expression of a battery of antioxidant and detoxifying genes, molecular chaperones and proteasome subunits (Sykiotis et al. 2010), with the objective of restoring redox homeostasis. This is the key cellular mechanism for defence against oxidative damage (Sykiotis et al. 2010). It has also become apparent that Nrf2 can negatively regulate many pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules, cyclooxygenase-2 and inducible nitric oxide synthase (Kim et al. 2010). Also, Nrf2 mediates inhibition of NF-KB activity, downregulating the expression of pro-inflammatory genes in innate immune cells (Thimmulappa et al. 2006; Kim et al. 2010). In **paper 3,** the association of two functional SNPs in the promoter region of the Nrf2 gene (-653A/G and -617C/A) with MS susceptibility, disease forms and progression was investigated. The -653A/G SNP was associated with disease presentation. A significantly higher frequency of the GG genotype among patients with RRMS was observed, even when adjusted for age at onset, gender, presence of HLA-DRB1*15, disease duration, EDSS and MSSS. Marzec et al reported that Nrf2 activity was low either in -653G or in -617A alleles carriers (Marzec et al. 2007). Our results show that patients with the -653GG genotype are more prone to develop a RRMS course, in which inflammation seems to have a pivotal role. This observation can be explained by the fact that this genotype is associated with low Nrf2 expression, and Nrf2 is a fundamental player in the control of inflammation. Multiple sclerosis has an inflammatory and a neurodegenerative phase that is reflected in its clinical course (RRMS vs. Progressive MS). Different theories try to explain how these two phases occur. One suggests that brain damage is driven by inflammatory processes similar to those observed during RRMS; however during the progressive disease stage, a microenvironment is created within the CNS that favours homing and retention of inflammatory cells (Frischer et al. 2009). A second possibility is that Multiple Sclerosis starts out as an inflammatory disease, but after several years, a neurodegenerative process, independent of inflammatory responses, becomes the key

mechanism responsible for disease progression (Meuth et al. 2008). Finally, MS could primarily be a neurodegenerative disease, with inflammation occurring as a secondary response, amplifying progressive states (Barnett et al. 2004; Kassmann et al. 2007).

The emerging role of miRNAs in innate and adaptive immunity strongly suggests an association with regulation of inflammatory diseases (O'Connell et al. 2012). MicroRNAs are a class of endogenous small non-coding RNAs (Lytle et al. 2007), that play a critical role in biological processes such as cellular proliferation and differentiation, development and apoptosis (Ebert et al. 2012). In recent years, a lot of attention has been drawn toward the identification of diagnostic, prognostic, process-specific, and treatment-related biomarkers for MS. The use of miRNAs as biomarkers in MS is still evolving. The few studies that are published to date have used either whole blood, peripheral blood mononuclear cells, plasma, serum, cerebrospinal fluid, T cells, or affected tissues for miRNA expression analysis (Gandhi 2015). The stable expression of miRNAs in serum and plasma makes them an important biomarker candidate. In this thesis (**paper 4**) we analysed the expression levels of circulating miRNA-155 in the serum of MS patients, and found that miR-155 expression was 13 fold higher in MS patients relative to controls. Deregulated miRNA levels in biological fluids could represent a new source of biomarkers in MS that could be helpful for disease prognosis and for discrimination of clinical subtype (Keller et al. 2009), thereby helping therapeutic decisions or monitoring of therapeutic effects. Also, miRNAs could themselves constitute new therapeutic targets (Zare-Shahabadi et al. 2013; Aslani et al. 2017). For example, several lines of evidence have shown that the suppression of miR-155 inhibit the development of Th1 and Th17 cells (Zhang et al. 2014). Supporting these observations, anti-miR-155 treatment was reported to inhibit EAE development in mice (Murugaiyan et al. 2011). MicroRNAs regulate gene expression at the post-transcriptional level. Experimentally validating the target genes of these miRNA will help to further understand the underlying mechanisms of disease, and this will allow us to further understand the pathogenesis of MS. For example, it would be interesting to assess whether miRNAs affect the expression of genes associated with an increased risk for MS development (e.g. HLA-DRB1*15).

4.1.2 – Vitamin D

The influence of migration (Cabre et al. 2005) and latitude (Simpson et al. 2011) on MS prevalence strongly suggests a role for the environment in disease susceptibility. It is known that several environmental factors are capable of causing MS in a genetically susceptible individual. In this thesis we evaluated in more detail vitamin D, which has been proposed to be an important environmental risk factor for MS development (Pierrot-Deseilligny et al. 2010).

It is widely accepted that vitamin D has pleiotropic properties affecting the regulation of immune responses. If we assume that vitamin D status is associated with MS risk the literature shows that the risk may start as early as in the prenatal period. In fact, the majority of MS patients from studies in the Northern hemisphere were born in April or May, just after the very low winter levels of vitamin D (Templer et al. 1992; Willer et al. 2005; Bayes et al. 2010; Torkildsen et al. 2012; Dobson et al. 2013; Grytten et al. 2013). This suggests that exposure to vitamin D insufficiency in utero could be relevant for the risk of MS development. In the Southern hemisphere, a reverse pattern was detected, with an excess of births in November–December and a decrease in May–June (Staples et al. 2010). Like in most studies we also found an incidence peak at the end of winter (February) and less MS births than expected in the autumn, with the lowest point being in October **(paper 5)**. Our results are not consistent with a previous study from the Portuguese MS population (Barros et al. 2013), where a group of 421 MS patients, also from the North of Portugal, was studied. Some of the discordant results between the positive and negative studies in the literature can be explained by the fact that, while the month of birth effect is more prominent in high-risk areas for MS, especially in areas with low sunlight exposure (north hemisphere), this effect seems to be negligible or non-existent in areas with high sunlight exposure (Torkildsen et al. 2012; Grytten et al. 2013). Also, Fiddes et al. stated that, in the absence of adequate control for confounding factors, such as year of birth and place of birth, the reported associations of MS with month of birth are probably false positives (Fiddes et al. 2013). In our study we took those confounding factors into account and we only included control statistics from the same districts and with the same years of birth as those of our patients. The association of the month of birth with the risk of developing MS is relatively consistent; however a correlation with disease phenotype is more controversial. In our study we didn't find any association between MS birth and age at onset. Interestingly, the median MSSS also has a peak in spring months and the lowest point in October; however the difference was not statistically significant. This is in agreement with Koch et al (Koch et al. 2008) and with a more recent study from Italy that also found no association between month of birth and

MS progression (Lucenti et al. 2014). As a conclusion we can say that MS seems to be more frequent in individuals born in January-June but birth month does not seem to influence disease progression.

Winter levels of circulating vitamin D in pregnant women and newborns are low (Newhook et al. 2009), and low concentrations of neonatal vitamin D are associated with an increased risk of MS (Munger et al. 2016; Nielsen et al. 2017). These observations suggest a role for the intrauterine environment in disease susceptibility. Experimental data on animal fetal development suggest that low maternal vitamin D has important implications for the developing brain of the fetus (Harms et al. 2011). Compelling data from in vitro experiments and animal models implicates vitamin D in brain proliferation, differentiation, neurotropism, neuroprotection, neurotransmission, myelination, and neuroplasticity (DeLuca et al. 2013). Furthermore, vitamin D also helps tune the fetal immune system by suppressing inflammatory cytokines and promoting self-tolerance (Smolders et al. 2008a). Data have emerged demonstrating that vitamin D supplementation during pregnancy alters transcriptome, and epigenetic modifications through DNA methylation in genes that regulate metabolic processes, antigen processing, inflammation, regulation of cell death, cell proliferation, transmission of nerve impulse, neurogenesis, neuron differentiation and sensory organ development is also seen (Hollis *et al*. 2017; Wagner *et al*. 2017).

Concerning vitamin D levels, several studies have reported inadequacy of 25-hydroxyvitamin D all over Europe (Pludowski et al. 2014; Cashman et al. 2016; Quraishi et al. 2016). In Portugal, no epidemiological studies of vitamin D deficiency in adult healthy individuals existed so far. In **paper 6**, a cohort of 198 participants (18-67 years) living in the north of Portugal, Porto was investigated. In this study we observed that almost half of the studied population (48%) presented serum 25(OH)D levels suggestive of deficiency, reaching 74% in winter, reflecting the well-known 25(OH)D seasonal fluctuation (Maxwell 1994). Also, no association between 25(OH)D levels and gender or age was observed. There is compelling evidence indicating that lower levels of vitamin D are associated with increased risk of MS. Lower serum 25(OH)D levels were observed in Portuguese MS patients, including in patients with a recent disease onset (**paper 7**). These results are in agreement with several previous studies of other populations (Gelfand et al. 2011; Hatamian et al. 2013; Kirbas et al. 2013; Mazdeh et al. 2013; Niino et al. 2015; Behrens et al. 2016; Brola et al. 2016; Karampoor et al. 2016; Zhang et al. 2016). More than half of our patients (66%) presented 25(OH)D deficiency compared to 48% in the healthy controls. Restricted mobility, due to advanced MS-related disability, may limit sunlight exposure, and could confound the efforts of establishing causality

between vitamin D deficiency and disease susceptibility or outcome, as the levels of vitamin D are rarely known before disease onset and diagnosis. In this setting, a sub-group of patients with recent disease onset was studied, demonstrating 25(OH)D deficits already at this early stage of the disease. This may have a particular value and is in accordance with several data from other studies that described decreased levels of vitamin D in new diagnosed patients (Kirbas et al. 2013; Mazdeh et al. 2013). Accordingly, Behrens et al. showed that “clinically hardly affected patients”, in the earliest phases of MS, also present low 25(OH)D levels. (Behrens et al. 2016).

The immunomodulatory effects of vitamin D give support to the hypothesis that a higher vitamin D status could prevent relapses in RRMS and reduce MS disability progression. Nevertheless, there are contradictory evidences regarding whether vitamin D insufficiency has an adverse effect on MS outcomes. Longitudinal studies have shown a relationship between low serum vitamin D levels and disease activity - as observed on magnetic resonance imaging (Loken-Amsrud et al. 2012; Mowry et al. 2012; Fitzgerald et al. 2015), a higher relapse risk (Simpson et al. 2010; Runia et al. 2012) and increased disability progression (Ascherio et al. 2014; Thouvenot et al. 2015). In our study low vitamin D levels were associated with higher disability, assessed by EDSS and by MSSS, which is in accordance with several studies already published (van der Mei et al. 2007; Smolders et al. 2008b; Thouvenot et al. 2015; Zhang et al. 2016). Nevertheless, this association was not confirmed in other populations (Yildiz et al. 2011; Fitzgerald et al. 2015; Muris et al. 2015; Becker et al. 2016). Some of the strengths of our study are the inclusion of a relatively large group of patients with MS and controls, and the consideration of seasonal variation. It should be noted that 25(OH)D levels may not reflect its true biological activity, as downstream events in the vitamin D signalling, or other factors that could modulate vitamin D bioavailability were not taken into account. In conclusion, vitamin D levels in patients with MS were significantly lower than in healthy subjects, also a significant association was found between vitamin D level and disability.

In the present thesis, evidence has been obtained to support a causal link between vitamin D insufficiency and MS. So, an important question that can be made is whether provision of vitamin D would modify MS disease activity or risk. A debate related to the optimal levels of 25(OH)D is ongoing (Whiting et al. 2005; Aloia et al. 2008). Available evidence suggests that children and adults should maintain a blood level of 25(OH)D above 50 nmol/L to prevent rickets and osteomalacia, respectively. To maximize vitamin D effect on calcium, bone, and muscle metabolism, the 25(OH)D blood level should be above 75 nmol/L. Numerous epidemiological studies have suggested that a 25(OH)D blood level above 75 nmol/L may have additional health benefits in reducing the risk of

common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases (Holick et al. 2011). Consideration of vitamin D as a therapeutic agent for established MS will require further studies on dose and efficacy (Dorr et al. 2012). Apart from the potential disease-modifying effects of vitamin D, there is a good rationale to encourage vitamin D supplementation for MS patients: low 25(OH)D levels are observed in patients, and many of them have low bone mineral density, with increased risk of fracture and other multiple risk factors for osteoporosis.

As stated before, calcitriol initiates its signalling cascade by binding to the vitamin D receptor (Haussler et al. 2013). Results of previous VDR gene association studies in MS are conflicting (Fukazawa et al. 1999; Niino et al. 2000; Steckley et al. 2000; Partridge et al. 2004; Yeo et al. 2004; Tajouri et al. 2005; 2009; Smolders et al. 2009b; Smolders et al. 2009a). These may be due to clinical heterogeneity, differences in ethnicity, geographical factors, interactions with other genetic or environmental factors and/or low statistical power. Smolders et al (Smolders et al. 2009b) hypothesized that an association of VDR polymorphisms with MS might only be detected in a population with a sufficient vitamin D status, meaning that these polymorphisms are likely to influence the response of vitamin D metabolism only in the presence of sufficient amounts of vitamin D, which could also explain some of the reported negative results. In **paper 8** we investigated the association between MS and the VDR FokI polymorphism, in both disease development and progression, in a Portuguese population. An association between FokI ff genotype and MS susceptibility was found, but no associations with disease form or progression were observed. This is in agreement with a study from Cierny and colleagues that also did not find significant association between this SNP and the rate of disease disability progression (Cierny et al. 2015). One strength of our study is the relatively large sample size of both populations. It has also some limitations, namely only one polymorphism of the VDR gene was studied, and we cannot exclude that other genetic variants of the VDR gene could also be associated with MS susceptibility. Further clinical and experimental studies on vitamin D metabolites and MS should take the FokI VDR polymorphism into account (Smolders et al. 2009b). Assessment of this and other SNPs of the VDR gene could assist in the identification of individuals at risk of vitamin D insufficiency and may also be useful to adjust treatment in patients with an insufficient response to vitamin D supplementation (Herrmann et al. 2017).

Vitamin D receptor is a nuclear transcription factor that binds to VDREs in target genes. A genetic study in humans showed a direct functional interaction of VDR with the major gene (HLA-DRB1*15) that determines susceptibility to Multiple Sclerosis (Ramagopalan

et al. 2009; Berlanga-Taylor et al. 2011). Taking into consideration that vitamin D, or its metabolites, alters the levels of some miRNAs, and canonical VDR-mediated regulation of miRNAs via VDREs has been demonstrated for several different miRNAs, vitamin D can regulate the transcription of miRNA genes. This can be achieved through VDR binding to VDREs in the promoter of target miRNA genes, or indirectly through regulating genes involved in miRNA processing or stability (Giangreco et al. 2013). Also, there are suggestions that vitamin D not only increases specific miRNAs, but up-regulates miRNAs expression globally, by VDR-dependent chromatin opening and increased pri-miRNA expression (Giangreco et al. 2013).

4.2 – Conclusions and Future Perspectives

Multiple Sclerosis seems to occur in genetically predisposed individuals who are exposed to certain environmental factors, especially during childhood (Ascherio et al. 2012). In this thesis, besides confirming the well-known association with HLA-DRB1*15, we have shown that the KIR2DS1 allele is a protective factor for the development of MS. As already stressed, genetic factors only have a small effect on MS susceptibility, documented by the 60% discordance observed in monozygotic twins. However, the growing number of known risk genes (>100) and gene-gene interaction will lead us to a better understanding of the pathogenesis of MS. The main inference that can be made from genetic studies so far has been the recognition that most of the identified risk loci are involved in immune function.

Although genetic influence on the risk of MS is well established, less is known about gene associations with MS disease course and/or severity. Evidence that MS severity or disease course is altered by HLA or non-HLA alleles is controversial (Hauser et al. 2000; Barcellos et al. 2003; Jensen et al. 2010; Lundstrom et al. 2011). A recent study confirms and extends previous observations linking HLA MS susceptibility alleles with disease progression and specific clinical and MRI phenotypic traits (Isobe et al. 2016). In this thesis we observed that the Nrf2 -653GG genotype is a genetic marker for the development of a relapse-remitting course of the disease. This knowledge could be of potential interest for a better understanding of the mechanisms driving disease progression, contributing in this way for the development of novel therapeutic approaches.

Multiple epidemiological studies have highlighted the role of environmental factors on the prevalence of MS and demonstrated that vitamin D deficiency play a significant role in the initiation of the disease. With this thesis we have observed that vitamin D deficiency

is prevalent in the North of Portugal, affecting almost half of the adult healthy population and even more in MS patients. An effective strategy to prevent vitamin D deficiency and insufficiency should be envisaged and implemented. This will be particularly important for individuals who have family history of MS and are at increased risk of developing the disease. The evaluation of other environmental factors, including sodium intake, BMI during adolescence, alcohol and coffee consumption, and the gut microbiota is underway (Olsson et al. 2017). Several studies were set out to identify the differential abundance of intestinal bacteria between individuals with MS and healthy controls and to find a common MS microbiota signature (Miyake et al. 2015; Chen et al. 2016; Jangi et al. 2016; Tremlett et al. 2016). Potential immunopathogenic links between the gut microbiota and MS emphasize the need for further systematic studies in this emerging field (Budhram et al. 2017).

Studying each risk factor separately is, in our opinion, not likely to bring fundamental changes in the understanding of MS pathogenesis. The interaction dynamics between vitamin D genes, other immune-related MS susceptibility genes, and environmental factors could contribute to elucidate the understanding of MS mechanisms (Correale et al. 2009). Future studies should combine genetic, environmental, and clinical assessments (Olsson et al. 2017).

It is believed nowadays that environmental factors can break tolerance through post-translational modifications triggering a range of immune responses (Selmi et al. 2012). Epigenetic phenomena are remarkably important for controlling the patterns of gene expression during normal physiological functions, like cell cycle and development, as well as in response to environmental factors providing an explanation for the link observed between these risk factors and the development of autoimmune diseases (Grolleau-Julius et al. 2010). Epigenetic mechanisms regulate transcription of the majority of the genes associated with MS onset and perpetuation (Aslani et al. 2017). In this thesis microRNA-155 expression levels were overexpressed in MS patients, which confirm the existence of a chronic inflammatory state. Other immune relevant microRNAs may also be involved in this process, and the influence of other epigenetic mechanisms, like DNA methylation, can’t be rule out.

Concluding remarks

Our understanding of the (immuno) pathogenesis and genetics of MS has considerably improved in the last years. Several large scale studies are now underway aiming to unravel the complex genetic architecture of the human disease. These projects will accelerate the discovery of disease-associated genetic variants. Increasing technological possibilities, large scale studies assessing the transcriptome, metabolome, proteome and the human microbiome will enable the identification of anomalous pathways and interactions between genes and gene-environment interactions (Bhargava et al. 2016; Del Boccio et al. 2016; Villoslada et al. 2017). A large number of new drugs for MS are in the therapeutic pipeline, all of them modulating the immune system. This would not have been possible without the contribution of the recent advances in the knowledge of the role of genes and gene targets in Multiple Sclerosis. One of the goals from all on-going research efforts is the development of new tools to understand the underlying mechanisms of neurodegeneration and the subsequent development of drugs targeting the degenerative component of MS.

References

(2009). "Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20." Nat Genet **41**(7): 824-828.

Aloia, J. F., M. Patel, R. Dimaano, et al. (2008). "Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration." Am J Clin Nutr **87**(6): 1952-1958.

Ascherio, A., K. L. Munger and J. D. Lunemann (2012). "The initiation and prevention of multiple sclerosis." Nat Rev Neurol **8**(11): 602-612.

Ascherio, A., K. L. Munger, R. White, et al. (2014). "Vitamin D as an early predictor of multiple sclerosis activity and progression." JAMA Neurol **71**(3): 306-314.

Aslani, S., N. Jafari, M. R. Javan, et al. (2017). "Epigenetic Modifications and Therapy in Multiple Sclerosis." Neuromolecular Med **19**(1): 11-23.

Barcellos, L. F., J. R. Oksenberg, A. B. Begovich, et al. (2003). "HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course." Am J Hum Genet **72**(3): 710-716.

Barnett, M. H. and J. W. Prineas (2004). "Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion." Ann Neurol **55**(4): 458-468.

Barros, P., J. M. de Sa and M. J. Sa (2013). "Month of birth and risk of multiple sclerosis in a Portuguese population." Clin Neurol Neurosurg **115**(9): 1762-1765.

Bashirova, A. A., M. P. Martin, D. W. McVicar, et al. (2006). "The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense." Annu Rev Genomics Hum Genet **7**: 277-300.

Bayes, H. K., C. J. Weir and C. O'Leary (2010). "Timing of birth and risk of multiple sclerosis in the Scottish population." Eur Neurol **63**(1): 36-40.

Becker, J., D. Callegaro, M. A. Lana-Peixoto, et al. (2016). "Hypovitaminosis D association with disease activity in relapsing remitting multiple sclerosis in Brazil." J Neurol Sci **363**: 236-239.

Behrens, J. R., L. Rasche, R. M. Giess, et al. (2016). "Low 25-hydroxyvitamin D, but not the bioavailable fraction of 25-hydroxyvitamin D, is a risk factor for multiple sclerosis." Eur J Neurol **23**(1): 62-67.

Berlanga-Taylor, A. J., G. Disanto, G. C. Ebers, et al. (2011). "Vitamin D-gene interactions in multiple sclerosis." J Neurol Sci **311**(1-2): 32-36.

Bettencourt, A., C. Carvalho, B. Leal, et al. (2015). "The Protective Role of HLA-DRB1(*)13 in Autoimmune Diseases." J Immunol Res **2015**: 948723.

Bettencourt, A., A. Martins da Silva, E. C. P. Pinho, et al. (2012). "Molecular genetic studies of multiple sclerosis in the portuguese population." Acta Med Port **25**(4): 224-230.

Bhargava, P. and P. A. Calabresi (2016). "Metabolomics in multiple sclerosis." Mult Scler **22**(4): 451-460.

Bielekova, B., M. Catalfamo, S. Reichert-Scrivner, et al. (2006). "Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis." Proc Natl Acad Sci U S A **103**(15): 5941-5946.

Bielekova, B., T. Howard, A. N. Packer, et al. (2009). "Effect of anti-CD25 antibody daclizumab in the inhibition of inflammation and stabilization of disease progression in multiple sclerosis." Arch Neurol **66**(4): 483-489.

Brola, W., P. Sobolewski, W. Szczuchniak, et al. (2016). "Association of seasonal serum 25-hydroxyvitamin D levels with disability and relapses in relapsing-remitting multiple sclerosis." Eur J Clin Nutr **70**(9): 995-999.

Browne, P., D. Chandraratna, C. Angood, et al. (2014). "Atlas of Multiple Sclerosis 2013: A growing global problem with widespread inequity." Neurology **83**(11): 1022-1024.

Brynedal, B., K. Duvefelt, G. Jonasdottir, et al. (2007). "HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis." PLoS ONE **2**(7): e664.

Budhram, A., S. Parvathy, M. Kremenchutzky, et al. (2017). "Breaking down the gut microbiome composition in multiple sclerosis." Mult Scler **23**(5): 628-636.

Cabre, P., A. Signate, S. Olindo, et al. (2005). "Role of return migration in the emergence of multiple sclerosis in the French West Indies." Brain **128**(Pt 12): 2899-2910.

Cashman, K. D., K. G. Dowling, Z. Skrabakova, et al. (2016). "Vitamin D deficiency in Europe: pandemic?" Am J Clin Nutr **103**(4): 1033-1044.

Chen, J., N. Chia, K. R. Kalari, et al. (2016). "Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls." Sci Rep **6**: 28484.

Cierny, D., J. Michalik, E. Kurca, et al. (2015). "FokI vitamin D receptor gene polymorphism in association with multiple sclerosis risk and disability progression in Slovaks." Neurol Res **37**(4): 301-308.

Correale, J., M. C. Ysrraelit and M. I. Gaitan (2009). "Immunomodulatory effects of Vitamin D in multiple sclerosis." Brain **132**(Pt 5): 1146-1160.

Del Boccio, P., C. Rossi, M. di Ioia, et al. (2016). "Integration of metabolomics and proteomics in multiple sclerosis: From biomarkers discovery to personalized medicine." Proteomics Clin Appl **10**(4): 470-484.

DeLuca, G. C., S. M. Kimball, J. Kolasinski, et al. (2013). "Review: the role of vitamin D in nervous system health and disease." Neuropathol Appl Neurobiol **39**(5): 458-484.

Dobson, R., G. Giovannoni and S. Ramagopalan (2013). "The month of birth effect in multiple sclerosis: systematic review, meta-analysis and effect of latitude." J Neurol Neurosurg Psychiatry **84**(4): 427-432.

Dorr, J., S. Ohlraun, H. Skarabis, et al. (2012). "Efficacy of vitamin D supplementation in multiple sclerosis (EVIDIMS Trial): study protocol for a randomized controlled trial." Trials **13**: 15.

Ebert, M. S. and P. A. Sharp (2012). "Roles for microRNAs in conferring robustness to biological processes." Cell **149**(3): 515-524.

Fiddes, B., J. Wason, A. Kemppinen, et al. (2013). "Confounding underlies the apparent month of birth effect in multiple sclerosis." Ann Neurol **73**(6): 714-720.

Fitzgerald, K. C., K. L. Munger, K. Kochert, et al. (2015). "Association of Vitamin D Levels With Multiple Sclerosis Activity and Progression in Patients Receiving Interferon Beta-1b." JAMA Neurol **72**(12): 1458-1465.

Frischer, J. M., S. Bramow, A. Dal-Bianco, et al. (2009). "The relation between inflammation and neurodegeneration in multiple sclerosis brains." Brain **132**(Pt 5): 1175-1189.

Fugger, L., M. A. Friese and J. I. Bell (2009). "From genes to function: the next challenge to understanding multiple sclerosis." Nat Rev Immunol **9**(6): 408-417.

Fukazawa, T., I. Yabe, S. Kikuchi, et al. (1999). "Association of vitamin D receptor gene polymorphism with multiple sclerosis in Japanese." J Neurol Sci **166**(1): 47-52.

Gandhi, R. (2015). "miRNA in multiple sclerosis: search for novel biomarkers." Mult Scler.

Gelfand, J. M., B. A. Cree, J. McElroy, et al. (2011). "Vitamin D in African Americans with multiple sclerosis." Neurology **76**(21): 1824-1830.

Giangreco, A. A. and L. Nonn (2013). "The sum of many small changes: microRNAs are specifically and potentially globally altered by vitamin D3 metabolites." J Steroid Biochem Mol Biol **136**: 86-93.

Grolleau-Julius, A., D. Ray and R. L. Yung (2010). "The role of epigenetics in aging and autoimmunity." Clin Rev Allergy Immunol **39**(1): 42-50.

Gross, C. C., A. Schulte-Mecklenbeck, H. Wiendl, et al. (2016). "Regulatory Functions of Natural Killer Cells in Multiple Sclerosis." Front Immunol **7**: 606.

Grytten, N., O. Torkildsen, J. H. Aarseth, et al. (2013). "Month of birth as a latitude-dependent risk factor for multiple sclerosis in Norway." Mult Scler **19**(8): 1028-1034.

Harms, L. R., T. H. Burne, D. W. Eyles, et al. (2011). "Vitamin D and the brain." Best Pract Res Clin Endocrinol Metab **25**(4): 657-669.

Hatamian, H., E. Bidabadi, S. M. Seyed Saadat, et al. (2013). "Is serum vitamin D levels associated with disability in patients with newly diagnosed multiple sclerosis?" Iran J Neurol **12**(2): 41-46.

Hauser, S. L., J. R. Oksenberg, R. Lincoln, et al. (2000). "Interaction between HLA-DR2 and abnormal brain MRI in optic neuritis and early MS." Am J Ophthalmol **130**(5): 690-691.

Haussler, M. R., G. K. Whitfield, I. Kaneko, et al. (2013). "Molecular mechanisms of vitamin D action." Calcif Tissue Int **92**(2): 77-98.

Herrmann, M., C. L. Farrell, I. Pusceddu, et al. (2017). "Assessment of vitamin D status - a changing landscape." Clin Chem Lab Med **55**(1): 3-26.

Holick, M. F., N. C. Binkley, H. A. Bischoff-Ferrari, et al. (2011). "Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline." J Clin Endocrinol Metab **96**(7): 1911-1930.

Hollis, B. W. and C. L. Wagner (2017). "Vitamin D supplementation during pregnancy: Improvements in birth outcomes and complications through direct genomic alteration." Mol Cell Endocrinol.

Isobe, N., A. Keshavan, P. A. Gourraud, et al. (2016). "Association of HLA Genetic Risk Burden With Disease Phenotypes in Multiple Sclerosis." JAMA Neurol **73**(7): 795-802.

Itoh, K., T. Chiba, S. Takahashi, et al. (1997). "An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements." Biochem Biophys Res Commun **236**(2): 313-322.

Jangi, S., R. Gandhi, L. M. Cox, et al. (2016). "Alterations of the human gut microbiome in multiple sclerosis." Nat Commun **7**: 12015.

Jensen, C. J., J. Stankovich, A. Van der Walt, et al. (2010). "Multiple sclerosis susceptibility-associated SNPs do not influence disease severity measures in a cohort of Australian MS patients." PLoS One **5**(4): e10003.

Karampoor, S., H. Zahednasab, S. Ramagopalan, et al. (2016). "25-hydroxyvitamin D levels are associated with multiple sclerosis in Iran: A cross-sectional study." J Neuroimmunol **290**: 47-48.

Kassmann, C. M., C. Lappe-Siefke, M. Baes, et al. (2007). "Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes." Nat Genet **39**(8): 969-976.

Keller, A., P. Leidinger, J. Lange, et al. (2009). "Multiple sclerosis: microRNA expression profiles accurately differentiate patients with relapsing-remitting disease from healthy controls." PLoS One **4**(10): e7440.

Kim, J., Y. N. Cha and Y. J. Surh (2010). "A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders." Mutat Res **690**(1-2): 12-23.

Kirbas, A., S. Kirbas, O. Anlar, et al. (2013). "Investigation of the relationship between vitamin D and bone mineral density in newly diagnosed multiple sclerosis." Acta Neurol Belg **113**(1): 43-47.

Koch, M., J. De Keyser and H. Tremlett (2008). "Timing of birth and disease progression in multiple sclerosis." Mult Scler **14**(6): 793-798.

Loken-Amsrud, K. I., T. Holmoy, S. J. Bakke, et al. (2012). "Vitamin D and disease activity in multiple sclerosis before and during interferon-beta treatment." Neurology **79**(3): 267-273.

Lucenti, A., S. Galimberti, N. Barizzone, et al. (2014). "Multiple sclerosis progression is not associated with birth timing in Italy." J Neurol Sci **346**(1-2): 194-196.

Lundstrom, W., E. Greiner, F. Lundmark, et al. (2011). "No influence on disease progression of non-HLA susceptibility genes in MS." J Neuroimmunol **237**(1-2): 98-100.

Lytle, J. R., T. A. Yario and J. A. Steitz (2007). "Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR." Proc Natl Acad Sci U S A **104**(23): 9667-9672.

Martin, R. (2012). "Anti-CD25 (daclizumab) monoclonal antibody therapy in relapsing-remitting multiple sclerosis." Clin Immunol **142**(1): 9-14.

Martin, R., M. Sospedra, M. Rosito, et al. (2016). "Current multiple sclerosis treatments have improved our understanding of MS autoimmune pathogenesis." Eur J Immunol **46**(9): 2078-2090.

Marzec, J. M., J. D. Christie, S. P. Reddy, et al. (2007). "Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury." FASEB J **21**(9): 2237-2246.

Maxwell, J. D. (1994). "Seasonal variation in vitamin D." Proc Nutr Soc **53**(3): 533-543.

Mazdeh, M., S. Seifirad, N. Kazemi, et al. (2013). "Comparison of vitamin D3 serum levels in new diagnosed patients with multiple sclerosis versus their healthy relatives." Acta Med Iran **51**(5): 289-292.

Meuth, S. G., O. J. Simon, A. Grimm, et al. (2008). "CNS inflammation and neuronal degeneration is aggravated by impaired CD200-CD200R-mediated macrophage silencing." J Neuroimmunol **194**(1-2): 62-69.

Miyake, S., S. Kim, W. Suda, et al. (2015). "Dysbiosis in the Gut Microbiota of Patients with Multiple Sclerosis, with a Striking Depletion of Species Belonging to Clostridia XIVa and IV Clusters." PLoS One **10**(9): e0137429.

Morandi, B., P. Bramanti, I. Bonaccorsi, et al. (2008). "Role of natural killer cells in the pathogenesis and progression of multiple sclerosis." Pharmacol Res **57**(1): 1-5.

Moretta, A., E. Marcenaro, S. Parolini, et al. (2008). "NK cells at the interface between innate and adaptive immunity." Cell Death Differ **15**(2): 226-233.

Moutsianas, L., L. Jostins, A. H. Beecham, et al. (2015). "Class II HLA interactions modulate genetic risk for multiple sclerosis." Nat Genet **47**(10): 1107-1113.

Mowry, E. M., E. Waubant, C. E. McCulloch, et al. (2012). "Vitamin D status predicts new brain magnetic resonance imaging activity in multiple sclerosis." Ann Neurol **72**(2): 234-240.

Munger, K. L., J. Aivo, K. Hongell, et al. (2016). "Vitamin D Status During Pregnancy and Risk of Multiple Sclerosis in Offspring of Women in the Finnish Maternity Cohort." JAMA Neurol **73**(5): 515-519.

Muris, A. H., L. Rolf, K. Broen, et al. (2015). "A low vitamin D status at diagnosis is associated with an early conversion to secondary progressive multiple sclerosis." J Steroid Biochem Mol Biol.

Murugaiyan, G., V. Beynon, A. Mittal, et al. (2011). "Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis." J Immunol **187**(5): 2213-2221.

Newhook, L. A., S. Sloka, M. Grant, et al. (2009). "Vitamin D insufficiency common in newborns, children and pregnant women living in Newfoundland and Labrador, Canada." Matern Child Nutr **5**(2): 186-191.

Nielsen, N. M., K. L. Munger, N. Koch-Henriksen, et al. (2017). "Neonatal vitamin D status and risk of multiple sclerosis: A population-based case-control study." Neurology **88**(1): 44-51.

Niino, M., T. Fukazawa, I. Yabe, et al. (2000). "Vitamin D receptor gene polymorphism in multiple sclerosis and the association with HLA class II alleles." J Neurol Sci **177**(1): 65-71.

Niino, M., S. Sato, T. Fukazawa, et al. (2015). "Decreased serum vitamin D levels in Japanese patients with multiple sclerosis." J Neuroimmunol **279**: 40-45.

O'Connell, R. M., D. S. Rao and D. Baltimore (2012). "microRNA regulation of inflammatory responses." Annu Rev Immunol **30**: 295-312.

Olsson, T., L. F. Barcellos and L. Alfredsson (2017). "Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis." Nat Rev Neurol **13**(1): 25-36.

Parham, P. and A. Moffett (2013). "Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution." Nat Rev Immunol **13**(2): 133-144.

Partridge, J. M., S. J. Weatherby, J. A. Woolmore, et al. (2004). "Susceptibility and outcome in MS: associations with polymorphisms in pigmentation-related genes." Neurology **62**(12): 2323-2325.

Pierrot-Deseilligny, C. and J. C. Souberbielle (2010). "Is hypovitaminosis D one of the environmental risk factors for multiple sclerosis?" Brain **133**(Pt 7): 1869-1888.

Pludowski, P., W. B. Grant, H. P. Bhattoa, et al. (2014). "Vitamin d status in central europe." Int J Endocrinol **2014**: 589587.

Quraishi, S. A., C. A. Camargo, Jr. and J. E. Manson (2016). "Low vitamin D status in Europe: moving from evidence to sound public health policies." Am J Clin Nutr **103**(4): 957-958.

Ramagopalan, S. V., N. J. Maugeri, L. Handunnetthi, et al. (2009). "Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D." PLoS Genet **5**(2): e1000369.

Ransohoff, R. M., D. A. Hafler and C. F. Lucchinetti (2015). "Multiple sclerosis-a quiet revolution." Nat Rev Neurol **11**(3): 134-142.

Runia, T. F., W. C. Hop, Y. B. de Rijke, et al. (2012). "Lower serum vitamin D levels are associated with a higher relapse risk in multiple sclerosis." Neurology **79**(3): 261-266.

Schaeffer, J., C. Cossetti, G. Mallucci, et al. (2015). Chapter 30 - Multiple Sclerosis A2 - Zigmond, Michael J. Neurobiology of Brain Disorders. L. P. Rowland and J. T. Coyle. San Diego, Academic Press**:** 497-520.

Selmi, C., P. S. Leung, D. H. Sherr, et al. (2012). "Mechanisms of environmental influence on human autoimmunity: a National Institute of Environmental Health Sciences expert panel workshop." J Autoimmun **39**(4): 272-284.

Shahsavar, F., S. Mapar and S. A. Ahmadi (2016). "Multiple sclerosis is accompanied by lack of KIR2DS1 gene: A meta-analysis." Genom Data **10**: 75-78.

Shi, F. D. and L. Van Kaer (2006). "Reciprocal regulation between natural killer cells and autoreactive T cells." Nat Rev Immunol **6**(10): 751-760.

Silva, A. M., A. Bettencourt, C. Pereira, et al. (2009). "Protective role of the HLA-A*02 allele in Portuguese patients with multiple sclerosis." Mult Scler **15**(6): 771-774.

Silva, A. M., C. Pereira, A. Bettencourt, et al. (2007). "The role of HLA-DRB1 alleles on susceptibility and outcome of a Portuguese Multiple Sclerosis population." J Neurol Sci **258**(1-2): 69-74.

Simpson, S., Jr., L. Blizzard, P. Otahal, et al. (2011). "Latitude is significantly associated with the prevalence of multiple sclerosis: a meta-analysis." J Neurol Neurosurg Psychiatry **82**(10): 1132-1141.

Simpson, S., Jr., B. Taylor, L. Blizzard, et al. (2010). "Higher 25-hydroxyvitamin D is associated with lower relapse risk in multiple sclerosis." Ann Neurol **68**(2): 193-203.

Smolders, J., J. Damoiseaux, P. Menheere, et al. (2008a). "Vitamin D as an immune modulator in multiple sclerosis, a review." J Neuroimmunol **194**(1-2): 7-17.

Smolders, J., J. Damoiseaux, P. Menheere, et al. (2009a). "Association study on two vitamin D receptor gene polymorphisms and vitamin D metabolites in multiple sclerosis." Ann N Y Acad Sci **1173**: 515-520.

Smolders, J., J. Damoiseaux, P. Menheere, et al. (2009b). "Fok-I vitamin D receptor gene polymorphism (rs10735810) and vitamin D metabolism in multiple sclerosis." J Neuroimmunol **207**(1-2): 117-121.

Smolders, J., P. Menheere, A. Kessels, et al. (2008b). "Association of vitamin D metabolite levels with relapse rate and disability in multiple sclerosis." Mult Scler **14**(9): 1220-1224.

Staples, J., A. L. Ponsonby and L. Lim (2010). "Low maternal exposure to ultraviolet radiation in pregnancy, month of birth, and risk of multiple sclerosis in offspring: longitudinal analysis." BMJ **340**: c1640.

Steckley, J. L., D. A. Dyment, A. D. Sadovnick, et al. (2000). "Genetic analysis of vitamin D related genes in Canadian multiple sclerosis patients. Canadian Collaborative Study Group." Neurology **54**(3): 729-732.

Sykiotis, G. P. and D. Bohmann (2010). "Stress-activated cap'n'collar transcription factors in aging and human disease." Sci Signal **3**(112): re3.

Tajouri, L., M. Ovcaric, R. Curtain, et al. (2005). "Variation in the vitamin D receptor gene is associated with multiple sclerosis in an Australian population." J Neurogenet **19**(1): 25-38.

Templer, D. I., N. H. Trent, D. A. Spencer, et al. (1992). "Season of birth in multiple sclerosis." Acta Neurol Scand **85**(2): 107-109.

Thimmulappa, R. K., H. Lee, T. Rangasamy, et al. (2006). "Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis." J Clin Invest **116**(4): 984-995.

Thouvenot, E., M. Orsini, J. P. Daures, et al. (2015). "Vitamin D is associated with degree of disability in patients with fully ambulatory relapsing-remitting multiple sclerosis." Eur J Neurol **22**(3): 564-569.

Torkildsen, O., N. Grytten, J. Aarseth, et al. (2012). "Month of birth as a risk factor for multiple sclerosis: an update." Acta Neurol Scand Suppl(195): 58-62.

Tremlett, H., D. W. Fadrosh, A. A. Faruqi, et al. (2016). "Gut microbiota in early pediatric multiple sclerosis: a case-control study." Eur J Neurol **23**(8): 1308-1321.

van der Mei, I. A., A. L. Ponsonby, T. Dwyer, et al. (2007). "Vitamin D levels in people with multiple sclerosis and community controls in Tasmania, Australia." J Neurol **254**(5): 581-590.

Villoslada, P., C. Alonso, I. Agirrezabal, et al. (2017). "Metabolomic signatures associated with disease severity in multiple sclerosis." Neurol Neuroimmunol Neuroinflamm **4**(2): e321.

Wagner, C. L., B. W. Hollis, K. Kotsa, et al. (2017). "Vitamin D administration during pregnancy as prevention for pregnancy, neonatal and postnatal complications." Rev Endocr Metab Disord.

Whiting, S. J. and M. S. Calvo (2005). "Dietary recommendations to meet both endocrine and autocrine needs of Vitamin D." J Steroid Biochem Mol Biol **97**(1-2): 7-12.

Willer, C. J., D. A. Dyment, A. D. Sadovnick, et al. (2005). "Timing of birth and risk of multiple sclerosis: population based study." BMJ **330**(7483): 120.

Yeo, T. W., M. Maranian, S. Singlehurst, et al. (2004). "Four single nucleotide polymorphisms from the vitamin D receptor gene in UK multiple sclerosis." J Neurol **251**(6): 753-754.

Yildiz, M., B. Tettenborn and N. Putzki (2011). "Vitamin D levels in Swiss multiple sclerosis patients." Swiss Med Wkly **141**: w13192.

Zare-Shahabadi, A., Y. Renaudineau and N. Rezaei (2013). "MicroRNAs and multiple sclerosis: from physiopathology toward therapy." Expert Opin Ther Targets **17**(12): 1497-1507.

Zhang, J., Y. Cheng, W. Cui, et al. (2014). "MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with multiple sclerosis and experimental autoimmune encephalomyelitis." J Neuroimmunol **266**(1-2): 56-63.

Zhang, Y., G. Liu, X. Han, et al. (2016). "The association of serum 25-hydroxyvitamin D levels with multiple sclerosis severity and progression in a case-control study from China." J Neuroimmunol **297**: 127-131.