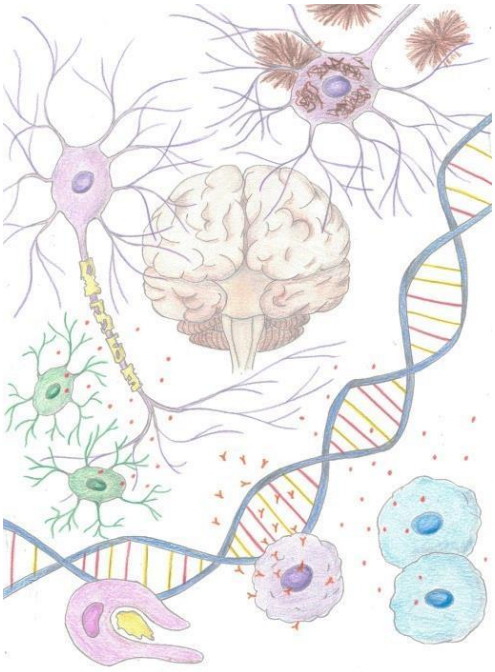
UNIVERSITA' DEGLI STUDI DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Neuroinflammation in neurodegenerative diseases: an immunogenetic study on risk factors in Multiple Sclerosis and Alzheimer's disease



Cristiana Pistono

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXX – A.A. 2014-2017

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Supervised by Prof. Mariaclara Cuccia
Prof. Elena Raimondi

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Abstract

The world of neurodegenerative disorders is highly complex. In fact, these disorders are extremely different from one other, but all of them are characterized by neuroinflammation and by progressive CNS dysfunction. 80% are sporadic and determined by the interplay of genetic and environmental factors. Our attention was focused on possible genetic risk factors that could contribute to the development of two neurodegenerative disorders: multiple sclerosis (MS) and Alzheimer's disease (AD).

Based on these considerations, at present, our research investigates possible risk factors for the pathogenesis of MS and AD. In particular, we are interested in investigating whether the polymorphisms of genes known to be involved in immunomodulation may have a functional impact on the pathogenic mechanisms of these diseases.

Most of the present work is focused on MS, a disimmune disease characterised by an auto-immune attack to CNS. In MS, neuroinflammation and neurodegeneration are placed side by side, with several processes contributing to neurodegeneration, including oxidative stress, energy deficiency and ionic imbalances.

Our research group has previously shown that a SNP (rs1061581) in the HSPA1B gene, which encodes a stress inducible chaperone called Hsp70-2, is related to the risk of developing MS: G allele frequency is increased in MS patients. In this work, genetic variants in HSP70 genes, in the MHC class III region (chromosome 6p21.3), were analysed, considering the SNP HSP70-HOM rs2227956, located in the HSPA1L gene encoding for the constitutively expressed Hsp70-Hom. The C allele confers a twofold increased risk of developing MS (OR = 2.13). Disease severity, measured by the Multiple Sclerosis Severity Score (MSSS), distributes differently, depending on the HSP70-HOM genotype; CC patients exhibited an increased MSSS of 1.22 on average. These two polymorphic variants are in linkage disequilibrium. The multilocus analysis shows that the combination of the two risk alleles increases MS risk by more than three times (OR=3.49). Hsp70-Hom protein levels were investigated *in vitro* on PBMCs. The protein expression does not correlate with the HSP70-HOM rs2227956 genotype. However, a direct and significant relationship between Hsp70-Hom protein expression and MS severity has been reported. These results suggest that Hsp70-Hom probably plays a role in promoting immune system activation, thus influencing MS risk and progression.

Considering the importance of HSP70-2 rs1061581 polymorphism for MS susceptibility, the possible role of the Hsp70-2 protein in the response to oxidative stress was investigated, treating *in vitro* PBMCs from MS patients and healthy controls with hydrogen peroxide (H_2O_2) and measuring mitochondrial activity, Hsp70-2 protein expression, and intracellular ROS production. No significant differences were found. The data regarding the mitochondrial activity and the Hsp70-2 protein expression levels were stratified by the HSP70-2 rs1061581 genotype to verify whether the presence of an allelic variant may influence the response to oxidative stress. The correlation of MTT and Hsp70-2 protein

levels with the HSP70-2 rs1061581 genotype does not show any significant difference. Two polymorphisms of the vitamin D receptor (VDR) gene (chromosome 12q13.11) were considered because of the importance of the interaction between genetic and environmental factors. Vitamin D is involved in the correct regulation of the immune system. Low levels of vitamin D constitute an important risk factor for developing MS. Its biological actions are mediated by its receptor (VDR). This observation leads us to hypothesize that polymorphisms in the VDR gene might influence MS susceptibility. Genotypic and allelic frequencies of two SNPs, VDR rs731236 (Taq-I) and rs4334089 (HpyCH4V), were determined. MS patients and healthy controls do not show significant differences. We investigated whether an altered expression of VDR or a change in the distribution of this receptor between the cytoplasm and nucleus could be associated with MS risk; the total, cytosolic and nuclear VDR protein expression in PBMCs from MS patients and healthy controls was determined. These levels do not significantly change between MS patients and healthy controls and are not correlated to the two polymorphisms. The environment probably acts in a stronger way compared to genetics; for this reason, the possible implication of vitamin D plasma levels on VDR protein expression in MS patients was analysed. Our patients have a mean $25(OH)D_3$ plasma levels of 21.82 ± 13.61 ng/ml. Vitamin D plasma levels can modulate the total VDR protein expression: the increase of 1 unit of vitamin D significantly decreases the total VDR protein expression by 10 units.

In the second part of the thesis, data about AD are reported. Literature evidence suggests a role of the innate immune system in the pathogenesis of AD. Neuroinflammation seems to be an important contributor to neurodegeneration. C4A and C4B copy number variations (CNVs) were investigated. The two C4 genes (MHC class III), encode the serum complement component 4. Our analysis reveals a significant increase in C4A and C4B copy number in AD patients compared to healthy donors. The increase in C4A and C4B copy numbers is consistent with the increased protein levels found in the CSF and plasma of AD patients and could influence neuroinflammation. Furthermore, three SNPs in the gene for the CR1 (chromosome 1q32.2) were investigated, since their potential impact on AD susceptibility. Genotypic and allelic frequencies of the three polymorphisms were analysed. AD patients and healthy controls do not show any significant difference for all three polymorphisms.

Acknowledgements

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Abbreviations

$1\alpha,25(OH)_2D_3$	1α,25-dihydroxyvitamin D3
$25(OH)D_3$	25-hydroxyvitamin D_3
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APC	Antigen presenting cell
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ARR	Annualized relapse rate
Aβ	Amyloid beta
BBB	Blood brain barrier
BDNF	Brain-derived neutrophic factor
BSA	Bovine serum albumin
C4	Fourth complement component
CI	Confidence interval
CIS	Clinically isolated syndrome
CMA	Chaperone-mediated autophagy
CMV	Cytomegalovirus
CNS	Central nervous system
CNV	Copy number variation
CR1	Complement receptor 1
CSF	Cerebrospinal fluid
DAMP	Danger-associated molecular pattern
DBP	Vitamin D-binding protein
DHODH	Dihydroorotate dehydrogenase
DMF	Dimethyl fumarate
DMT	Disease modifying therapy
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
ER	Endoplasmic reticulum
GWAS	Genome-wide association study
HD	Huntington's disease
HLA	Human leukocyte antigen
HMOX1	Heme oxygenase 1
HRP	Horseradish peroxidase
HSE	Heat shock elements
HSF1	Heat shock transcription factor 1
Hsp	Heat shock protein

IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ISF	Interstitial fluid
Keap1	Kelch-like ECH-associated protein 1
LHR	long homologous repeat
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MBL	Mannose binding lectin
MBP	Myelin basic protein
MCI	Mild cognitive impairment
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSSS	Multiple sclerosis severity score
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBD	Nucleotide binding domain
NFT	Neurofibrillary tangle
NK	Natural killer
NO	Nitric oxide
NOX	NADPH oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
OPC	Oligodendrocyte precursor cell
OR	odds ratio
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction - restriction fragment length polymorphism
PD	Parkinson’s disease
PLP	Myelin proteolipid protein
PMS	Progressive MS
PSEN	Presenilin
qPCR	Quantitative PCR
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycosylation end-product
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRMS	Relapsing-remitting MS
RXR	Retinoid X receptor
S1P	Sphingosine 1-phosphate
SBD	Substrate binding domain

SCR	short consensus repeat
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SOD	Superoxide dismuntase
TCR	T cell receptor
TEMED	N,N,N',N'-tetrametiletilendiammine
Th	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TREM	Triggering receptor expressed on myeloid cells
VDR	Vitamin D receptor
VDRE	Vitamin D response element
WT	Wild-type

Contents

1. Introduction

Neurodegenerative diseases are complex and heterogeneous disorders; they are multifactorial pathologies and it is difficult at the moment to completely understand their etiology and pathogenesis.

Multiple Sclerosis (MS) and Alzheimer's disease (AD) are two multifactorial neurodegenerative diseases which have a great impact on the society: MS is a disabling neurological disease affecting approximately 2.3 million people worldwide, in particular young adults, while over 36.5 million people worldwide are affected by dementia, and the majority of the cases are AD-related. These disorders represent a cost for the individual, the family and the society, putting great pressure on the need to better understand the risk factors that contribute to onset and progression. The research is necessary to finally find an effective therapy, which has not been achieved to date.

Although different in the pathogenesis, MS and AD, like other neurodegenerative diseases, are characterized by a great neuroinflammatory process; however, in AD it is not always clear if this process is a cause or a consequence of the disease. Alteration in genes involved in the immune system function may contribute to these disorders. However, analysing the complex scenario of neuroinflammation is not easy, since a plethora of genes is involved in this process. Not only the classical HLA gene located in class I and II regions are essential for the immune function: many genes in class III and also those located on other chromosomes take part in the orchestration and regulation of the neuroinflammation.

Based on these considerations, at present, our research investigates possible risk factors for the pathogenesis of MS and AD. In particular, we are interested in investigating whether the polymorphisms of genes known to be involved in immunomodulation may have a functional impact on the pathogenic mechanisms of these diseases.

In the case of MS the neuroinflammation plays a prominent role and is combined with neurodegeneration. The altered adaptive immune system plays a fundamental role in MS pathogenesis contributing to the demyelination. Oxidative stress, energy deficiency, ionic imbalance, and the failure of neuroprotective mechanisms also contribute to neurodegeneration. We focus the attention on MHC class III genes HSPA1L and HSPA1B coding for Hsp70 proteins, because of their role in the protection of cells against damages induced by several type of stress, including oxidative stress, and their involvement in immunity. These genes are polymorphic and we are indeed interested in studying whether particular polymorphisms may have a functional role in MS pathogenesis. Furthermore, environmental factors and their interplay with genetic factors are also important for MS susceptibility and progression. Among environmental factors, vitamin D deficiency is

clearly a risk factor. For this reason, in parallel, the polymorphic gene for the vitamin D receptor (VDR) (chromosome 12) which has a role in immunomodulation, is also analysed.
Regarding AD, in which the involvement of the innate immune system has been described, the complement system seems to play an important but controversial role. For this reason we focus the attention on the possible involvement of two particular complement components, the C4 and CR1 proteins. The coding genes C4A and C4B are located in the MHC class III, while the CR1 gene maps on chromosome 1. These gene are highly polymorphic and their variants may influence the neuroinflammatory process.

2. Review of the literature

2.1. Neurodegenerative diseases

The central nervous system (CNS) is one of the most elaborate and fragile element of our body. Neurodegenerative diseases alter and degrade the structure and the function of this complex and important system. Neurodegeneration consists in progressive CNS dysfunction characterized by damage and loss of specific neuronal subtypes, which ultimately leads to function impairments. Neuronal loss or functional degeneration manifests itself in a multiplicity of symptoms: memory impairments, locomotor dysfunction, cognitive deficits, emotional and behavioural problems. Neurodegeneration is the main pathological hallmark of several disorders affecting the CNS, such as Multiple Sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS) (Amor S et al., 2010). Neurodegenerative diseases are disabling disorders affecting millions of people worldwide, and they are becoming increasingly prevalent, due in part to the increase in life expectancy. Currently, no effective treatments for any of these disorders exist, although several therapies can relieve some of the physical or mental symptoms.

Neurodegenerative disorders are very different from each other; moreover, the same disease can show a strong heterogeneity in clinical features, highlighting their complexity (Lam B et al., 2013; Sabatelli M et al., 2013). However, neurodegenerative diseases show common features. 80% of them occur sporadically (Prusiner SB, 2012): unique combinations of conditions can trigger the pathology. In fact, these disorders are complex and multifactorial diseases, determined by the interplay between genetics and environmental factors, which not only influences the risk of developing the disease but also its progression (Martin et al., 2017; Delamarre and Meissner, 2017). Furthermore, alterations in post-transcriptional gene regulation may influence processes that are relevant to the pathogenesis (Cookson 2017). Only a few neurodegenerative disorders are genetic diseases, such as Huntington's disease (HD) (Bertram L and Tanzi RE, 2005). An important etiological factor is aging (Maynard S et al., 2015), although some neurodegenerative diseases occur at a younger age, like MS. Furthermore, the presence of infections has been associated with the risk of developing the disease and seem to change its progression (De Chiara G et al., 2012; Zhou L et al., 2013).

In neurodegenerative disorders, dendritic spine loss, and eventually neuronal death, are common features, together with cognitive defects (Halpain S et al., 2005; Amato MP et al., 2006; Das NR and Sharma SS, 2016). Several cellular and molecular events contribute to the neurodegenerative processes: protein aggregation, impaired mitochondrial function,

neuronal apoptosis, oxidative stress, altered gene expression (Jellinger KA 2001; Chiurchiù V et al., 2016). A common feature of several neurodegenerative disorders is the presence of protein aggregates within, and in some cases outside, the CNS cells (Knowles TP et al., 2014). However, the misfolded proteins are different in different pathologies; for example, in AD, amyloid beta (Aβ) peptide accumulates in senile plaques in the brain parenchyma, and neurofibrillary tangles (NFTs) of tau proteins are present inside the cells (Scheltens P et al., 2016), whereas in PD the protein that accumulates is α-synuclein (Braak H et al., 2003).
Mitochondrial dysfunction is an early pathological feature of several neurodegenerative diseases (Golpich M et al., 2017). Mitochondrial function is very important for neurons, which are extremely metabolically active cells (Kann O and Kovács R, 2007): mitochondria produce ATP and they are also involved in Ca^{2+} homeostasis, apoptosis and reactive oxygen species (ROS) production (Filosto M et al., 2011). Mitochondrial functions are altered in the brains of individuals with specific neurodegenerative disorders, such as PD and AD (Chen H and Chan DC, 2009; Su B et al., 2010). Evidence suggests that mitochondria become dysfunctional by reducing ATP supply, changing calcium buffering, opening the mitochondrial transition pore, and enhancing ROS production (Lin MT and Beal MF, 2006; Reddy PH and Reddy TP, 2011). In this way, mitochondrial dysfunction is linked to oxidative damage: ROS are normally created by mitochondrial respiration, but when their production exceeds a threshold, oxidative damage of proteins, carbohydrates, nucleic acids and lipids can occur (Bhat AH et al., 2015).
Furthermore, the involvement of glia cells in the progression of neurodegenerative diseases has been described (Heneka MT et al., 2010), including microglia. All these disorders are associated with neuroinflammatory processes, which can contribute to oxidative stress and involve components of the peripheral immune system (Amor S et al., 2014).

2.1.1. Inflammation in the central nervous system

The brain has long been considered an immunologically privileged site, so called an immune sanctuary. The limited penetration of immune cells, antibodies and immune mediators from the systemic circulation into the CNS, the absence of a lymphatic system, the low levels of major histocompatibility complex (MHC) molecules expression, and the limited number of antigen-presenting cells (APCs) are elements suggesting the concept of an immune privileged site (Wilson EH et al., 2010). The structural isolation is a consequence of the presence of the blood-brain barrier (BBB) (Figure 1). This barrier is formed mainly by specialized endothelial cells tightly sealed together, pericytes, basal membrane and astrocytes, and has the fundamental role of regulating the passage of nutrients and other important molecules to the brain (Abbott NJ et al., 2010). The BBB controls the movements of molecules and cells from the peripheral blood to protect the

CNS from potentially harmful compounds, such as microorganisms, toxins and immune cells (Abbott NJ et al., 2010).

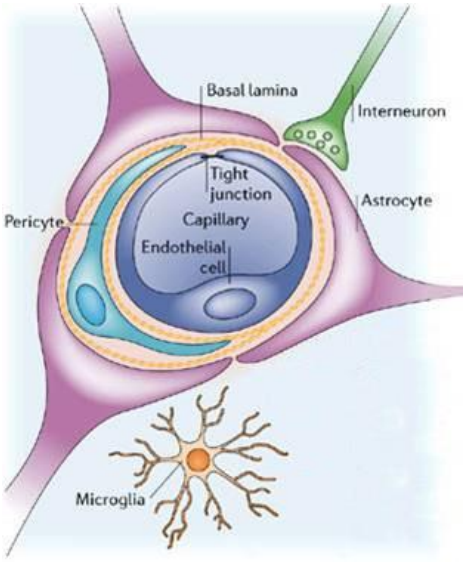

Figure 1. Components of the blood brain barrier. The capillary endothelial cells form tight junctions at their margin. Pericytes are distributed discontinuously and partially surround the endothelium. Both endothelial cells and pericytes are enclosed by basal lamina. Astrocytic perivascular endfeet surround the capillaries and the cell association is important in maintaining the barrier properties. Axonal projections from neurons contribute to the regulation of permeability. The figure also shows the microglia. Modified from: Abbott NJ et al., 2006.

However, in recent years it has become clear that the CNS is an immunologically active organ: the brain is monitored for infection or tissue injury. The presence of immune surveillance is important in physiological conditions for maintaining tissue homeostasis, neuronal integrity and network functioning in the CNS, and it is critical for correct brain functioning and recovery after injury (Heneka MT et al., 2014; Kipnis J, 2016). CNS integrity is guarded through the innate immune system: the activation of resident innate immune cells is a first line of defense, protecting the brain from pathogens and clearing the parenchyma from cellular debris, with an adaptive immunity only present in specific conditions.

Innate immune responses are usually initiated thanks to the recognition of pathogen-associated molecular patterns (PAMPs), conserved structures expressed by microorganism, or danger-associated molecular patterns (DAMPs), endogenous molecules that appear in

case of tissue damage or in situations of stress (Amor S et al., 2014). These structures are recognized by pattern-recognition receptors, including Toll-like receptors (TLRs), expressed by several CNS cells (Amor S et al., 2014).

Microglial cells are the principal constituents of a dedicated neuroimmune system present throughout the CNS and represent the major cellular component of the innate immune system of the brain. Under physiological conditions, microglia exhibit anti-inflammatory propriety and can release neurotrophic factors (Streit WS, 2002). However, like macrophages, microglial cells express TLRs and are able to produce pro-inflammatory mediators in response to pathogens or tissue damage (Rivest S, 2003). The inflammatory response at the level of the CNS not only involves microglia but also implicates several cell types both inside the CNS and from the periphery (Lampron A et al., 2013). By producing inflammatory factors, microglia can influence surrounding astrocytes, which in turn respond by releasing pro-inflammatory molecules and ROS, thus supporting the development of an inflammatory reaction (Hanisch UK and Kettenmann H, 2007; Glass CK et al., 2010). Astrocytes also express innate immune receptors that can directly bind DAMPs and PAMPs (Bowman CC et al., 2003). In addition, neurons play a role in the inflammatory responses in the CNS: neuronal membrane proteins, cell adhesion molecules and secreted soluble mediators, such as chemokines, neuropeptides and neurotransmitters, contribute to the regulation of inflammation (Tian et al., 2009).

In the inflammatory response, the interface between the CNS and the periphery is also important; the BBB contributes actively to the immune response in the CNS (Heneka MT et al., 2014). This structure can be crossed by peripheral immune cells (Carson MJ et al., 2006), modulates the function and controls the fate of the infiltrating cells (Ifergan I et al., 2008).

The activation of the innate immune system involves the production of pro-inflammatory cytokines and chemokines, which are the pivotal elements for the recruitment of the adaptive immune system. Thanks to the induction of adhesion molecules on the BBB, immune cells, including T lymphocytes, enter the brain (Amor S et al., 2010) (Figure 2). Under physiological conditions, T lymphocytes do not enter the brain parenchyma. However, T cells can be found in healthy meninges and seem to be important for correct brain functioning. It seems that T cells release cytokines that can affect neurons and other CNS cells through paracrine signalling, thus influencing CNS function and behaviour, although their exact role is not completely clear (Filiano AJ et al., 2017). In case of infections or tissue damage, T lymphocytes readily cross the BBB and target infectious agents. The CNS system actively interacts with these cells to regulate T cell responses (Tian L et al., 2009).

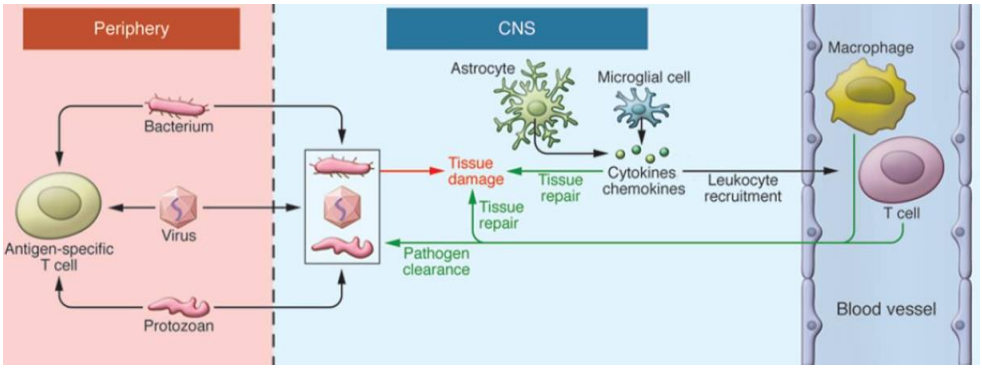

Figure 2. Immune response in the CNS. Resident microglia and astrocytes are involved in the protection of CNS against infections or injuries. They secrete cytokines and chemokines to recruit immune system cells from the periphery of the body for assistance in pathogen clearance. Modified from: Ransohoff RM and Brown MA, 2012.

2.1.2. Alterations of the immune response may contribute to neurodegeneration

The immune system is involved in the protection and maintenance of CNS homeostasis. Innate and adaptive immune responses are important in shaping the brain during its development, controlling viral and bacterial infection, reducing tumour growth, removing necrotic cells after ischemia, and promoting regeneration following damage (Amor S et al., 2014). The immune response is usually self-limiting and resolved once the infection has been eradicated or the damaged tissue repaired. However, in some cases the inflammation cannot be resolved and continues, overwhelming normal resolution mechanisms and leading to uncontrolled inflammation. The inflammation may contribute to the production of neurotoxic factors that amplify tissue damage and contribute to neurodegenerative diseases (Glass CK et al., 2010).

The imbalance between the pro-inflammatory and reparatory functions of neuroimmune cells may result in CNS injury. The physiological functions of microglia are important for the brain, but the loss, alteration or functional perturbation of microglia may occur in response to neurodegeneration and may contribute to pathogenesis and disease progression (Heneka MT et al., 2014). In neurodegenerative diseases, microglia are active. In several cases, this is due to the exposure to excessive levels of immune mediators, including pro-inflammatory cytokines; in others, self-molecules present in degenerating brains may activate microglia TLRs (Heneka MT et al., 2014). The activation of the microglia promotes the release of immune mediators, ROS and reactive nitrogen species (RNS),

affecting in a complex and unclear way the neighbouring cells, including neurons. Furthermore, microglia-driven inflammation may cause harmful feedback effects on the microglia themselves in the diseased tissue (Heneka MT et al., 2014). The activation of microglia, together with the production of cytokines and nitric oxide (NO), seems to correlate with the impaired ability to remodel synapses and with the decreased synaptic plasticity observed in neurodegenerative diseases (Heneka MT et al., 2014). The sustained release of pro-inflammatory molecules is known to be involved in the suppression of axonal transport and adult neurogenesis (Monje ML et al., 2003). Furthermore, the presence of neuroinflammation reduces the supply of neurotrophic factors to glial cells (Nagatsu T and Sawada M, 2005) and may affect physiological processes involved in intraneuronal protein handling (Alirezaei M et al., 2008).

2.1.3.Neuroinflammation in neurodegenerative disorders

Neuroinflammation is a common feature of neurodegenerative diseases. Activation of microglia and astrocytes and increased levels of proinflammatory cytokines into the CNS are the universal components of neuroinflammation (Heneka MT et al., 2014; Heppner FL et al., 2015). Sustained inflammatory responses that contribute to neurodegeneration are driven, at least in part, by positive feedback loops: damaged and dead neurons activate microglia, and the crosstalk between microglia and astrocytes may amplify the inflammatory processes. Furthermore, inflammation itself may influence the production of disease-specific inducers (Glass CK et al., 2010).

MS is caused by a primary defect in the immune system which targets components of the myelin sheath, resulting in the secondary damage of neurons, but for other neurodegenerative diseases it is typically thought that inflammation does not represent an initiating event (Glass CK et al., 2010). However, emerging evidence suggests that inflammation may have a role in the risk of developing other neurodegenerative diseases. For example, epidemiological studies show that the use of anti-inflammatory drugs, precisely non-steroidal anti-inflammatory drugs, reduced the risk of developing PD (Rees K et al., 2011; Noyce AJ et al., 2012). Additional evidence comes from genetic studies. For example, several risk loci for the development of AD have been identified in genes involved in inflammatory pathways, including HLA-DRB1, and genes encoding for the complement receptor 1 (CR1) and the triggering receptor expressed on myeloid cells 2 (TREM2) (Lambert JC et al., 2009; Lambert JC et al., 2010; Guerreiro R et al., 2013). The involvement of inflammation in neurodegenerative diseases is supported by observations that MHC class III genes also play a role in the susceptibility of these disorders. For example, the frequency of peculiar genotypes of polymorphisms in RAGE (receptor for advanced glycosylation end-product) (rs1800624) and TNF-α (tumor necrosis factor-α)

(rs1800629 and rs361525) significantly differs between AD patients and healthy controls. Moreover, their haplotype shows significant differences between AD patients and healthy subjects (Maggioli E et al., 2013).

Neuroinflammation mainly involving innate immune responses at the level of the CNS in neurodegenerative diseases such as AD, PD and ALS contributes to disease progression (Glass CK et al., 2010). In these disorders, the inflammatory process is likely promoted by the accumulation of protein aggregates and by the presence of damaged cells, which activate microglia. MS is also distinguished from other neurodegenerative disorders by the absence of protein aggregates and the strong involvement of the adaptive immune system (Glass CK et al., 2010). However, a growing number of observations suggest that adaptive immune mechanisms might also play a role in the development of other neurodegenerative disorders. Increased levels of CD8+ T lymphocytes and natural killer (NK) cells were detected in ALS patients (Rentzos M et al., 2012). However, T cells may also play a protective role in this pathology: CD4+ T lymphocytes support glial neuroprotection, thus slowing disease progression in mSOD1 transgenic mice (Beers DR et al., 2008). Innate as well as adaptive immune systems may be involved in the development of PD. Brochard and collaborators suggested that infiltrated CD4+ lymphocytes may mediate dopaminergic toxicity (Brochard V et al., 2009).

2.2. Multiple sclerosis

MS is a neurodegenerative disease that affects 2.3 million people worldwide, with a global median prevalence of 33 cases per 100,000 and with an important geographical distribution. The prevalence is highest in North America (140 per 100,000) and Europe (108 per 100,000) and lowest in sub-Saharan Africa (2.1 per 100,000) and East Asia (2.2 per 100,000) (Atlas of MS 2013) (Figure 3). MS usually appears between 20 and 40 years of age and is the most common cause of neurologic disability in young adults (Compston A and Coles A, 2008). This disease affects more women than men; the female:male ratio has increased over the past decades, most likely due to an augmented incidence in women, not a decreased incidence in men (Koch-Henriksen N and Sørensen PS, 2010). Furthermore, the ratio varies from region to region, ranging from 2:1 to 3:1 (Koch-Henriksen N and Sørensen PS, 2010).

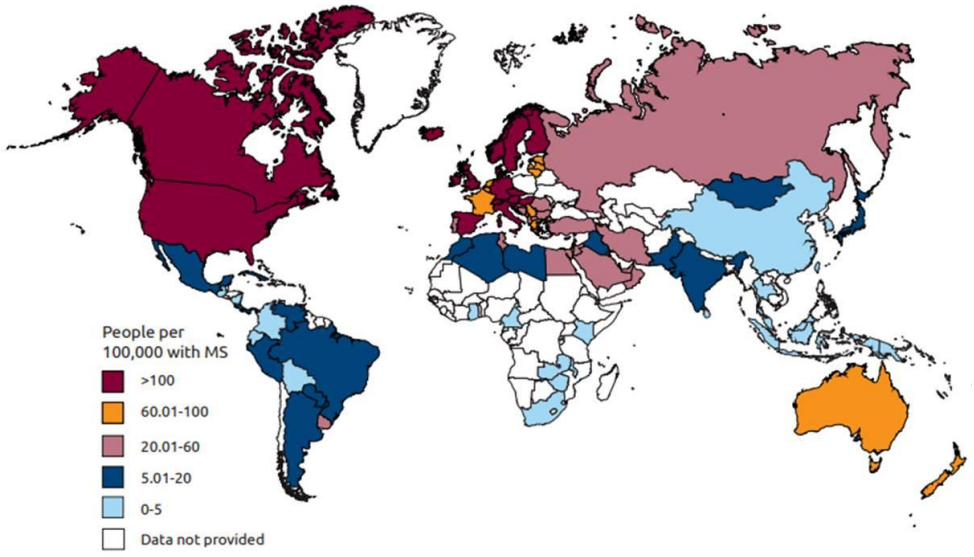

Figure 3. Global multiple sclerosis prevalence. MS prevalence greatly varies in different regions of the world. From: Atlas of MS 2013.

MS is a heterogeneous disorder with several disease courses and symptomatologies. Two main types of disease course are recognizable: relapsing-remitting MS (RRMS) and progressive MS (PMS) (Lublin FD et al., 2014). RRMS, the most common form of the disease, affects approximatively 85% of patients and is characterized by the alternation of relapses, which coincide with episodes of neurological dysfunction lasting at least 24 hours,

in the absence of fever or infection (Polman CH et al., 2011), and with remissions, periods of clinical recovery (Dendrou CA et al., 2015). The PMS is characterized by progressive neurological decline with CNS atrophy and axonal loss (Figure 4).

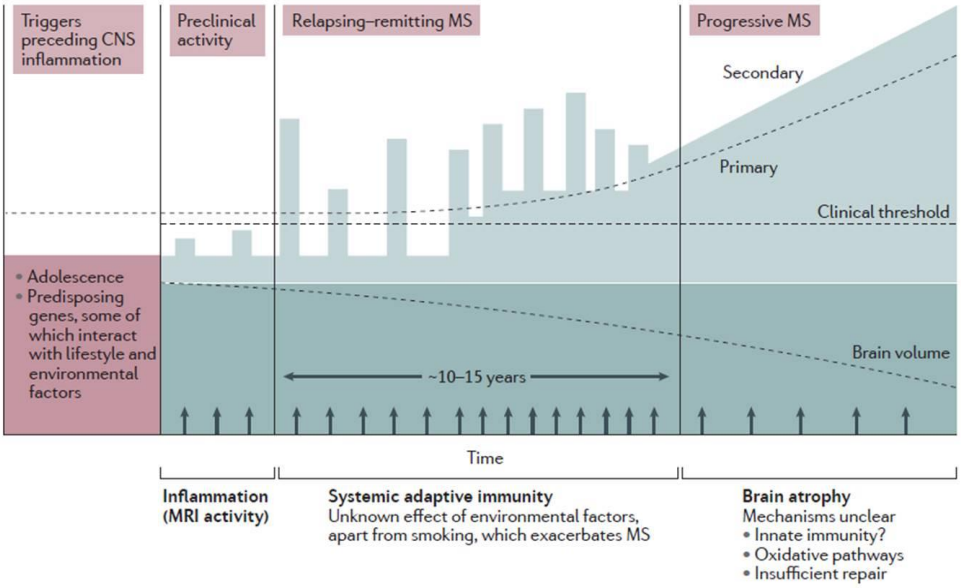

Figure 4. Heterogeneity of multiple sclerosis course. The light green area represents the progression of MS. The bars represent the episodes of CNS system inflammation typical of the relapsing-remitting form and the arrows indicate episodes of new CNS inflammation recorded by MRI. The graph also shows the progressive forms and the reduction in brain volume. Before the disease onset, several factors predispose to MS; after disease onset, several mechanisms contribute to disease progression. From: Olsson T et al., 2017.

However, the symptoms are similar, including visual and sensory disturbances, fatigue, motor impairments, pain and cognitive deficits (Compston A and Coles A, 2008). The presence of clinical symptoms, together with signs and findings from a magnetic resonance imaging (MRI), leads to the diagnosis of MS (Polman CH et al., 2011). The diagnosis is based on the 2010 revisions of the McDonald diagnostic criteria. Of importance in this regard is the demonstration of the dissemination of lesions in space and time and the exclusion of alternative diagnoses (Polman CH et al., 2011). The diagnosis of MS can be further reinforced by neurophysiological testing of evoked potentials in visual, sensory or auditory pathways, which identify clinically silent lesions (Leocani L and Comi G, 2014), and by cerebrospinal fluid (CSF) findings. Oligoclonal immunoglobulin IgG bands in CSF are present in up to 90% of MS patients (Dobson R et al., 2013).

The heterogeneity of symptoms depends on the spatiotemporal dissemination of the lesion within the CNS (Kearney H et al., 2015). The lesions are called plaques and are a hallmark of the disease. These plaques are areas of demyelination, also characterized by oligodendrocyte damage, which can be located both in the white and in the grey matter of the brain and the spinal cord (Frischer JM et al., 2009). During the early stage of the disease, the neurons and their axons are mostly preserved. However, with the progression of the disease, the neuro-axonal loss starts and then exacerbates, leading to brain atrophy (Figure 5). Furthermore, astrocytes form glial scars in the CNS. In the initial phase of MS, remyelination can occur in the plaques, but with the progression of the disease the repair mechanisms decrease (Popescu BF and Lucchinetti CF, 2012).

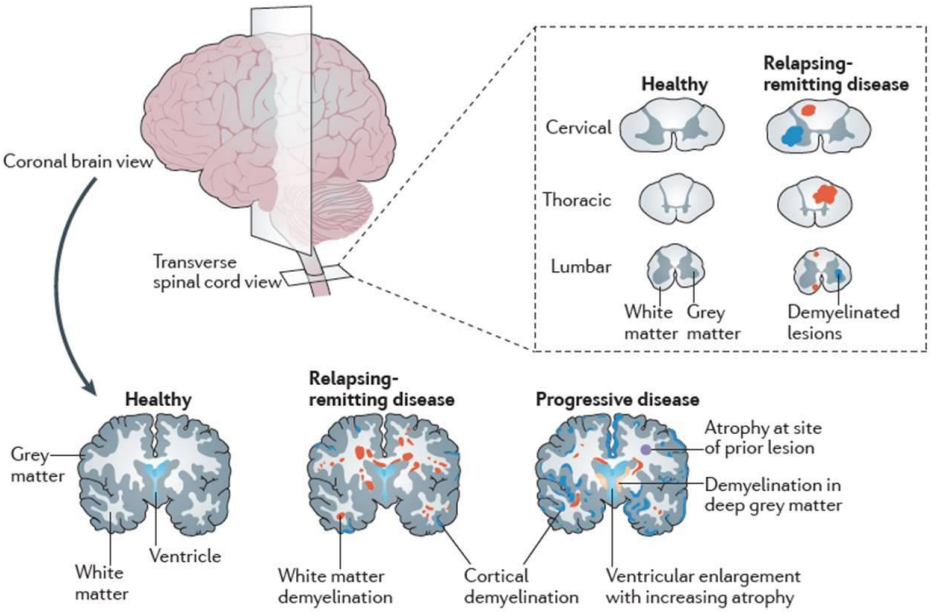

Figure 5. Formation of plaques during multiple sclerosis progression. MS is characterized by demyelinated areas, called plaques, in the white and grey matter of the brain and in the spinal cord. The progression of the disease is also characterized by brain atrophy. From: Dendrou CA et al., 2015.

The exact cause of MS is not well-defined, but the disease seems to arise from the combination of genetic, environmental and epigenetic factors. Genetic variation accounts for about 30% of the risk, with polymorphic genes located in the Human Leukocyte Antigen (HLA) region playing an important role (International Multiple Sclerosis Genetics Consortium et al., 2011). More than 100 genetic regions outside the HLA are involved in MS susceptibility (International Multiple Sclerosis Genetics Consortium et al., 2013).

It is known that the formation of plaques is due to an autoimmune attack: autoreactive T lymphocytes recognize CNS autoantigens, initiating the auto-inflammatory process against the myelin (Popescu BF and Lucchinetti CF, 2012). However, MS is not only an inflammatory disease; neuroinflammation and neurodegeneration occur side by side. Several processes contribute to neurodegeneration, including oxidative stress, energy deficiency and ionic imbalances.

Based on the main role of the immune system in MS pathogenesis, the therapy consists in the administration of immunomodulating agents that reduce and postpone the risk of relapses (Table 1).

	DRUG	MECHANISMS OF ACTION
Fist-line DMTs	**Interferon-β**	Regulates the immune system, inhibiting T cell proliferation and balancing the expression of pro-inflammatory and anti-inflammatory cytokines (Dhib-Jalbut S and Marks S, 2010).
	Glatiramer acetate	Competes with short antigenic MBP peptides to form complexes with MCH class II molecules. GA promotes Th2 differentiation and activates Treg cells (Liblau R, 2009).
	Dimethyl fumarate	Reduces the number of circulating T cells, decreases the expression of adhesion molecules on lymphocyte, and reduces the secretion of pro-inflammatory cytokines. DMF promotes the production of anti-oxidant enzymes (Pistono C et al., 2017).
	Teriflunomide	Inhibits the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), important for *de novo* synthesis of pyrimidines, thus having a cytostatic effect on activated T and B lymphocytes. It also affects T and B cell functioning. It decreases the production of pro-inflammatory cytokines and interferes with the interaction between T lymphocytes and APCs (Pistono C et al., 2017).
Second-line DMTs	**Natalizumab**	Targets the α4-integrin expressed on the surface of immune cells, thus reducing immune cell migration across the BBB (Sheremata WA et al., 2005).
	Alemtuzumab	Targets CD52, which is present on the surface of T and B lymphocytes, and acts to deplete circulating lymphocytes (Brown JW and Coles AJ, 2013).
	Fingolimod	The phosphorylated form competes with the sphingosine 1-phosphate (S1P) (essential for the maturation of T cells and for their transition from lymph node to lymphatic circulation) for the binding to S1P receptors on the surface of T lymphocytes. T remain sequestered inside the lymph nodes. Fingolimod can directly act inside the CNS (Pistono C et al., 2017).

Table 1. Mechanisms of action of DMTs used in MS therapy.

In RRMS, acute treatments using corticosteroids are important to decrease the duration of relapses and allow for a faster recovery; but they have no effects on long-term disability (Morrow SA et al., 2009). Disease modifying therapies (DMTs) are long-term treatments that mainly act to decrease the immune response that promotes lesion formation, thus drastically reducing the number of attacks and decreasing disease progression (Damal K et al., 2013). Patients with a mild-to-moderately active disease start the therapy with a first-line DMT, whereas second-line DMT are administered to patients with an aggressive form. When patients treated with a first-line DMT do not show a positive response, they are switched to a second-line DMT (Gajofatto A and Benedetti MD, 2015).
Until the approval by the FDA of the first oral therapy in 2010 (fingolimod), MS treatments mainly consisted of subcutaneous or intramuscular injectable agents. Monoclonal antibodies can be administered by intravenous infusion. Currently, the number of available treatments has increased, including three orally administered drugs: dimethyl fumarate (DMF) (first-line treatment), teriflunomide (first-line treatment) and fingolimod (second-line treatment). In addition to the approved DMTs, several agents and new approaches are at different developmental stages, although their effectiveness in human clinical trials remains to be determined (Dargahi N et al., 2017).

2.2.1.Pathogenesis

The formation of the plaques typical of MS is due to an autoimmune attack. Neuroinflammation characterises all stages of the disease, but it is more pronounced in the acute phases, promoting demyelination, gliosis and neuroaxonal degeneration, ultimately leading to the disruption of neuronal signalling (Frischer JM et al., 2009). Autoreactive T lymphocytes directed against CNS autoantigens, in particular components of myelin, can migrate inside the CNS by crossing the BBB to initiate the formation of the plaques. Infiltrates in the plaques are mainly composed of T lymphocytes, in particular CD8 T cells, and macrophages, although the presence of CD4 T cells and B cells is assessed. B lymphocytes can produce auto-antibodies against myelin and other CNS components, such as neurons and glial cells, highlighting the complexity of the autoimmune response typical of MS (Fraussen J et al., 2014) (Figure 6).
Although the role of adaptive immunity in MS is central, the involvement of the complement system has been hypothesised. Several studies suggest that complement proteins are dysregulated in MS; alterations in the plasma and CSF levels of some components have been detected in MS patients (Li Y et al., 2011; Ingram G et al., 2012). Moreover, some lesions display prominent complement activation (Lucchinetti C et al., 2000). Further evidence comes from genetic studies on the copy number variations (CNVs) of the genes for the forth complement component (C4A and C4B), with a significantly

higher frequency of C4AQ0 allele in patients with the RRMS being reported (Franciotta D et al., 1995).

The infiltration of immune cells from the periphery is the main feature of the early stages of MS. Microglia and astrocytes become activated thanks to the secretion of pro-inflammatory cytokines by infiltrated immune cells, contributing to demyelination, oligodendrocyte and neuro-axonal injuries (Popescu BF and Lucchinetti CF, 2012) (Figure 6).

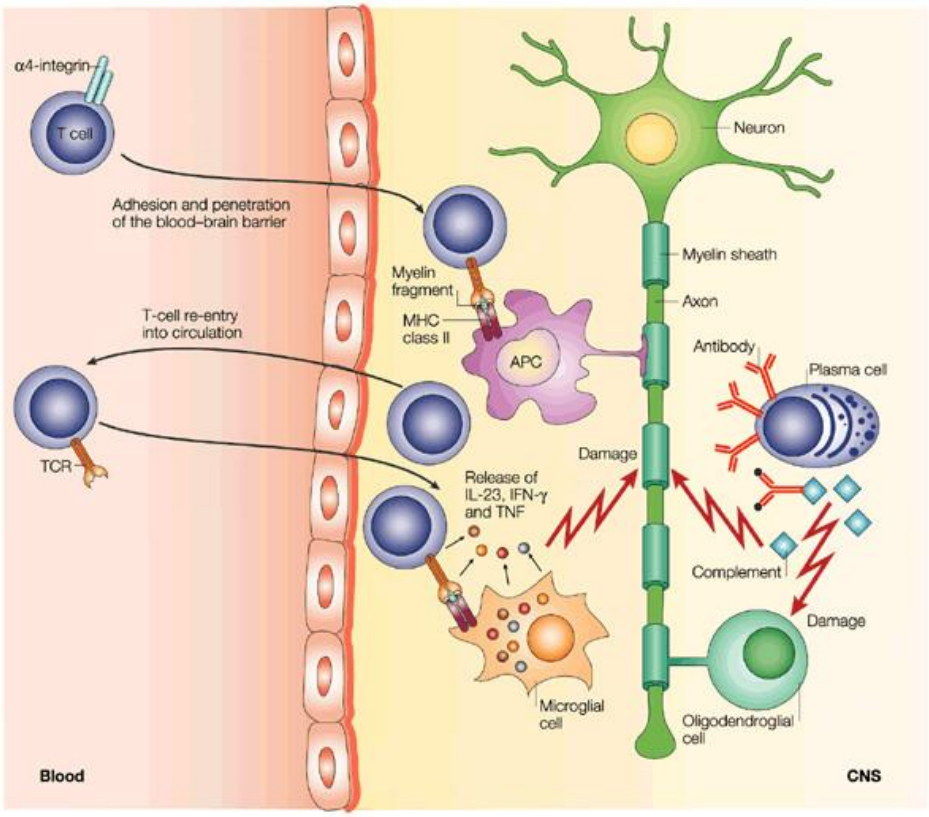

Figure 6. Auto-inflammatory attack in multiple sclerosis. T cells, B cells and APCs, including macrophages, migrate into the CNS. Lymphocytes enter the CNS through the BBB thanks to the use of α4-integrin. Inside the CNS, these cells accumulate and secrete pro-inflammatory molecules, thus contributing to the damage of myelin and oligodendrocytes. The neurons with injured myelin cannot conduct electrical impulses efficiently. B cells, differentiated into plasmacells, produce antibodies against myelin peptides, which interact with complement system components to produce membrane-attack complexes that further damage the myelin and oligodendroglial cells. Modified from: Steinman L and Zamvil S, 2003.

The immune system and CNS-resident cells contribute to the damage through direct cell contact-dependent mechanisms and the action of secreted inflammatory and neurotoxic mediators. After the first manifestation of the disease, an increase in the number and in the activation of both microglia and macrophages have been observed in lesions and also in the normal-appearing white matter (Giannetti P et al., 2015). As neuro-axonal degeneration spreads, microglia in the vicinity of the damage become activated, which may lead to the formation of new lesions (Kolasinski J et al., 2012) and contribute to the brain atrophy (Chard DT et al., 2002).

It seems that in the initial phases of the disease, macrophages can initiate the demyelination, whereas microglia are involved in debris clearing (Yamasaki R et al., 2014) and in the production of neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF) (Chen Z et al., 2014), producing a neuroprotective effect. However, with the progression of the disease it is clear that resident microglia have a pro-inflammatory effect.

Immune system cells seem to mainly target myelin proteins. Circulating CD4+ T cells of MS patients recognize myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG). However, it has been noticed that they can be recognised also in healthy individuals, and evidence for potential differences in the frequency and avidity of these cells between the two groups is conflicting (Hellings N et al., 2001; Bielekova B et al., 2004).

Furthermore, other molecules can be recognised. For example, it has been hypothesized that an autoimmune response against αβ-crystalline may exacerbate inflammation. This protein is the most abundant gene transcript in early active MS lesions, whereas it is absent in normal brains. It has anti-apoptotic and neuroprotective functions and acts to reduce several inflammatory pathways. The immune response against this negative regulator of inflammation may increase inflammation and demyelination (Ousman SS et al., 2007). Furthermore, antibodies against neurofascine, a cell adhesion molecule important for maintaining the structural and functional integrity of myelinated fibres, may contribute to axonal injuries (Mathey EK et al., 2007).

During the earlier stages of MS, new inflammatory lesions form frequently, and the infiltration of immune system cells into the CNS is considered the main driver of demyelination. Demyelination of axons eliminates the nerve impulse transmission; the nerve conduction velocity decreases, resulting in neurological impairment. Furthermore, with the lack of myelin, the physical protection of axons and metabolic support are also reduced (Lee Y et al., 2012), predisposing axons to neurodegeneration. Axonal degeneration and loss is already present in the earlier stages of MS (De Stefano N et al., 2002). In early MS stages, spontaneous remylination (the generation of new myelin sheaths around denuded axons) can occur. The oligodendrocytes precursors can migrate and surround the lesions, trying to remyelinate axons without myelin, thereby contributing to recovery (Scolding N et al., 1998) (Figure 7).

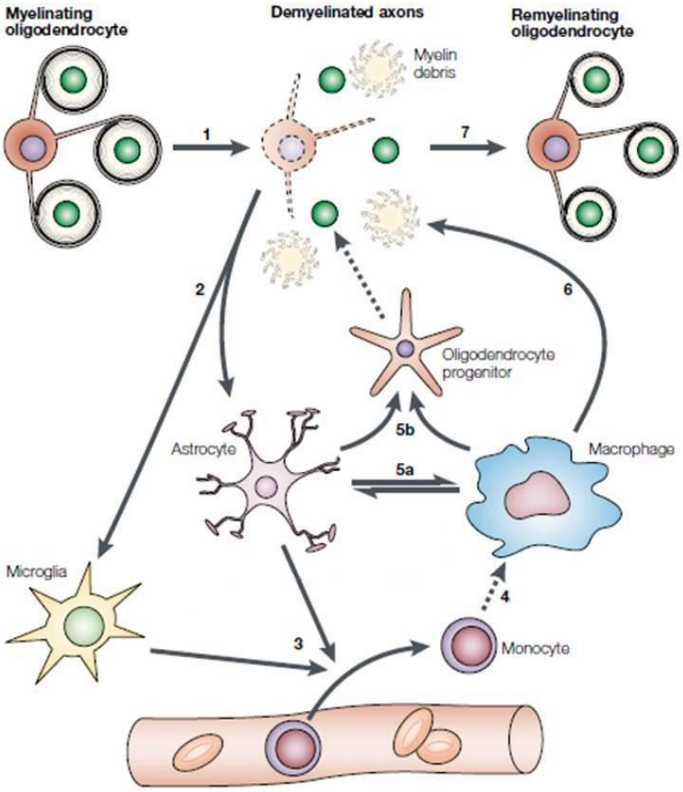

Figure 7. Remyelination in MS. Remyelination involves a sequence of steps, the dysregulation of which will result in impairments of this process. During demyelination, (1) myelin debris are produced. (2) Demyelination causes the activation of astrocytes and microglia (3) which, in turn, produce factors that contribute to the recruitment of monocytes from blood vessels. (4) The recruited monocytes differentiate into macrophages. (5a) Activated astrocytes and macrophages produce factors that activate each other, as a result of which (5b) both produce growth factors that act on oligodendrocyte progenitor cells. (6) Macrophages also contribute to remove myelin debris. (7) Under the influence of factors that are produced by astrocytes and macrophages, recruited oligodendrocyte progenitors differentiate into remyelinating oligodendrocytes, contributing to axons remyelination. Modified from: Franklin RJ, 2002.

However, histopathological analysis shows that axonal damage may continue to be present in lesions with signs of remyelination (Bitsch A et al., 2000). Remyelination increasingly fails with disease progression. For a successful remyelination, oligodendrocyte precursor cells (OPCs) may respond to demyelination, migrate to the lesion site, and proliferate and

differentiate in oligodendrocytes. The failure of one of these steps leads to deficient remyelination. Some studies show that around 70% of MS lesions that remain demyelinated contain OPCs, suggesting the failure of differentiation, while 30% contain only a few OPCs, indicating a failure in their recruitment (Lucchinetti C et al., 1999). Moreover, the process of remyelination involves other CNS cells, such as microglia and astrocytes (Figure 7). Microglia and macrophages are important for cleaning myelin debris (Kotter MR et al., 2006) and secreting molecules essential to induce OPC maturation (Miron VE et al., 2013). Astrocytes participate in the creation of a permissive environment for remyelination by their action on OPC migration and on oligodendrocyte proliferation and differentiation (Williams A et al., 2007) (Figure 7). Remyelinated axons seem to regain their function with a proper redistribution of ion channels at the nodes of Ranvier and with the restoration of saltatory conduction. However, it has been noted that remyelinated fibres tend to have thinner myelin sheaths compared to normal axons and that myelin displays alterations in composition (Manrique-Hoyos N et al., 2012).

With the progression of the disease, BBB permeability is increasingly reduced and the infiltration of immune cells decreases; the areas of demyelination coexist with diffuse axonal and neuronal degeneration. The lesions expand radially, in particular with the progressive forms of the disease, and diffuse microglia activation occurs, resulting in extensive abnormalities in the white matter (Kutzelnigg A et al., 2005).

After the initial phase, the inflammatory response triggers a self-sustaining chronic neurodegenerative process which can continue even in the absence of the autoimmune reaction. It seems that the neurodegeneration in MS is the culmination of a cascade of events: chronic inflammation, oxidative stress, energy deficiency, ionic imbalance, combined with the failure of neuroprotective and regenerative mechanisms (Friese MA et al., 2014) (Figure 8).

CNS resident cells play a key role in supporting neurodegeneration. Microglia and astrocytes can perceive homeostatic disturbances and, in turn, can produce inflammatory and neurotoxic mediators, such as cytokines, chemokines and ROS, which further damage the axons and neurons (Friese MA et al., 2014). The production of ROS and RNS probably promotes injuries to mitochondria, which in turn increases the production of ROS, worsening the oxidative stress present in the brain parenchyma. The damage to mitochondria contributes to energy deficiency, which has a negative implication for the normal neuro-axonal function. To help maintain the correct ion homeostasis, important for the conduction of the nerve impulse, several ion channels, including Na^+ and Ca^{2+} channels, and glutamate receptors become redistributed along the demyelinated axons (Kornek B et al., 2001; Craner MJ et al., 2004; Ouardouz M et al., 2009). However, the alteration in their expression and/or activity can lead to the accumulation of glutamate and Ca^{2+}, which promotes ionic imbalance, worsening the axonal degeneration (Friese MA et al., 2014).

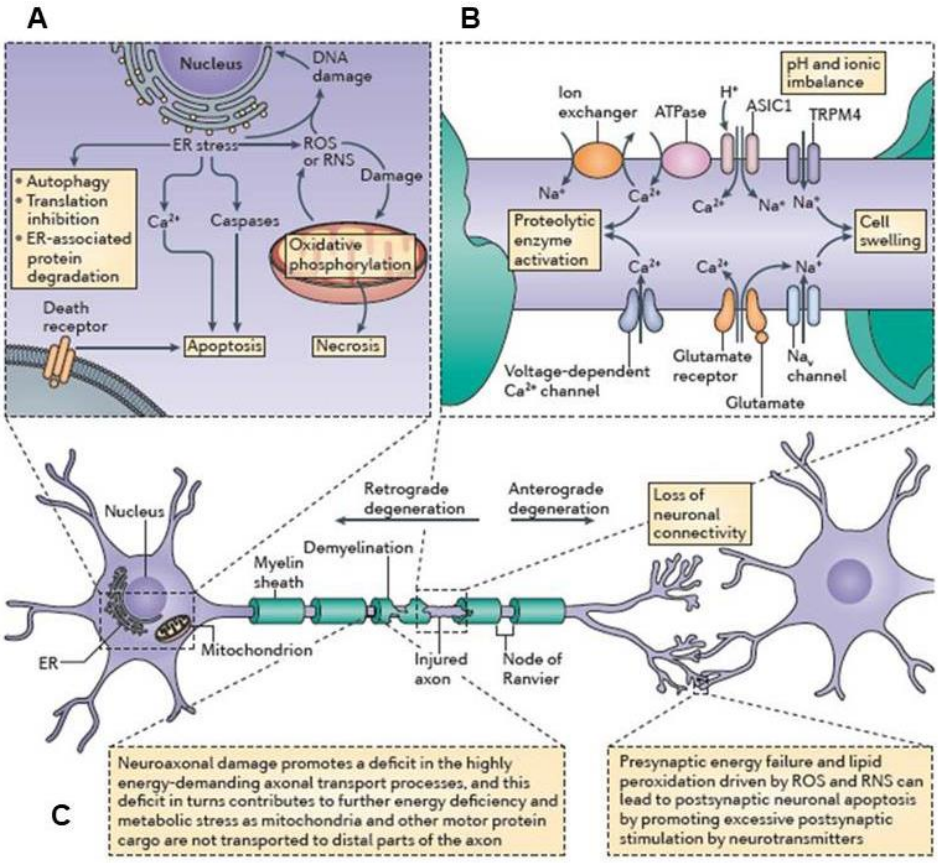

Figure 8. Neurodegenerative processes in multiple sclerosis. A| Chronic inflammation results in the production of ROS and RNS, which may promote mitochondrial injuries. The oxidative environment leads to metabolic stress, protein misfolding, energy deficiency and, ultimately, a loss of neuronal function. **B|** Several neuronal ion channels display a compensatory redistribution along the demyelinated neurons to try to maintain ionic homeostasis. However, this redistribution promotes an ionic imbalance that, together with the excessive accumulation of glutamate, contributes to tissue damage. **C|** The degenerative mechanisms can spread towards the neuronal cell body and the axon terminal, influencing presynaptic and postsynaptic neurons, respectively. Furthermore, neuronal death can occur. From: Dendrou CA et al., 2015.

Several mechanisms are triggered by CNS cells to contrast neuro-axonal injuries, such as the increased expression of pro-survival genes. However, the neurodegenerative mechanisms are able to override the protective mechanisms, contributing to CNS damage.

Furthermore, in the later stages of MS, microglia stimulate astrocytes, which, in turn, can produce CC-chemokine ligand 2 and granulocyte-macrophage colony-stimulating factor, further stimulating microglia activation. Astrocytes can also prevent remyelination by inhibiting the development of mature oligodendrocytes from progenitor cells (Mayo L et al., 2014). Moreover, long-term inflammation can be present thanks to previously infiltrating adaptive immune system cells, in particular B lymphocytes, which can form tertiary lymphoid structures within the CNS (Howell OW et al., 2011), underlining the complexity of the processes involved in MS pathogenesis.

2.2.1.1. Role of immune system cells against the central nervous system

In MS the inflammation seems to be driven by two main CD4+ T cell subsets: T helper 1 (Th1) and Th17 cells, that secrete pro-inflammatory cytokines, mainly interferon-γ (IFN-γ) and interleukin-17 (IL-17), respectively (O'Garra A et al., 1997; Becher B and Segal BM, 2011) (Figure 9). T cell differentiation is deviated away from the Th2 subset, which exhibits anti-inflammatory functions, secreting mainly IL-4 and IL-13 (Oreja-Guevara C et al., 2012). CD8+ T cells are found at higher frequency compared to CD4+ lymphocytes both in white and grey matter cortical demyelinating lesions; it has been noticed that their number correlates with axonal damage (Frischer JM et al., 2009). CD8+ T lymphocytes are thought to play a key role in MS pathogenesis: CD8+ T cells produce cytolytic proteins, such as perforin, directly contributing to neuronal cell destruction and triggering oligodendrocyte death (Kasper LH and Shoemaker J, 2010).
Moreover, B lymphocytes are able to infiltrate into the CNS and, compared to T cells, their number varies more throughout disease progression (Figure 9). Clonally expanded B cells have been detected in meninges, parenchyma and CSF; intrathecal B cells produce antibodies, which are found in the CSF and are of diagnostic value (Frischer JM et al., 2009).
The pathological cascade may arise from defects in the immune system regulation, which allows immune cells to initiate the immune response within the brain (Figure 9). It has been shown that regulatory T (Treg) lymphocytes from MS patients are not able to efficiently suppress effector cells (Venken K et al., 2008). Additionally, effector T cells may be actively resistant to suppressive mechanisms. For example, CD4+ T cells of MS patients overexpress β-arrestin 1, which is critical for naïve and effector cell survival. In this way, the autoreactive cells do not effectively undergo apoptosis after proper stimulation (Shi Y et al., 2007). Together these observations suggest that the dysregulation of both effector and regulatory cells has a role in MS, leading to the activation of autoreactive adaptive immune cells able to infiltrate and cause damage within the CNS.

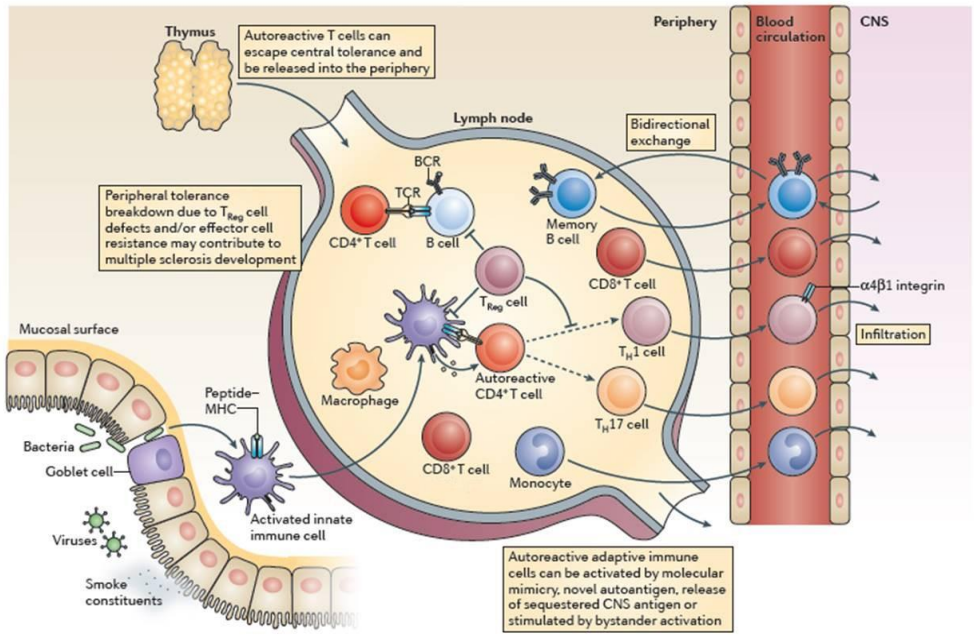

Figure 9. Immune system dysregulation in multiple sclerosis. During the establishment of central tolerance in the thymus, some autoreactive T cells can survive and reach the periphery. In healthy individuals, peripheral tolerance mechanisms inactivate these cells. If a reduction in the function of Treg cells and/or an increase in the resistance of effector B and T lymphocytes to suppressive mechanisms occur, tolerance is broken. In this way, autoreactive B and T lymphocytes can be activated in the periphery by molecular mimicry, novel autoantigen presentation, recognition of sequestered CNS antigen, or bystander activation. Genetic and environmental factors, including infectious agents and smoke constituents, contribute to these events. Once activated, CD8+ T cells, differentiated Th1 and Th17 cells, B cells and innate immune cells, can migrate into the CNS, creating an inflammatory response and inducing tissue damage. B cells can traffic out of the CNS and then undergo affinity maturation in the lymph nodes. Subsequently, they can re-enter the CNS and promote further damage. Modified from: Dendrou CA et al., 2015.

It is not clear whether the disease is triggered in the periphery or in the CNS. The CNS-extrinsic (peripheral) model suggests that autoreactive T lymphocytes are activated in peripheral areas and then reach the CNS, together with activated B cells and monocytes. The peripheral activation of T cells may occur due to several mechanisms: molecular mimicry, bystander activation, or co-expression of T cell receptors (TCRs) with different specificities (Ji Q et al., 2010). Molecular mimicry occurs when a peptide from a foreign antigen presented to a T cell closely resembles part of a self-protein; in MS, it seems that

viruses can mimic CNS peptides, allowing the initiation of the autoimmune reaction (Olson JK et al., 2001). The bystander activation leads to the proliferation and expansion of unrelated polyclonal T cells called heterologous T cells. The heterologous T cells are thought to be activated not through a strong TCR ligation but via cytokines, which results from the excessive activation of immune cells during the immunological response (Tough DF et al., 1996).

On the other hand, unknown intrinsic events in the CNS may trigger MS, and the infiltration of autoreactive immune cells is only a secondary phenomenon. The causes for this triggering are for now only hypotheses, among which CNS viral infections and primary neurodegenerative processes (Tsunoda I et al., 2003).

The most accepted hypothesis is that the disease starts with the activation of specific T lymphocytes in the periphery. This hypothesis is supported in mice by the experimental autoimmune encephalomyelitis (EAE). In this model of MS, the injection of myelin- or CNS-derived antigens results in the formation of CNS-specific CD4+ T cells (Mokhtarian F et al., 1984; Mendel I et al., 1995). However, the site of activation of these autoreactive lymphocytes is still unclear. A study on EAE mice, published in 2012, suggests that intravenously transferred T cells gain the ability to enter into the CNS after residing transiently within lung tissues (Odoardi F et al., 2012). They move to the bronchus-associated lymphoid tissues and draining lymph nodes to re-enter the blood circulation and ultimately reach the CNS. On the way, a switch towards a migratory mode occurs before the onset of the inflammatory process in the CNS. T lymphocytes reprogram the expression of their genes, upregulating membrane receptors, such as the sphingosine 1-phosphate 1 receptor ($S1P_1$), important in guiding the lymphocytes outside the lymph nodes, chemokines receptors and adhesion molecules. Furthermore, the researchers noticed that the lungs are a niche not only for activated T cells; myelin-reactive memory T lymphocytes strongly proliferate in the lungs and assume migratory properties. It is important to consider that this site of activation is in direct contact with the outer environment and colonized by a microbiota. Environmental factors may directly influence a pathogenic response (Odoardi F et al., 2012).

2.2.2. Genetic risk factors

MS is a complex disease, and the increased risk that relatives of affected individuals will develop MS is evidence of susceptibility on a genetic basis, but the familial risk is only modest. Monozygotic twins have a concordance rate of around 30%, whereas dizygotic twins have a concordance of about 5% (Ebers GC, 2008) (Figure 10). However, a more recent study of the Swedish population suggests lower concordance rates: 17% for monozygotic twins (Westerlind H et al., 2014). The risk is higher for children both of

whose parents are affected by MS compared to those having only one parent with the disease (Ebers GC et al., 2000) (Figure 10).

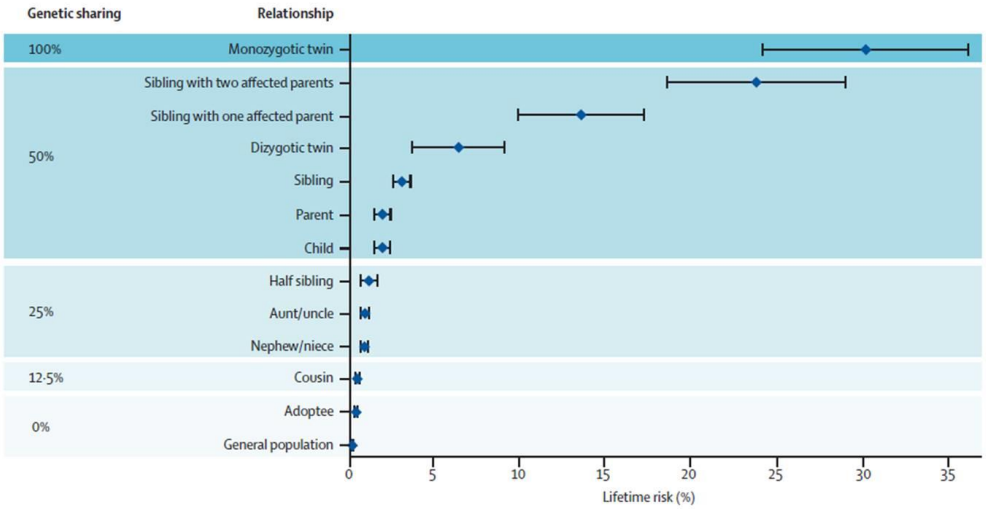

Figure 10. Recurrence risk for multiple sclerosis. Age-adjusted recurrence risk of MS for different relatives. The degree of genetic sharing increases the risk of MS. Error bars indicate the estimated 95% confidence interval. From: Compston A and Coles A, 2008.

Therefore, MS risk is determined by the combined effects of several genetic variants, by environmental factors, and by their interaction. Furthermore, epigenetic alterations are probably important in determining the risk of developing MS.

The fact that MS risk is determined by the combination of environmental and genetic factors is a characteristic shared with autoimmune diseases. Furthermore, some loci involved in MS susceptibility include immune-related genes, which are also involved in the risk of developing other autoimmune diseases (Cotsapas C et al., 2011).

Important in determining MS risk are the genes located in the HLA region, a genomic region of about 4,000 kb located on the short arm of chromosome 6, which includes more than 240 highly polymorphic genes. These genes are essential for immune system function and are divided into three classes (Figure 11).

The first association between MS and alleles of the MHC was proposed in the 1970s (Compston DA et al., 1976; Terasaki PI et al., 1976). HLA-DRB1*15:01 confers the highest genetic risk for MS, with an OR of about 3 (Olerup O and Hillert J, 1991; International Multiple Sclerosis Genetics Consortium et al., 2011). HLA-DRB1, as like the other genes in MHC class II, is expressed on APCs, presenting peptides to CD4+ T lymphocytes, cells important in the pathogenesis of MS.

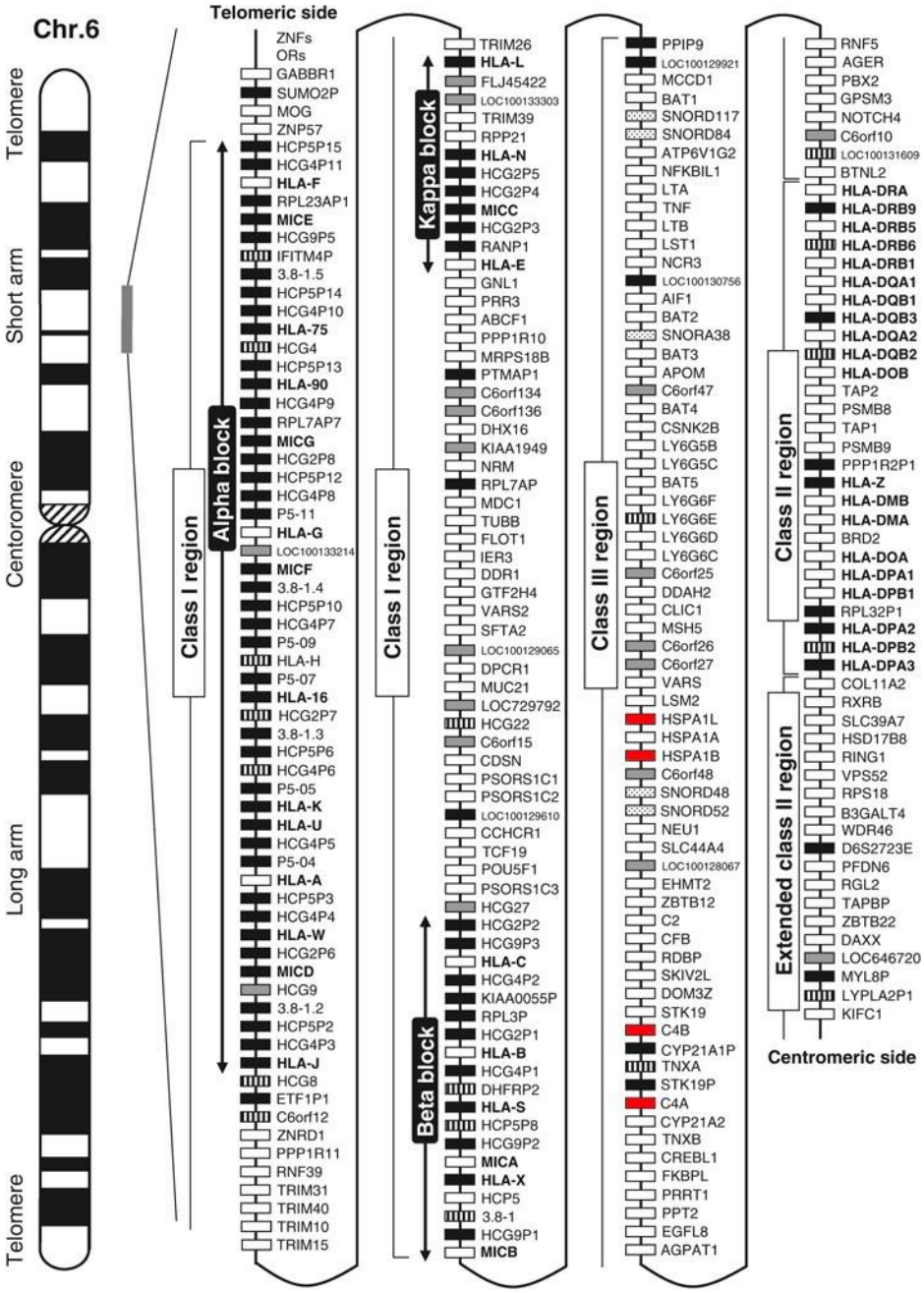

Figure 11. Gene map of the human leukocyte antigen region. In red are highlighted the genes that will be analysed in the thesis. Modified from: Shiina T et al., 2009.

HLA allelic heterogeneity among populations is well documented in MS. For example, HLA-DRB1*15:01 has been confirmed to be the strongest genetic risk factor, not only in Caucasian Europeans and North Americans, but also in African Americans and in the Northern Han Chinese population (Oksenberg JR et al., 2004; Qiu W et al., 2011 a). However, HLA-DRB1*04:05 seems to be the primary risk allele in the Japanese population, whereas HLA-DRB1*15:01 is the top risk variant in Japanese individuals without HLA-DRB1*04:05 (McElroy JP et al., 2011). Other alleles in the MHC class II region have been associated with MS in different populations; for example, HLA-DRB1*03:01, HLA-DQB1* 02:01 and HLA-DRB1*13:03 are secondary risk variants for Northern European populations (International Multiple Sclerosis Genetics Consortium et al., 2011).

Several MHC class I alleles have also been identified. These genes code for molecules which are necessary to present peptides to $CD8^+$ T cells, other important cells in the pathogenesis of MS. HLA-A*02 is associated with protection against MS (International Multiple Sclerosis Genetics Consortium et al., 2011). In fact, the absence of HLA-A*02 and the presence of HLA-DRB1*15:01 lead to a five-times-greater risk of developing MS (Brynedal B et al., 2007).

In addition, polymorphisms in MHC class III seem to influence MS risk. This region is located between class I and class II and has an extension of about 1,100 kb, including more than 60 genes. Some of these genes are responsible for complement components (i.e., C2, Bf, C4A, C4B), pro-inflammatory cytokines (TNF-α) and heat shock proteins (HSP70 genes) (Milner CM and Campbell RD, 2001). Our research group reported that a single nucleotide polymorphism (SNP) (rs1061581) in the HSPA1B gene is related to the risk of developing MS; the G allele frequency is higher in MS patients compared to healthy controls, leading to a MS estimated risk of 1.31 (Boiocchi C et al., 2014).

Genes located outside the MHC region can also modify MS risk. Genome-wide association studies (GWAS) have identified about 110 non-HLA SNPs that contribute to influence MS risk, although their effect is only modest (International Multiple Sclerosis Genetics Consortium et al., 2013). Important in this regard are the SNPs located in genes involved in the regulation of the immune system. The first GWAS from the International Multiple Sclerosis Genetics Consortium identified variants in the genes encoding for the α-chain of IL-2 receptor (IL2RA) and for the α-chain of IL-7 receptor (IL7RA) associated with MS (International Multiple Sclerosis Genetics Consortium et al., 2007). Their effect is only modest: the two SNPs (rs12722489 and rs2104286) in IL2RA confer an OR of 1.25 and 1.19, respectively, and the SNP (rs6897932) in IL7RA gives an OR of 1.18.

Moreover, HLA genes seem not only to have an impact on MS susceptibility but also on the disease course. For example, in MS patients the protective HLA-B*44 allele shows an association with a better radiological outcome, preserving brain volume and reducing the burden of lesions observed with MRI (Healy BC et al., 2010). HLA-DRB1*15:01 carrier

status correlates with disease severity (Okuda DT et al., 2009), and another study reported a correlation between the carriage of HLA-DRB1*15:01 and the presence of diffuse cord lesions and higher disability (Qiu W et al., 2011 b). Moreover, HLA-DRB1*15:01 is associated with a lower age of onset (Masterman T et al, 2000).

2.2.3. Environmental risk factors: the complex interplay with genetics

Non-genetic factors seems to make a larger contribution than genetic factors to MS risk and to the heterogeneous disease course. Over the years, the association between MS and several risk factors has been analysed. Epstein-Barr virus (EBV) infections, smoking and low vitamin D levels were among the first to be investigated; today, these represent well established risk factors for MS. More recently adolescent obesity has been added to them. However, it is not always easy to assess the importance of environmental factors; several factors need further investigation to allow us to exactly assess their role. Among these factors, exposure to organic solvents and night shift work seem to be linked with an increased risk of developing MS, whereas coffee seems to decrease the risk (Olsson T et al., 2017).

Several **infectious agents** have been analysed as possible risk factors for MS. Virus and bacteria seem to be involved in triggering autoreactive T lymphocytes, thanks to molecular mimicry (Olson JK et al., 2001). Peripheral inflammation due to infections may directly influence the CNS (Konsman JP et al., 2004). Furthermore, CNS infections may promote the release of CNS antigens into the periphery (Miller SD et al., 1997).

One of the most studied viruses is EBV. There is a lot of evidence to support its role in increasing MS risk. A meta-analysis showed that people that have had a clinically overt infectious mononucleosis have more than twice the risk of developing MS (Handel AE et al., 2010). However, the exact role of EBV in MS is not completely clear. The difficulty in establishing its role is also due to the fact that EBV is common both in individuals with other neuroinflammatory diseases and in healthy subjects, although the role of EBV in MS susceptibility may be supported by the reported interaction between infectious mononucleosis and HLA-DRB1*15:01. Individuals with HLA-DRB1*15 with a history of infectious mononucleosis have an increased risk of developing MS compared to people with HLA-DRB1*15 but without infectious mononucleosis (Nielsen TR et al., 2009).

Another infective agent analysed is cytomegalovirus (CMV), a common virus from the same family as EBV. In this case, infection from CMV seems to reduce the risk of MS (Sundqvist E et al., 2014). However, it remains to be proven whether this negative association is due to a true protective effect of CMV infection on MS risk.

Smoking is a risk factor for developing MS (Handel AE et al., 2011); several studies have shown that the risk augments with increasing cumulative exposure to smoke (Hernán MA

et al., 2001; Hedström AK et al., 2009). Furthermore, passive exposure to smoking has been associated with increased risk of developing the disease, and in this case as well the risk grows with the increasing duration of exposure. The authors of the study have suggested that the negative effect of passive smoking may be due to lung irritation, which could trigger immune responses (Hedström AK et al., 2011 a) (Figure 12). The possible link between lung irritation and MS risk seems to be supported also by studies regarding air pollution (Heydarpour P et al., 2014). Particulate matter smaller than 10 µm seems to also be related to the risk of relapses in MS patients (Angelici L et al., 2016).
Interestingly, the interaction between smoking and HLA risk alleles for MS has been reported: in Scandinavian population the combined OR of about 5 resulting from the presence of HLA-DRB1*15:01 and the absence of HLA-A*02 in non-smokers increases to almost 14 in smokers (Hedström AK et al., 2011 b). The interaction of smoking with HLA variants has also been reported in rheumatoid arthritis (RA), although the alleles involved are different (Mahdi H et al., 2009). Smoking is able to activate enzymes in the lung that can post-translationally modify peptides, so that T cells can recognized them and become activated, inducing autoimmunity (Klareskog L et al., 2009) (Figure 12).

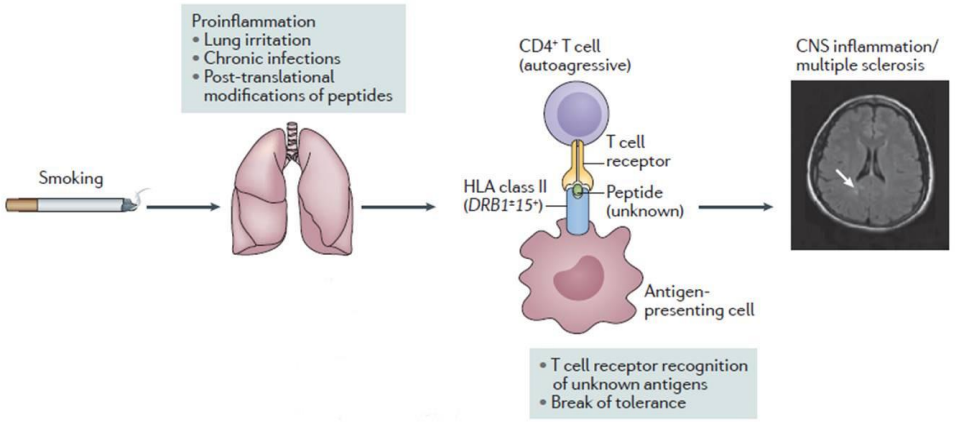

Figure 12. Smoking can influence multiple sclerosis risk through several potential mechanisms. Smoking may promote activation of pro-inflammatory pathways: it may induce lung irritation and alter peptides thanks to post-translational modification. Resident $CD4^+$ T cells may be activated through the recognition of these peptides presented by HLA-DRB1*15:01 molecules and become reactive against CNS-antigens. Modified from: Olsson T et al., 2017.

Growing evidence suggests a role for **obesity** in the risk of developing MS. Obesity during adolescence has been associated with an increased risk of developing MS in adult life (Hedström AK et al., 2014); furthermore, childhood obesity increases the risk of paediatric

MS (Langer-Gould A et al., 2013). However, it is not clear how obesity influences MS risk. It may be significant that obesity is associated with a low-grade inflammation (Lumeng CN et al., 2007) and with increased levels of leptin, which is related to pro-inflammatory processes (Matarese G et al., 2005). In this way, obesity may contribute to enhance the activation of the adaptive immune system. As for smoking, the body max index interacts with HLA variants important for MS risk, and obese subjects with the HLA-DRB1*15:01 and without the protective HLA-A*02 are fourteen times as likely to develop the disease (Hedström AK et al., 2014).

In recent years, **gut microbiota** has gained attention. Changes in the gut microbiota alter the mucosal immune system, cause gut inflammation and alter the intestinal immunity, thus influencing the correct functioning of the immune system (Kuhn KA and Stappenbeck TS, 2013; Chassaing B and Gewirtz AT, 2014).

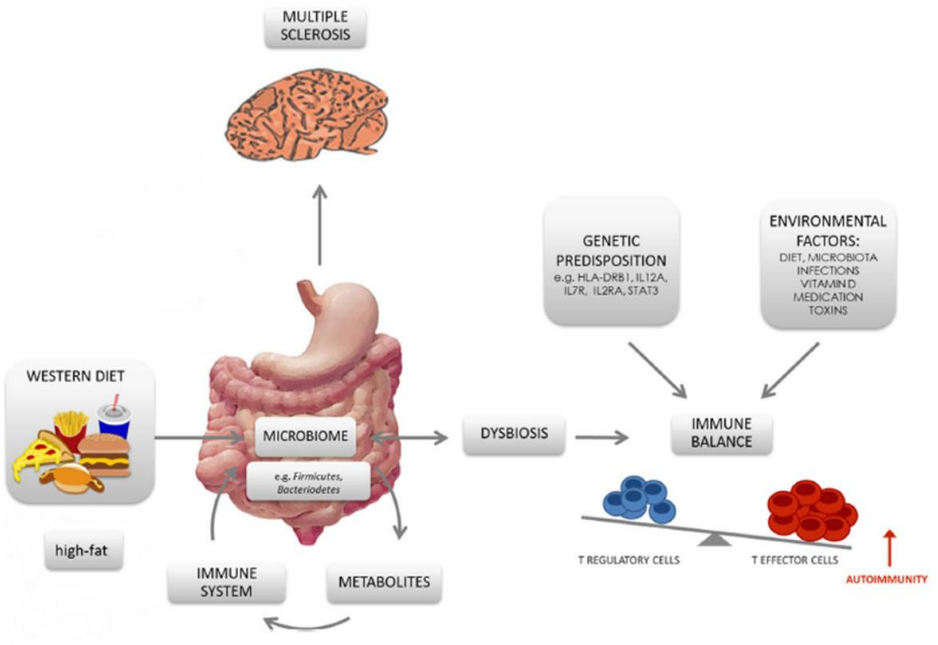

Figure 13. Role of gut microbiota in multiple sclerosis risk. Alterations of gut microbiota may influence the correct functioning of the immune system, leading to Treg deficiency and an activation of proinflammatory Th17 cells. In this way, the dysbiotic microbiome can influence MS risk, together with genetic and environmental factors. Diet can also influence the gut microbiota: the "western diet", rich in fatty acids, increases inflammation and might negatively affect gut-immune homeostasis. Modified from: Jörg S et al., 2016.

Studies on EAE suggest that the gut microbiota may also play a role in MS (Ochoa-Repáraz J et al., 2010; Berer K et al., 2011) (Figure 13). In mice with EAE, the replacement of some bacterial population in the gut can lead to a reduction of the pro-inflammatory state, suppressing the progression of the clinical symptoms. The suppressive activity seems to correlate with a shift from a Th1 to a Th2 response (Lavasani S et al., 2010). Several factors contribute to alterations in gut microbiota, such as diet, medication or stress, thereby influencing intestinal immunity and increasing the incidence of autoimmune diseases, including MS (Peterson CT et al., 2015; Riccio P and Rossano R, 2015).

Diet can act not only on the gut microbiota, but also directly on the cells of the body; nutrients can interact with enzymes, transcription factors and receptors, and this may influence cellular metabolism, thus altering inflammatory responses (Desvergne B et al., 2006).

Besides the well-established role of vitamin D in varying MS risk, other nutrients have been analysed, among them vitamin A. Vitamin A is essential for several functions, including the maintenance of immune system integrity. The administration of retinoids before or after EAE onset improves the clinical course (Racke MK et al., 1995). In the mouse model of EAE, dietary supplementation of all-trans retinoic acid, the primary mediator of biological actions of vitamin A, attenuated clinical symptoms and reduced the expression of MHC class II molecules on dendritic cells (Zhan XX et al., 2013). Furthermore, low levels of vitamin A are markers of inflammation, without being specific for MS (Schweigert FJ, 2001). An increase in vitamin A intake leads to a reduction of the *in vitro* proliferation of MOG-reactive lymphocytes from MS patients (Honarvar NM et al., 2013).

2.2.4.Role of vitamin D in multiple sclerosis

The importance of vitamin D in MS is suggested by two observations: 1) MS prevalence and incidence vary with the latitude (Simpson S Jr et al., 2011) and 2) increased exposure to ultraviolet radiation is related to a decreased risk of developing MS (Bäärnhielm M et al., 2012), thus suggesting the importance of vitamin D in this disorder. In fact, solar light is important for the production of vitamin D at the level of the skin. Direct evidence of the importance of vitamin D derives from the observation that the oral supplementation of vitamin D reduced the risk of developing the disease (Cortese M et al., 2015). A recent meta-analysis confirmed that people with MS have lower vitamin D levels in the serum compared to healthy controls (Duan S et al., 2014). In patients with MS, high vitamin D levels correlate with a reduction in the neurofilament light chain in CSF (Sandberg L et al., 2016). Neurofilaments are the main products of neuroaxonal breakdown and are used as biomarkers of neurodegeneration; the reduction reported in the study indicates a decrease in

axonal damage. Vitamin D levels may also be important for disease progression; severe vitamin D deficiency is associated with an increased risk of conversion from clinically isolated syndrome (CIS) to clinically defined MS (Martinelli V et al., 2014). Furthermore, in RRMS higher vitamin D levels reduced MS relapse activity, slowed the rate of progression, and lowered the disability (Ascherio A et al., 2014; Thouvenot E et al., 2015).
However, evidence suggests important benefits for MS from sun exposure, not only through vitamin D but also thanks to non-vitamin D pathways. In the animal model, ultraviolet radiation exposure gives protection against neuroinflammation, reducing clinical signs of EAE, independently of vitamin D (Becklund BR et al., 2010). A study reported that higher levels of sun exposure, but not higher vitamin D levels, are associated with a low fatigue score and less depressive symptoms in MS patients (Knippenberg S et al., 2014). It has been observed that ultraviolet radiation exposure reduces inflammation in experimental mice, modulating CD8+ T cell immunity (Rana S et al., 2011). Studies on mice showed that ultraviolet radiation reduces systemic inflammation thanks to the induction of tolerogenic Treg and dendritic cells (Breuer J et al., 2014).
Since vitamin D seems important to slow down disease progression, several trials in which cholecalciferol was administered to MS patients were conducted. However, the results are contradictory. Some trials showed beneficial effects from cholecalciferol supplementation. For example, when added to IFN-β treatment, cholecalciferol reduces the lesions detected by MRI, with patients showing a tendency to reduce disability accumulation (Soilu-Hänninen M et al., 2012). However, other trials did not find any improvements in the clinical parameters (Mosayebi G et al., 2011; Kampman MT et al., 2012).

2.2.4.1. Vitamin D production

The precursor vitamin D_3, also called cholecalciferol, can be obtained through diet, but it is mainly formed from 7-dehydrocholesterol in the skin due to a ultraviolet light-mediated reaction (Figure 14). Vitamin D3 is bound by the vitamin D-binding protein (DBP) and transported to the liver, where it is hydrolysed by the microsomal vitamin D 25-hydroxylases CYP2R1 to produce 25-hydroxyvitamin D_3 ($25(OH)D_3$), the major circulating form. $25(OH)D_3$ is transported by DBP to the kidney, where the mitochondrial 1α-hydroxylase CYP27B1 forms the 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), also known as calcitriol. This reaction occurs mainly in the kidney, but several other cell types, such as immune cells, can form $1\alpha,25(OH)_2D_3$ (Figure 14). This form circulates in the bloodstream, bound to the DBP, reaching several tissues and acting as a nuclear hormone to exert several functions: $1\alpha,25(OH)_2D_3$ regulates calcium and phosphate metabolism, raising their blood levels via intestinal absorption and renal reabsorption to facilitate bone mineralization, as well as activating bone resorption as part of the skeletal remodelling cycle (Carmeliet G et

al. 2015). However, vitamin D not only is implicated in the regulation of calcium levels and bone metabolism but also regulates metabolism, cellular growth and immune system functioning (DeLuca HF, 2004).

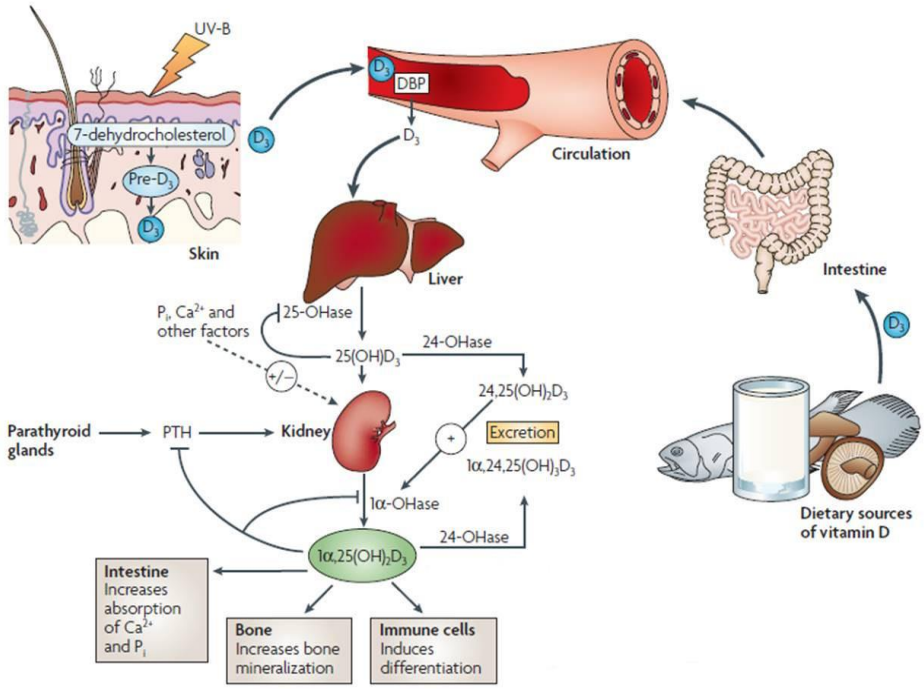

Figure 14. Vitamin D metabolism. Vitamin D_3 can be obtained from diet, but most of it is produced by photochemical synthesis at the level of the skin thanks to ultraviolet B (sunlight) exposure. Vitamin D_3 binds to vitamin D-binding protein (DBP) in the bloodstream and is transported to the liver. Vitamin D_3 is hydroxylated by liver 25-hydroxylases (25-OHase) to form 25-hydroxyvitamin D_3 ($25(OH)D_3$), which is then hydroxylated in position 1 in the kidney by the 1α-hydroxylase (1α-OHase), forming the active $1\alpha,25(OH)_2D_3$, which has different effects on various target tissues. The synthesis of $1\alpha,25(OH)_2D_3$ is stimulated by the parathyroid hormone (PTH) and suppressed by Ca^{2+}, inorganic phosphate (Pi) and $1\alpha,25(OH)_2D_3$ itself. The rate-limiting step in catabolism is the degradation of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ to $24,25(OH)_2D_3$ and $1\alpha,24,25(OH)_3D_3$, respectively, which occurs through 24-hydroxylation by 25-hydroxyvitamin D 24-hydroxylase (24-OHase), encoded by the CYP24A1 gene. $24,25(OH)D_3$ and $1\alpha,24,25(OH)_3D_3$ are then excreted. Modified from Deeb KK et al., 2007.

Vitamin D, in particular 1α,25$(OH)_2D_3$, exerts its effects by acting as a hormone by binding the vitamin D receptor (VDR). VDR is expressed in almost all the cell types of our body, with the highest expression in metabolic tissue, such as the kidneys, bones and intestine (Verstuyf A et al., 2010). It has been estimated that VDR directly or indirectly controls the transcription of thousands of protein-coding mRNAs as well as a huge amount of non-coding RNAs (Campbell MJ, 2014).

2.2.4.2. Vitamin D function on the immune system and its implication for diseases

The main known function of vitamin D is the regulation of calcium homeostasis and bone mineralization by controlling the blood levels of Ca^{2+} thanks to intestinal absorption and renal reabsorption (Carmeliet G et al. 2015). However, the fact that VDR is expressed in almost all the cells of our body highlights the importance of this vitamin beyond calcium homeostasis, suggesting that its deficiency is not only linked with bone health but also associated with the appearance of other diseases, such as cancer, cardiovascular disorders and autoimmune diseases.

Vitamin D is essential for a correct regulation of the immune system, since it controls both innate and adaptive immune responses. The importance of vitamin D is supported by the observation that VDR is expressed in several immune cells, especially macrophages and dendritic cells, but also in CD4+ and CD8+ T cells and B cells (Provvedini DM et al., 1983; Brennan A et al., 1987; Veldman CM et al, 2000). Vitamin D appears to control pathways which limit the antibacterial activity of the innate immune system, thus limiting potential inflammatory events. For example, *in vitro* vitamin D promotes the down-regulation of TLR2 and TLR4 expression on monocytes, reducing the responsiveness to PAMPs (Sadeghi K et al., 2006).

However, the role of vitamin D on immune function is highly complex and can also promote innate immune system responses. 1α,25$(OH)_2D_3$ can affect the innate response by upregulating the expression of the triggering receptor expressed on myeloid cells 1 (TREM1), a cell surface receptor that is expressed mainly on monocytes and neutrophils (Kim TH et al., 2013). 1,25$(OH)_2D_3$ may function as an enhancer of the innate immune response and also induce the antimicrobial peptide cathelicidin (Kim TH et al., 2013). Dendric cells also express VDR and vitamin D seems important in regulating cell maturation (Hewison M et al., 2003). VDR is also expressed in activated lymphocytes; after the activation by mitogens, *in vitro* 1α,25$(OH)_2D_3$ is a potent inhibitor of T cell proliferation (Bhalla AK et al., 1984). Vitamin D predominantly acts on the adaptive immune system by modulating the phenotype of T cells (Smolders J et al., 2009 a; Nanduri R et al., 2015).

The variety of functions exerted by vitamin D on several immune system cells suggests the

importance of this molecule in autoimmune diseases. Evidence supporting its implication in autoimmune disorders comes from *in vitro* studies and observations made directly on patients. *In vitro* 1α,25$(OH)_2D_3$ inhibits the expression of Th1 cytokines (Lemire JM et al., 1995) and promotes the development of a Th2 phenotype (Boonstra A et al., 2001). The administration of 1α,25$(OH)_2D_3$ in the mouse model of RA was observed to reduce the progression of the disease (Cantorna MT et al., 1998). VDR deficiency aggravated arthritis severity in human TNF-α transgenic mice (Zwerina K et al., 2011); in a mouse model of inflammatory bowel disease, the treatment with 1α,25$(OH)_2D_3$ decreased the symptoms thanks to its ability to down-regulate IL-17, TNF-α, IL-6, and IFN-γ levels and inhibit the activation Th1/Th17 cells in the colon and spleen (Zhang H et a., 2015). Moreover, treatment with 1α,25$(OH)_2D_3$ reduced the severity of systemic lupus erythematosus (SLE) in MRL/1 mice (Lemire JM et al., 1992). The supplementation of 1α,25$(OH)_2D_3$ could prevent the initiation and the progression of EAE, the experimental model of MS (Cantorna MT et al., 1996).

Furthermore, studies on patients suffering from autoimmune diseases support the importance of vitamin D in these disorders. In several autoimmune disorders, such as inflammatory bowel disease, type 1 diabetes and RA, patients have lower 25$(OH)D_3$ serum levels compared to heathy people (Lu C et al., 2015; Feng R et al., 2015; Lin J et al., 2016). Moreover, patients with lower 25$(OH)D_3$ levels have higher disease activity (Sahebari M et al., 2014; Lin J et al., 2016). It is not always easy to understand whether low vitamin D levels are a cause of autoimmune diseases or only a consequence. However, further evidence suggesting the importance of vitamin D levels in the susceptibility of autoimmune diseases comes from several studies that associate polymorphisms in the VDR genes to these disorders (Wang L et al., 2014; Tizaoui K et al., 2015; Zhou TB et al., 2015).

2.2.4.3. Vitamin D receptor

Vitamin D acts by binding to the VDR, with 1α,25$(OH)_2D_3$ having the highest affinity. VDR is an intracellular receptor that belongs to the steroid/thyroid nuclear receptor family. The N-terminal region includes the DNA binding domain; the ligand binding domain is located in the C-terminal region (Haussler MR et al., 1995) (Figure 15 A).

In the absence of 1α,25$(OH)_2D_3$, VDR can shuttle among the cytoplasm and the nucleus. Furthermore, it can form a heterodimer with the retinoid X receptor (RXR), which is able to bind to vitamin D response elements (VDRE). This heterodimer is not stable and can bind, only with low affinity, to the DNA; however, it allows the regulation of the basal level transcription of target genes. Only with the binding of 1α,25$(OH)_2D_3$ to the VDR does the dimer become stable and, based on the nature of VDRE, up or down regulates the transcription of the targeted genes (Smolders J et al., 2009 b) (Figure 15 B).

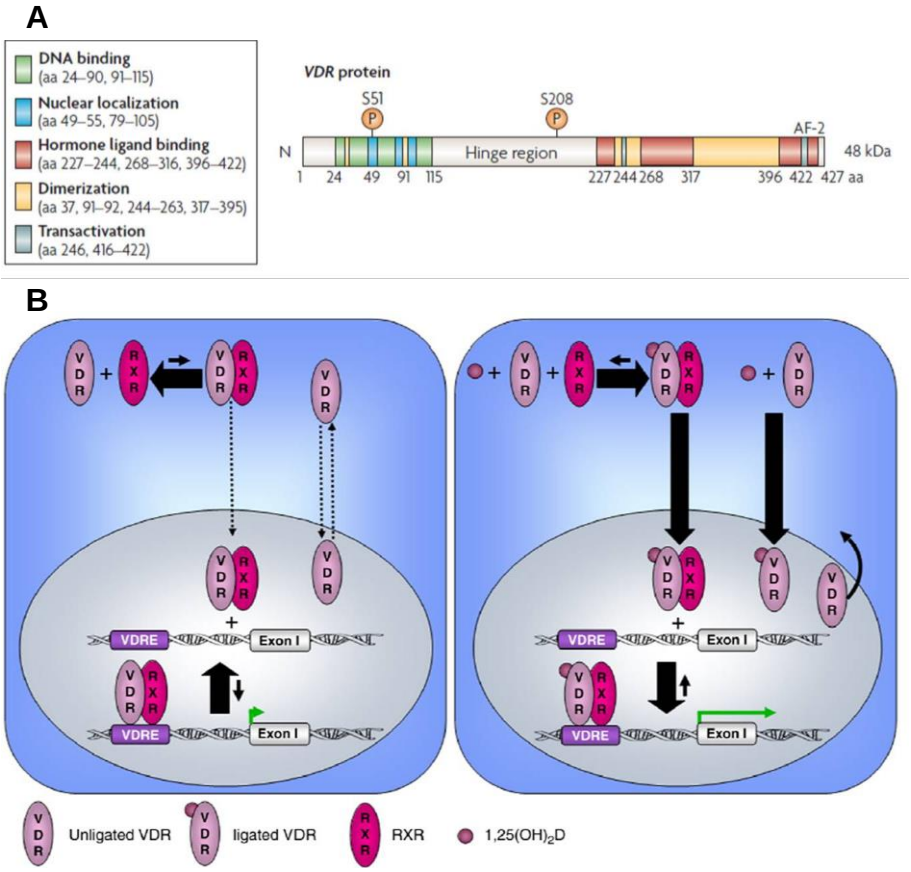

Figure 15. Structure and function of vitamin D receptor. A| Protein structure of VDR. The molecule has nuclear localization signals (blue) that direct the receptor into the nucleus. Upon 1α,25$(OH)_2D_3$ binding to the hormone ligand-binding domain (red), VDR is stabilized by the phosphorylation of serine 51 in the DNA-binding domain (green) and serine 208 in the hinge region. VDR associates with the retinoic acid receptor (RXR) through the dimerization domains (yellow). Modified from Deeb KK et al., 2007. **B|** Intracellular organisation of the VDR. On the left, in the absence of 1α,25$(OH)_2D_3$, VDR shuttles between the cytoplasm and nucleus. It dimerizes with RXR, forming an unstable heterodimer with low affinity for the VDRE. On the right, the binding of 1α,25$(OH)_2D_3$ activates VDR and translocates it to the nucleus. The VDR/RXR heterodimer is stabilised and has a high affinity for the VDRE, which results in an increased activation or repression of the controlled genes. From Smolders J et al., 2009 b.

The binding of VDR with 1α,25$(OH)_2D_3$ also increases the affinity with different cofactors

that act as a bridge between the heterodimer and the transcription machinery, thereby allowing transcription. These cofactors can be activators, which mediate the induction of the transcription, or repressors, which suppress the expression of genes (Tagami T et al., 1998). These proteins are important because they have chromatin-modifying enzymatic activities or act as a platform for the recruitment of histone-destabilizing/stabilizing enzymes.

The VDR gene is located on chromosome 12 (12q13.11) and contains 9 exons. More than 30 polymorphisms have been identified inside this gene (Smolders J et al., 2009 b). However, only a few have been studied in human disorders, and some have been related to the presence and/or the severity of many diseases, in particular autoimmune diseases (Figure 16).

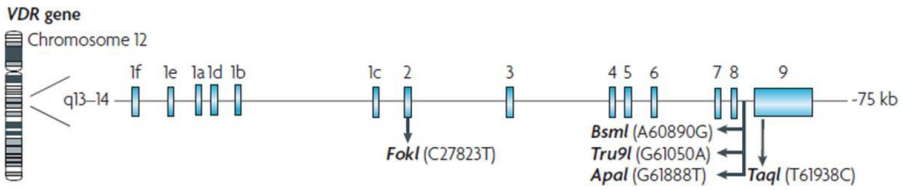

Figure 16. Vitamin D receptor gene. The human VDR gene is located on chromosome 12 and composed of promoter and regulatory regions (1a–1f) and exons 2–9, which encode the VDR protein. The figure also shows some SNPs in VDR studied in human diseases; these are defined by restriction enzymes, and the nucleotide change is indicated in parentheses. From: Deeb KK et al., 2007.

A polymorphism can potentially influence the effect of vitamin D on immune system function, as is the case of the Fok-I polymorphism (rs2228570). The polymorphism causes a substitution from T to C in exon 2, introducing a second start site which leads to the production of a longer protein (Gross C et al., 1998). The shorter isoform has been associated with a higher transcriptional activity (Jurutka PW et al., 2000), but not all the studies have obtained the same result (Gross C et al., 1998). The study by Jurutka and collaborators reported that the short isoform interacts more efficiently with a transcriptional coactivator (TFIIB) (Jurutka PW et al., 2000). Moreover, Fok-I polymorphism has been studied in relation to the ability of $1\alpha,25(OH)_2D_3$ to decrease the proliferation of peripheral blood mononuclear cells (PBMCs) *in vitro* (van Etten E et al., 2007). Furthermore, it suppresses IL-12 production by monocytes and dendritic cells (van Etten E et al., 2007). It seems that this polymorphism has consequences for both the VDR protein structure and its transcriptional activity.

However, understanding the consequences of a polymorphism for VDR function and

activity, and the possible implication for immune system function, is not easy. For example, Apa-I (rs7975232) and Bsm-I (rs1544410), which cause a substitution from G to T and from A to G respectively, are located in an intron flanked by exons 8 and 9 and do not have consequences on protein structure. A polymorphism can be located in an exon but cause a silent codon change without modifying the amino acid sequence. This is the case, for example, of the Taq-I (rs731236) polymorphism, which causes a substitution from T to C (Uitterlinden AG et al., 2004). Although these polymorphisms do not alter VDR protein, their variants can be in linkage disequilibrium with other polymorphisms, thus having potential effects (Uitterlinden AG et al., 2004).

The VDR polymorphisms have been studied in MS. The polymorphisms mentioned above are the most studied. Regarding the Fok-I polymorphism in MS, a British study reported an association between the long isoform and a decreased level of disability 10 years after MS onset (Mamutse G et al., 2008). However, the association was not found in a study on subjects from the Netherlands (Smolders J et al., 2009 c). Moreover, the short isoform was associated with lower $25(OH)D_3$ serum levels both in MS patients and in healthy controls (Smolders J et al., 2009 b). The association of Apa-I and Bsm-I with MS was reported in the Japanese population (Niino M et al., 2000). For these two polymorphisms, the presence of the restriction site is reported as a lowercase letter and its absence in an uppercase letter (a and A for Apa-I, b and B for BsmI). AA and bb genotypes are more frequent in MS patients compared with healthy controls. The involvement of Apa-I polymorphism in MS risk was also reported in an Australian population, with the A allele more frequent in MS patients compared to healthy controls (Tajouri L et al., 2005). However, the association of Apa-I and Bsm-I polymorphisms was not reported in other populations (Simon KC et al., 2010; Sioka C et al., 2011). Taq-I polymorphism was related to MS risk, with the t allele more frequent in MS patients (Tajouri L et al., 2005). However, in this case as well some studies did not find the association (Dickinson JL et al., 2009; Sioka C et al., 2011). Furthermore, a meta-analysis reported no association between MS and VDR polymorphisms (Fok-I, Apa-I, Bsm-I and Taq-I) (Huang J and Xie ZF, 2012).

Moreover, not only can VDR polymorphisms influence MS risk, but variants in other genes involved in vitamin D metabolism may also play a role, highlighting the complex effect of vitamin D on MS susceptibility. For example, alterations that modify vitamin D levels may be important for MS susceptibility. A GWAS identified CYP27B1, which codes for 1α-hydroxylase, and CYP24A1, which encodes the enzyme responsible for initiating calcitriol degradation, as genes involved in MS risk (International Multiple Sclerosis Genetics Consortium et al., 2011). Furthermore, polymorphisms in CYP27B1 seem to affect $1\alpha,25(OH)_2D_3$ circulating levels (Orton SM et al., 2008) and polymorphisms in DBP also influence circulating $25(OH)D_3$ concentrations (Sinotte M et al., 2009), having a potential effect on immune regulation. The variation of vitamin D circulating levels was also related to VDR polymorphisms: Fok-I was associated with serum $25(OH)D_3$ levels in twins with

MS (Orton SM et al., 2008) and with $1\alpha,25(OH)_2D_3$ levels (Smolders J et al., 2009 c).

2.2.5. Oxidative stress in multiple sclerosis pathogenesis

Oxidative stress is commonly implicated in the development of brain damage; it has been well demonstrated that ROS contribute to the pathogenesis of several neurodegenerative diseases, such as PD and AD (Lin MT and Beal MF, 2006). The brain is particularly vulnerable to oxidative stress, as it exhibits high oxygen consumption and is rich in polyunsaturated fatty acids, which are more susceptible to peroxidation. Furthermore, the brain expresses lower levels of antioxidant enzymes compared to other tissues (Montine KS et al., 2004). Oxidative stress also contributes to MS pathogenesis, taking part in lesion formation and disease progression.

The chemical compounds inducing oxidative stress and thus contributing to cell damage are classified as reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are unstable highly reactive molecules which exist mostly in a radical form, since they have an unpaired electron. The best studied ROS include radicals of oxygen, like superoxide anion (O^{2-}), hydroxyl radical ($OH\cdot$), peoxiradicals ($ROO\cdot$), and non-radical species, such as hydrogen peroxide (H_2O_2). Among RNS, nitric oxide ($NO\cdot$) can react with superoxide anion to form peroxynitrite ($ONOO^-$), the most deleterious free radical. At physiological concentrations, ROS and RNS act as second messengers, participating in intracellular signalling (Reth M, 2002); furthermore, ROS produced in higher concentrations by immune cells can kill pathogens (Leto TL and Geiszt M, 2006).

ROS are produced during oxidative phosphorylation in mitochondria, whereby a small percentage of electrons are transferred from the electron transport chain directly to oxygen, forming ROS as undesirable products (Cadenas E et al., 1977).

When ROS production exceeds a threshold, these compounds are toxic and induce oxidative stress, thus damaging cellular biomolecules (lipids, proteins, carbohydrates and nucleic acids), leading ultimately to cell death.

The cells can respond to oxidative stress thanks to several mechanisms that neutralize the excess of ROS and RNS, thus protecting cells against their deleterious effects.

The expression of antioxidant enzyme is induced by the elevated levels of ROS and RNS. The central player in the control of oxidative status is the nuclear factor erythroid 2-related factor 2 (Nrf2). In conditions of oxidative stress, the transcriptional factor Nrf2 can enter inside the nucleus and recognize the antioxidant response elements, thus inducing the expression of a multitude of antioxidant proteins, including enzymes involved in glutathione synthesis and in the thioredoxin system: superoxide dismuntases 1 and 2 (SOD1 and SOD2), catalase and heme oxygenase 1 (HMOX1) (Kensler TW et al., 2007). Furthermore, Nrf2 regulates the expression of molecular chaperons and is involved in

several other cellular processes, such as differentiation, proliferation, inflammation and lipid synthesis (Deshmukh P et al., 2017).

However, when metabolic processes or toxic insults outbalance anti-oxidant defences, oxidative stress is assessed, a condition found in MS plaques. Studies on *post mortem* brain tissues of MS patients revealed that, in the active lesions, demyelination and neurodegeneration are closely associated with the presence of oxidized lipids (Haider L et al., 2011; Fischer MT et al., 2013). Moreover, the nuclei of dystrophic glia cells and neurons contain oxidized DNA (Haider L et al., 2011). Neurons with intense cytoplasmic accumulation of oxidized phospholipids and DNA breaks are present in active cortical lesions (Fischer MT et al., 2013). A recent study described the presence of increased RNA oxidation in the normal-appearing cortex of the MS brain, leading the authors to suggest that RNA oxidation, induced by oxidative stress, may contribute to MS pathogenesis by producing aberrant proteins or by dysregulating the target gene expression (Kharel P et al., 2016). Furthermore, several proteins can be oxidised in MS.

Recent studies have focused on the search for potential markers of oxidative stress in order to correlate them with MS progression. A study reported increased levels of oxidative stress in erythrocytes in CIS, RRMS and SPMS patients, and this may correlate with the expanded disability status scale (EDSS) and radiological findings (Ljubisavljevic S et al., 2014). Furthermore, inflammation and oxidative and nitrosative stress biomarkers may be potential predictive biomarkers of high disability in MS; they have been associated with several aspects of disease progression (Kallaur AP et al., 2017). It has been observed that metabolites of NO (nitrites and nitrates) are significantly higher in the sera of patients with RRMS compared to healthy controls (Ortiz GG et al., 2009).

The importance of oxidative stress in MS pathogenesis has also been highlighted by studies on the animal model. Macrophages and microglial cells, isolated from the CNS of rats with clinical signs of EAE, exhibit elevated ROS levels compared to cells isolated from healthy rats or from animals sacrificed before clinical EAE manifestation (Ruuls SR et al., 1995). An *in vivo* imaging study revealed that the application of oxygen and nitrogen donors to the spinal cord of healthy mice was sufficient to induce EAE-like axonal injuries in the absence of demyelination, suggesting that demyelination may not be a prerequisite for axon damage (Nikić I et al., 2011). On the other hand, the treatment of EAE mice with scavengers, which reduced spinal ROS and RNS levels, limits focal axonal degeneration progression without altering the number of immune cells in acute EAE lesions (Nikić I et al., 2011).

2.2.5.1. Sources of oxidative stress during multiple sclerosis pathogenesis

The **inflammatory process** typical of MS is critically linked to oxidative stress, and thus to ROS-mediated tissue injury. Immune cells are an important source of ROS. It has been

observed that ROS are produced through the interaction of monocytes with the endothelium of the BBB, inducing alteration of tight-junction and cytoskeleton rearrangements, thus contributing to the loss of BBB integrity and the extravasation of immune system cells into the CNS (Van der Goes A et al., 2001) (Figure 17). Once infiltrated inside the CNS, leukocytes produce high amounts of ROS and RNS. Activated macrophages, together with the resident microglia, synthetize ROS and RNS thanks to enzymes such as myeloperoxidase, NADPH oxidases (NOX) and inducible nitric oxide synthase (iNOS) (Gray E et al., 2008; Fischer MT et al., 2012). The upregulation of NOX subunits has been described in the microglia present in active and slowly expanding lesions (Fischer MT et al., 2012); several studies reported that iNOS is upregulated in MS lesions and in the CSF of MS patients (Bagasra O et al., 1995; Calabrese V et al., 2002). ROS and RNS produced by immune cells during inflammatory response can damage the surrounding cells, a condition described in several autoimmune disorders (Kröncke KD et al., 1991; Fehsel K et al., 1993).

In the context of MS, ROS and RNS can trigger axonal injury, ultimately contributing to neuronal death (Nikić I et al., 2011). Olygodendrocytes are highly susceptible to oxidative damage, as they have high levels of polyunsaturated fatty acids that can be peroxidised. The lipid peroxidation is a chain reaction that, if not rapidly inhibited, can lead to the destruction of cellular membranes and myelin. Lipid peroxidation can play an important role in MS pathogenesis because it alters membrane structure and its chemical characteristics such as permeability (Jana A and Pahan K, 2007), further damaging neurons and olygodendrocytes, which are already influenced by the inflammatory process (Figure 17) (Gonsette RE, 2008).

ROS also have a central role in signalling transduction, which leads to the increased expression of pro-inflammatory genes; increased levels of cytokines and chemokine are able to recruit macrophages and stimulate myelin phagocytosis (Van der Goes A et al., 1998; Ortiz GG et al., 2013). ROS have also been described as inhibiting the expression of the genes for the myelin in human primary oligodendrocytes. Furthermore, oxidative stress compromises the differentiation of OPCs due to epigenetic mechanisms that alter the expression of genes that are important for maturation (French HM et al., 2009).

In the subsequent phase of MS pathogenesis, when the inflammatory process is reduced, other non-inflammatory mechanisms may contribute to ROS formation. Chronic inflammation increases the risk of mitochondrial damage. In turn, damaged mitochondria contribute to the development of an oxidative environment. **Mitochondrial injury** with subsequent energy failure is one of the main non-immune mechanisms contributing to MS pathogenesis (Figure 17).

The oxidative stress established in the area characterized by inflammation can lead to mitochondrial dysfunctions due to several mechanisms. ROS can alter the functioning of mitochondrial enzymes and modify their proteins, leading to degradation. ROS disrupt the

transport of adenosine triphosphate to the axons, contributing to neurodegeneration (Errea O et al., 2015). Furthermore, ROS can influence the synthesis of new components in the respiratory chain by inducing mutations in the mDNA (Campbell GR et al., 2011).

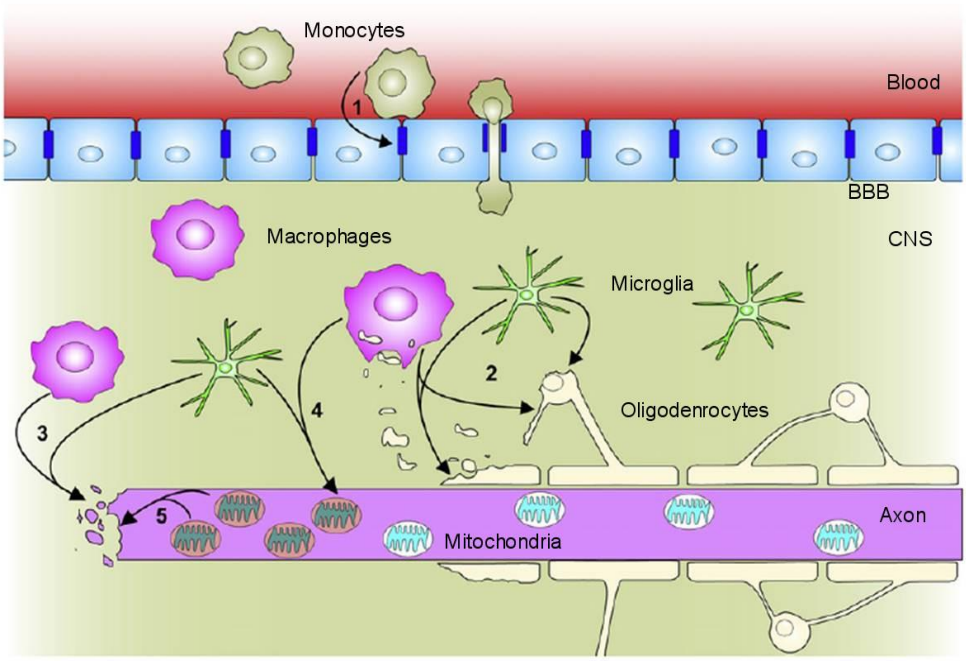

Figure 17. Role of ROS in multiple sclerosis pathogenesis. 1) Upon interaction with the BBB, monocytes produce ROS, which facilitate their migration. 2) Once inside the brain, macrophages, together with the activated microglia, produce vast amounts of ROS and RNS, which induce demyelination and oligodendrocyte cell death. Moreover, ROS facilitate myelin degradation by macrophages. 3) In the inflammatory phase of MS, ROS produced by macrophages and microglia mediate axonal degeneration. 4) Furthermore, they contribute to mitochondrial dysfunction, which ultimately leads to increased ROS production and decreased ATP production within demyelinated axons. 5) Due to increased axonal energy demand, mitochondria accumulate in chronically demyelinated axons. They continue to produce ROS also in chronically demyelinated axons, further contributing to axonal injury. Modified from: van Horssen J et al., 2011.

In chronic inactive lesions, the number of mitochondria is higher, possibly in response to a higher energy demand (Mahad DJ et al., 2009). The higher number of mitochondria in chronic lesions contribute to an increase in ROS formation, which in turn damages mitochondria and reduces ATP levels (Andrews HE et al., 2005), thus initiating a vicious

circle.
Therefore, oxidative stress may contribute to MS pathogenesis in both relapsing-remitting and progressive forms through the involvement in inflammation and in axonal degeneration, respectively.

2.2.6.Heat shock proteins

Oxidative stress can damage cellular proteins, thus leading to the formation of misfolded proteins and to the alteration in their stability and function. It is necessary for the cell to maintain the functional proteome, which is possible thanks to systems which control the proteome quality. Under conditions of proteotoxic stress, several proteins identify unfolded, misfolded and non-native polypeptides, in order to rescue them or, if this is not possible, degrade them. Important for these controls are the molecular chaperons, which prevent protein aggregation, refold unfolded proteins, or drive them to degradation through the proteasome. In conditions of oxidative stress, the expression and activity of molecular chaperons is tightly regulated, both at the transcriptional and post-translational levels (Niforou K et al., 2014).
In terms of oxidative stress, the heat shock proteins (Hsps), a family of proteins involved in protein folding and remodelling, which includes constitutive and stress-inducible members, are important. These proteins were first described in response to heat shock (Ritossa FA, 1962), but now it is clear that their expression can be induced by a plethora of stimuli, including ischemia, the presence of heavy metals, irradiation, oxidative stress, nutrient deprivation, infections and inflammation (Welch WJ, 1993; Jäättelä M, 1999). In general, they support the correct folding of newly synthesized proteins, contribute to protein translocation across different cellular compartments, mediate the assembly of protein complexes, and prevent protein aggregation (Saibil H, 2013). Hsps have cytoprotective functions, since they are involved in the refolding of misfolded proteins, protect against protein aggregates, and direct irreversibly damaged proteins to degradation (Turturici G et al., 2011).
Hsps are located in almost all the cellular compartments and can be secreted in the extracellular environment, where they have cytokinic functions (Asea A et al., 2000). Hsps are also expressed in the CNS. However, assessing their precise role is complicated: several cell types, including neurons, microglia, oligodendrocytes and astrocytes, express Hsp, but they exhibit distinct activation of different Hsps (Foster JA and Brown IR, 1997).
The transcription of HSP genes is under the control of the heat shock transcription factor 1 (HSF1) (Page TJ et al., 2006). HSF1 is constitutively expressed in most of the tissues; in the monomeric inactive form, it is located in the cytoplasm and interacts with Hsp90. Following stimulatory signals, HSF1 dissociates from Hsp90, forms an homo-trimer and,

after the phosphorylation of specific serine residues, migrates into the nucleus (Akerfelt M et al., 2010), where it can recognise specific sequences called heat shock elements (HSE), which are located in the promoter of specific genes (Akerfelt M et al., 2010).

Many HSP genes have alternative promoters which allow for their activation through other signalling pathways. Moreover, Hsp production is not only regulated at the transcriptional level: post-transcriptional modifications such as carbonylation, phosphorylation and protein-protein interaction are important for an immediate response to variations in the environment to permit cell survival (Niforou K et al., 2014).

Hsps are classified by molecular weight into different families: Hsp110, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsp families.

The Hsp110 family includes proteins that function mostly as nucleotide exchange factors for Hsp70 members, thus acting as co-regulators of the Hsp70 chaperone function (Raviol H et al., 2006).

The Hsp90 family includes both constitutive and stress-inducible members found in various subcellular locations, like cytosol, endoplasmic reticulum (ER) and mitochondria. Members of the Hsp90 and the Hsp70 families interact, influencing in a coordinated way several cellular functions (Stetler RA et al., 2010).

The Hsp70 family is the most studied and includes constitutively expressed proteins and inducible members located in several cellular compartments.

The majority of Hsp60 family members are in the mitochondria, where they are involved in the folding of a subset of mitochondrial proteins. Some members are found in the cytosol, on the surface of non-neuronal cells, and can also be secreted in the extracellular environment (Gupta RS et al., 2008; Stefano L et al., 2009).

Hsp40 family members act as co-chaperons, modulating and controlling Hsp90 and Hsp70 members (Stetler RA et al., 2010).

The small Hsp family includes 11 members poorly conserved in sequence, all of which have a conserved domain in the C terminus. In contrast to other chaperones, these proteins do not appear to require ATP for their functioning (Stetler RA et al., 2009). This family includes proteins like HMOX1 and the αB-crystalline.

Hsps are not only molecular chaperons, but they play several roles at nuclear level as well, acting also as a transcriptional element. Hsp90 members are important for the negative control of HSF1, inhibiting its binding to the DNA (Taylor DM et al., 2007). Furthermore,several Hsp members can have anti-apoptotic functions. Hsp70 and Hsp90 members form a complex with the apoptotic peptidase activating factor-1 to inhibit apoptosome formation (Pandey P et al., 2000; Saleh A et al., 2000).

Hsps are also involved in several pathologic states, like cerebral ischemia, trauma, epilepsy and neurodegenerative diseases (Yenari MA, 2002). In CNS disorders, Hsps seem to exert neuroprotective roles, preventing the aggregation of misfolded proteins and inducing anti-apoptotic mechanisms (Stetler RA et al., 2010).

2.2.6.1. Hsp70

The heat shock protein 70 (Hsp70) family represents the most widely studied group of Hsps and includes several monomeric proteins that are highly conserved in evolution with a molecular weight of about 70 kDa (Radons J, 2016). Hsp70s are found in all organisms, from archaebacterial to plants and humans; the prokaryotic DnaK shares approximately 50% amino acid identity with eukaryotic Hsp70s. Hsp70s have also conserved functional properties across the species; for example, experiments demonstrated that Drosophila Hsp70 expressed in mammalian cells efficiently protects them against heat stress (Pelham HR, 1984), and rodent Hsp70 can be functionally complimented by human Hsp70, allowing for protection against different types of stress (Li GC et al., 1991; Plumier JC et al., 1995).

The human Hsp70 family includes some constitutively expressed members, while others are stress-inducible. The different members have specific functions and differ with regard to their expression levels and localization; they play critical roles in mitochondria, ER, lysosomes, cytosol, and cell membranes and can also be secreted in the extracellular environment. For example, the constitutively expressed protein called Hsc70 is cytosolic, Grp75 is a mitochondrial protein, and Grp78 is located in the ER. Several members of the Hsp70 family can shuttle between compartments. Hsp70-1 is primarily cytosolic but can also be expressed in the luminal side of the lysosomal membrane, especially under stress conditions, to stabilize the membrane and facilitate the import of degraded proteins. Grp78 is the primary ER chaperone, but an alternative splice variant can also be found in the cytosol, enabling leukocytes to survive under stress conditions (Ni M et al., 2009). Similarly, Grp75, which is the major mitochondrial chaperone, can also be found in other cellular compartments such as ER and cytosol (Ran Q et al., 2000).

In addition to their central role as molecular chaperones, Hsp70 proteins have other functions. Hsp70s have anti-apoptotic functions, blocking apoptosis at various levels. Hsp70s act on Bax, blocking its translocation to mitochondria and the activation of this pro-apoptotic protein (Yang X et al., 2012). Furthermore, these proteins can inhibit the assembly of the death-inducing signalling complex (Guo F et al. 2005).

Hsp70s are also involved in the modulation of immune response. Increased intracellular Hsp70 levels augment cell tolerance to pro-inflammatory cytokines such as TNF-α and IL-1 (Jäättelä M and Wissing D, 1993). On the other hand, when Hsp70s are on the cell surface and released into the extracellular environment, for example, during viral infection or necrosis, they can stimulate immune response. The role of Hsp70 as a facilitator of the immune response has been demonstrated both *in vitro* and *in vivo* (Wells AD and Malkovsky M, 2000; Srivastava P, 2002). Hsp70s act as cytokines, inducing the maturation of APCs and the activation of dendritic cells, while also attracting NK cells (Multhoff G et al., 1999; Wan T et al., 2004). Furthermore, Hsp70s induce the immune response by interacting with Toll-like receptors (Asea A et al., 2002), permitting the activation of the

transcription factor Nf-kB and inducing the production of proinflammatory cytokines, chemokines and NO by macrophages (Panjwani NN et al., 2002). Hsp70s enhance antigen presentation by MHC class I and class II molecules (Mycko MP et al., 2004). Furthermore, the Hsp70-associated peptides bound to MCH class I and II molecules are more immunogenic than peptides alone (Chen D and Androlewicz MJ, 2001).

Hsp70s are highly conserved molecules with a common structure. They are composed of a 44 kDa N-terminal domain, called nucleotide binding domain (NBD), which binds and hydrolyses ATP, and a 28 kDa C-terminal domain, known as substrate binding domain (SBD), which binds polypeptides. Cytosolic Hsp70 contains a G/P-rich C-terminal region harbouring an EEVD motif involved in the binding of co-chaperones and other Hsps, whereas specialized proteins such as HspA5, located in the ER, and HspA9, located in the mitochondria, do not have this motif but instead a N-terminal localization signal (Hartl FU, 1996) (Figure 18).

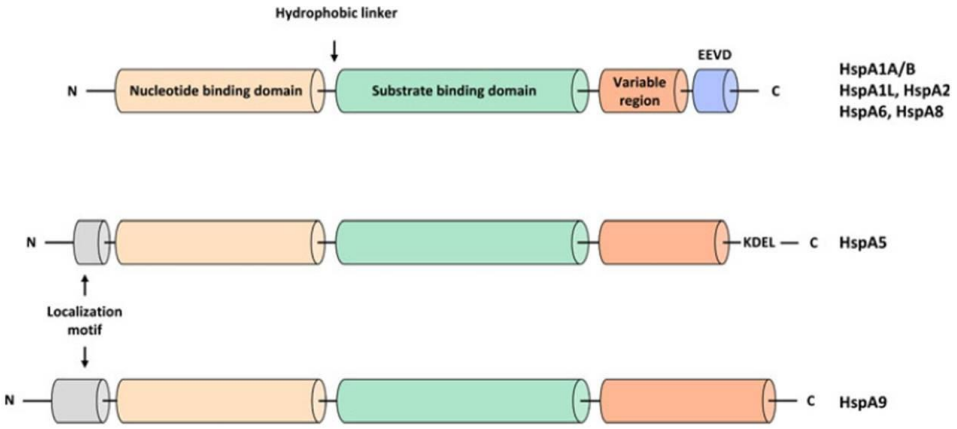

Figure 18. Hsp70 protein structure. Hsp70s are formed by an N-terminal nucleotide binding domain (NBD) and a substrate binding domain (SBD). The hydrophobic linker, connecting the NBD and the SBD, is found in most of the Hsp70 family members. The C-terminal EEVD motif is involved in the binding of co-chaperones and other Hsp proteins. Some Hsp70 proteins have a localization signal; for example, HspA5 is expressed in the endoplasmic reticulum and HspA9 is located in the mitochondria. The C-terminal ER retention signal (KDEL: lysine, aspartic acid, glutamic acid, leucine) is presented in HspA5. From: Radons J, 2016.

Many of the functions of Hsp70 depend on the crosstalk between NBD and SBD, since the binding with ATP influences the substrate binding. Hsp70s can have three different conformations: one in absence of ATP/ADP, one with ADP bound, and one with ATP bound. The ATP binding allows the interaction between the SBD and the polypeptide, but

the binding is not stable and the substrate can easily dissociate. ATP is then hydrolysed and the ADP-bound state increases the affinity for the substrate, closing the SBD (Mayer MP et al., 2000).

The activity and function of the chaperones are influenced by the binding of co-chaperons, including members of Hsp110 and Hsp40 families (Kelley WL, 1998; Dragovic Z et al., 2006).

Human Hsp70s are encoded by a multigene family, which includes up to 17 genes and 30 pseudogenes. Hsp70 genes express a high number of mRNA variants and protein isoform, but it is unclear whether these variants correspond to proteins with distinct functions (Brocchieri L et al., 2008). The HSP70 gene family is the result of multiple duplications facilitated by retrotransposition events of a single highly expressed gene: HSPA8 (Brocchieri L et al., 2008). HSP70 genes are distributed among several chromosomes: 1, 4, 5, 6, 9, 10, 11, 13, 14, 20 and 21, irrespective of their evolutionary relations, except for the pair of genes HSPA6 and HSPA7 on chromosome 1 and the triad of genes HSPA1A, HSPA1B and HSPA1L in close proximity on chromosome 6 (Brocchieri L et al., 2008).

HSPA1A, HSPA1B and HSPA1L are in the MHC class III region, on the short arm of chromosome 6p21.3, clustering between the genes for complement components and TNF (Milner CM and Campbell RD, 1990). HSPA1A and HSPA1B code for the major stress inducible Hsp70s, called Hsp70-1 and Hsp70-2 (Daugaard M et al., 2007). The two proteins, composed of 641 amino acids, differ only with respect to two amino acids and are more than 99% identical (Daugaard M et al., 2007). The two genes differ more regarding the upstream regulatory sequences, which influence basal expression and are required to provide an adequate response under stress conditions (Milner CM and Campbell RD, 1990). During stress conditions, HSPA1A and HSPA1B are activated by the binding of HSF1 to multiple copies of HSE in the upstream regulatory regions (Daugaard M et al., 2007). HSPA1L is an intronless gene which encodes a protein called Hsp70-Hom, 91% identical to Hsp70-1. HSPA1L does not contain HSE in its promoter and is constitutively expressed at high levels in testis and at lower levels in other tissues (Daugaard M et al., 2007).

These genes are less polymorphic than many other genes located in the MHC region. Most of the polymorphisms are located in the coding regions, but many cause silent mutations. However, some polymorphic variants may influence gene function and the response of genes to stress (Favatier F et al., 1997; Wu YR et al., 2004), suggesting also a possible relation with the altered immune response of MS. To underline the possible role of MHC class III polymorphisms, in particular of HSP70 genes, in MS, our research group has recently demonstrated that HSPA1B rs1061581 polymorphism is associated with an increased risk of developing MS (Boiocchi C et al., 2014). This polymorphism, located in the coding region, is silent (Goate AM et al., 1987), and several studies associated it to different autoimmune disorders, such as SLE and type II diabetes (Pablos JL et al., 1995;

Zouari Bouassida K et al., 2004).
Other polymorphisms can change the amino acid sequence; for example, polymorphisms in the HLA1L are mainly located in the region which encodes the SBD. Among these, the HSP70-HOM rs2227956 causes a Met to Thr amino acid substitution at position 493, located in the SBD (Milner CM and Campbell RD, 1992). This substitution seems to be associated with the variation in the peptide binding specificity, thus affecting the Hsp70-Hom biologic function. Data from the literature have shown a link between the HSP70-HOM polymorphism and the risk of autoimmune diseases such as SLE (Fürnrohr BG et al., 2010).

2.2.6.2. Role of Hsp70 proteins in multiple sclerosis

The role of Hsps in MS is not clear, but it is known that Hsp70s are related to neurodegeneration and to immune system regulation, key elements of MS pathogenesis.
Several studies have revealed that Hsp70s have a neuroprotective role in preventing protein aggregation and inducing anti-apoptotic mechanisms. The accumulation of abnormal protein aggregates is a common histopathological hallmark of several neurodegenerative disorders. Neurons are vulnerable to the accumulation of misfolded proteins; the aggregates are recognized by the neurons, which try to prevent the accumulation.
Following stress conditions, the induction of Hsp70 expression in the CNS, in particular in reactive astrocytes, oligodendrocytes and microglia, has been described (Satoh J and Kim SU, 1994; Foster JA and Brown IR, 1997). Several studies have demonstrated that the activation of cell responses to stress conditions and the subsequent Hsp70 overexpression have beneficial effects in several models of neurodegenerative disorders, such as AD and PD. When exogenous Hsp70s are administrated to rat microglial cultures, they induce microglia activation that may facilitate Aβ clearance (Kakimura J et al., 2002). Furthermore, a study points out that Hsp70s interact with several Aβ structures (oligomers, fibrils) and that Hsp70-mediated inhibition of protein aggregation is stronger in the early stages of the formation of Aβ aggregates (Evans CG et al., 2006). In mouse models of PD, Hsp70 overexpression reduced α-Syn accumulation and toxicity (Klucken J et al., 2004). *In vitro* aggregation experiments have demonstrated that Hsp70s inhibit fibril formation (Huang C et al., 2006).
Based on these experiments, the use of Hsp70s in the treatment of neurodegenerative disorders was hypothesised, and a way to pharmacologically induce Hsp70 overexpression was researched. Several compounds have been tested to induce Hsp70 expression; for example, arimoclomol, which acts as a co-inducer of Hsp expression, improves neuromuscular function and extends the lifespan of mSOD1 mice, a model of ALS (Kieran D et al., 2004). Geldanamycin can disrupt the complex between Hsp90 and HSF1, which

results in the induction of Hsp70 (Knowlton AA and Sun L, 2001). This compound can inhibit huntingtin aggregation in a cell culture model of HD (Sittler A et al., 2001). However, geldanamycin, even at low concentrations, is toxic for cells, and this may limit its potential use for long-term treatments (Supko JG et al., 1995). Celastrol is a Hsp90 inhibitor that can activate HSF1 and upregulate HSP gene expression (Zhang T et al., 2008). Its neuroprotective effects may be due to Hsp70 induction and the prevention of NF-κB activation, which reduce the release of pro-inflammatory cytokines and astrogliosis (Cleren C et al., 2005).

However, Hsp70 overexpression is not always beneficial in all instances. An increase in intracellular Hsp in vitro is not always beneficial for the survival of motoneurons (Kalmar B and Greensmith L, 2009). These findings are to be taken into consideration when the upregulation of Hsp levels is chosen as a potential therapy for neurodegenerative disorders.

In MS, the increased expression of several Hsps, including Hsp70, has been described in the lesions of MS patients and in EAE models, following the inflammation and the oxidative stress that is created (Aquino DA et al., 1993; Chabas D et al., 2001). Myelin isolated from active plaques contained three to four times more Hsc70 than did normal myelin; Hsp70s are present in MS myelin, although they are not detected in normal myelin (Aquino DA et al., 1997). These findings suggest that in MS the immune-mediated white matter destruction may be associated with the altered distribution and expression of Hsp70s.

It is important to consider that Hsp70 is also an extracellular protein. In the CNS, glial cells release Hsp70; neurones which express Hsc70, but are not able to produce high amount of Hsp70 following stress conditions (Brown IR, 1991), can take up the released Hsp70. In this way, glial cells protect adjacent neurons, inhibiting cell death during stress conditions (Guzhova I et al., 2001). This fact suggests that the supply of exogenous Hsp70 into the CNS may be a potential therapeutic strategy to reduce neuronal death in neurodegenerative diseases. It seems that the increased Hsp70 levels in MS plaques can protect neurons from the inflammatory environment (Figure 19). The inflammation characteristic of the early phase of MS may act as a stimulus to induce the release of Hsp70 from glial cells to protect neurons in the subsequent neurodegenerative phase. This hypothesis is supported by the observation that the stimulation of human oligodendrocytes with proinflammatory cytokines induces Hsp70 expression (D'Souza SD et al., 1994).

It is also essential to consider that Hsp70s can be released in the extracellular environment like cytokines, stimulating both the innate and the adaptive immune response, inducing the production of pro-inflammatory molecules (Fleshner M and Johnson JD, 2005). Hsp70s may lead to the formation of additional antigenic targets at the lesion level, with the subsequent amplification of the immune response. In MS lesions Hsp70s were associated with MBP and PLP in the CNS of MS patients, but not in the heathy tissue (Cwiklinska H et al., 2003).

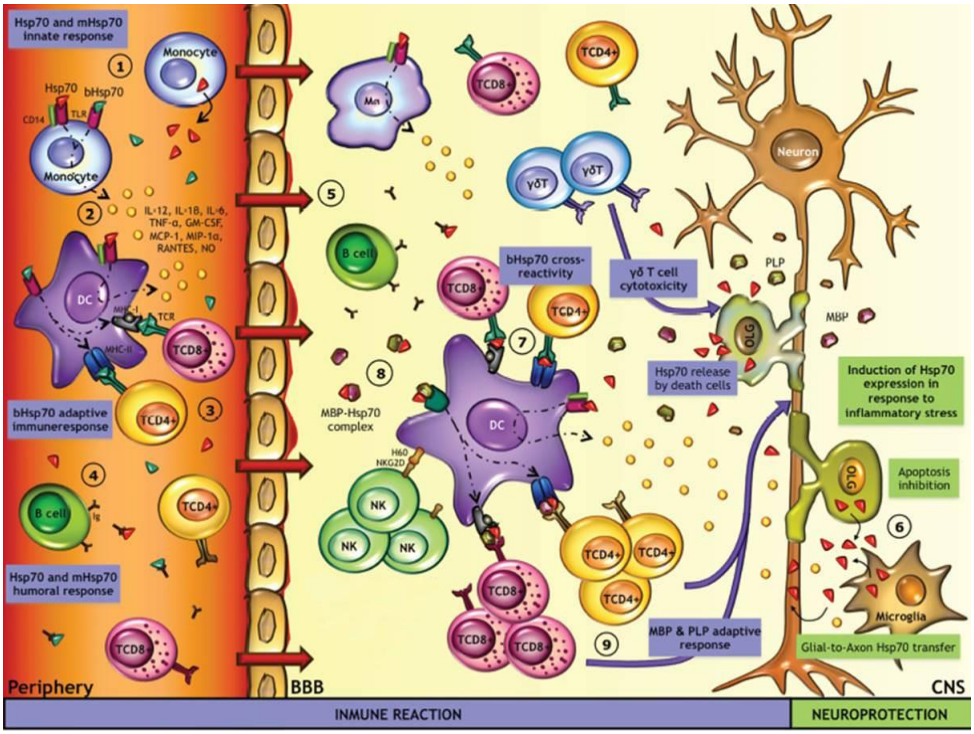

Figure 19. Proposed model for the role of Hsp70 in multiple sclerosis pathogenesis. The role of Hsp70 in MS is still unclear: it seems to have a negative effect, increasing the autoimmune response, but it can also have neuroprotective functions. 1) In the periphery, a stressful insult can induce Hsp70 expression and its release by immune cells, acting as a pro-inflammatory cytokine. 2) Endogenous Hsp70 and bacterial Hsp70 (bHsp70) may engage cell-surface signalling receptors, such as Toll-like receptors, to induce cytokines, chemokines and NO production by DC and monocyte. 3) bHsp70 is processed intracellularly and presented by MHC class I and II molecules by APCs, leading to the generation of bHsp70-specific T cells and 4) the production of anti-bHsp70 antibodies. 5) Despite the presence of the BBB, leukocytes can reach the CNS. 6) In the CNS, the inflammatory environment triggers Hsp70 expression. 7) bHsp70-specific lymphocytes may be directed against endogenous Hsp70, causing an autoimmune response. 8) In addition, myelin peptides, such as PLP and MBP, generated during myelin destruction may associate with Hsp70. Hsp70-PLP and Hsp70-MBP complexes can be recognized by APC, 9) triggering an adaptive immune response against them. Modified from: Mansilla MJ et al., 2012.

Hsp70s can act as adjuvants by binding immunogenic peptides. Hsp70s bind MBP and PLP

peptides to create highly immunogenic complexes, which are efficiently internalized by endocytosis, processed and presented by MHC class II molecules on APCs, thus stimulating specific CD4+ T cell responses (Cwiklinska H et al., 2003; Mycko MP et al., 2004). In patients with RRMS, the presence of a subpopulation of γδ T cells responsive for Hsp70 has been described (Battistini L et al., 1995). These cells have a potent cytotoxic activity and can destroy oligodendrocytes *in vitro* (Freedman MS et al., 1991). Furthermore, the abnormal increase of Hsp70 levels following heat shock and lipopolysaccharide (LPS) stimulation *in vitro* has been described in PBMCs from MS patients; the authors suggest a possible link between Hsp70 overexpression and the development of autoimmunity (Cwiklinska H et al., 2010).

To further complicate the role of Hsp70 in MS, Hsps are also produced by bacteria, and these proteins are highly conserved. Bacterial Hsp60 and Hsp70 seem to be the most immunogenic, being able to induce autoimmunity starting from infections. T lymphocytes and antibodies produced against microbial Hsp may target self-Hsp thanks to the recognition of conserved epitopes or by molecular mimicry (Figure 19).

In MS, Hsp70 seems to be the target of humoral immune response, with antibodies directed against both extracellular proteins and membrane proteins. However, the same levels of anti-Hsp70 antibodies have been described in the sera of healthy subjects and MS patients (Bustamante MF et al., 2011). These antibodies may have an immunoregulatory role in removing the excess of Hsp70. The response against Hsp70 can probably be increased in the CNS of MS patients; higher levels of antibodies for Hsp70 and Hsc70 have been found in the CSF of patients (Chiba S et al., 2006).

It is difficult to determine the exact role of Hsp70 in MS because of the pleiotropic functions of Hsp70s and the complex pathogenesis of this disease. The overexpression of Hsp70 in the CNS of MS patients can have a neuroprotective function, as happens in other neurodegenerative diseases. The insufficient Hsp70 production can be a factor for MS development, and the failure of Hsp70 overexpression may lead to MS progression.

However, MS is a disimmune disease and it is important to consider the role of Hsp70 in the immune function. Hsp70 overexpression may have a pro-inflammatory role (Figure 19).

A recent work suggests that, although Hsp70-1 could play a role in neuroprotection, in the MOG-induced EAE model this protein seems to be relevant in immune regulation. Hsp70-1 deficient mice are more resistant to developing EAE compared with wild-type (WT) mice, suggesting that this protein may play a role in promoting a specific T cell response (Mansilla MJ et al., 2014). Although the neuroprotective role has been suggested by several studies, Hsp70-1 deficient mice that develop EAE do not show increased demyelination (Mansilla MJ et al., 2014). The authors suggest that Hsp70 may be relevant in EAE, and specific therapies that down-regulate Hsp70 expression may be important in reducing the early autoimmune response in MS.

2.2.6.3. Hsp70 protection against oxidative stress

Oxidative stress has been described in MS lesions and correlates with the inflammatory process (Haider L et al., 2011). In case of oxidative stress, our cells can counteract the damages due to the presence of ROS, inducing several mechanisms controlled by different signalling pathways, all of which aim at preserving cellular homeostasis under oxidative stress conditions.

Particularly important are the genes encoding for antioxidant and anti-apoptotic proteins, which become activated when the cell needs to preserve homeostasis (Calabrese V et al., 2010). In addition to the genes encoding for anti-oxidant enzymes, the heat shock response genes are essential (Calabrese V et al., 2010).

In conditions of oxidative stress, misfolded proteins can aggregate and Hsp70 expression is increased, contributing to protein re-folding or to degradation. The observation that HSF1 is one of the transcription factors able to perceive the redox status of the cells further supports the involvement of heat shock response in the protection against oxidative stress (Zhang Y et al., 2011). This transcription factor coordinates cell survival, together with HIF1α (Majmundar AJ et al., 2010) and NF-kB (Morgan MJ e Liu ZG, 2011). HSF1 becomes activated in conditions of oxidative stress due to the oxidation of specific cysteine residue of the transcription factor (Trott A et al., 2008). HSF1 allows the transcription of different molecular chaperons, including members of the Hsp70 family.

Furthermore, Hsps can directly respond to the redox status of the cell, allowing also for a post-translational regulation. Hsp70s can perceive the redox status thanks to cysteine residue, which can be oxidised (Vignols F et al., 2003). Inducible Hsp70s have a redox-sensitive cysteine near their ATP binding pockets, and under conditions of oxidative stress, peptide binding to Hsp70 and complex stability are enhanced (Callahan MK et al., 2002). This fact was confirmed by a 2012 study (Miyata Y et al., 2012) that found that the oxidation of specific cysteine residues in Hsp70-1 may be important for the chaperone response to oxidative stress. However, the constitutive form of Hsc70 is not sensitive to this stress.

2.3. Alzheimer's disease

AD is a complex and multifactorial neurodegenerative disorder that accounts for 60-70% of all the cases of dementia in elderly adults (Alzheimer's Association, 2016). The disease is characterized by progressive loss of memory and consciousness and marked cognitive impairment (Alzheimer's Association, 2016). The first symptom is a gradual worsening of the ability to remember new information: patients show a slight deficit in working memory and a moderate deficit in short-term and episodic memory. As the disease progresses, cognitive and functional abilities decline and marked and irreversible mood alterations and loss of episodic memory function are observed (Albert MS, 1996; Perry RJ and Hodges JR, 1999). In the more advanced stages, patients need help with basic activities of daily living, such as bathing, dressing and eating, thereby losing their ability to communicate and failing to recognize people. Patients become more vulnerable to infections such as pneumonia, which often contributes to their death (Alzheimer's Association, 2016). The pace of the progression of the symptoms from mild to moderate to severe varies from person to person (Alzheimer's Association, 2016).

Clinically, only a probable diagnosis of AD is possible, based on NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) criteria, which has good sensitivity and specificity (>80%) for distinguishing patients with AD disease from people without dementia; however, it is less accurate in distinguishing between Alzheimer's disease and other dementias such vascular dementias (23–88%) (Ballard CG and Bannister C, 2005). Only the *post mortem* analysis of the brain can definitively permit the diagnosis of AD (Ballard C et al., 2011). The presence of senile plaques, formed by extracellular deposition of Aβ peptides, and NFTs from the aggregation of the hyper-phosphorylated tau protein, within a patient's brain are hallmarks of AD (Ballard C et al., 2011) (Figure 20). Aβ peptides accumulate in senile plaques from the proteolytic cleavage of the amyloid precursor protein (APP), mediated by the secretases, a family of enzymes. Accumulation of Aβ may interfere with neuron communication and contribute to neuronal death. NFTs block the transport of nutrients and essential molecules inside the neurons and play a role in cell death. All these changes result in the onset of the symptoms, including memory loss (Alzheimer's Association, 2016). It has been noticed that brain changes associated with AD may begin 20 years before the first symptoms appear (Villemagne VL et al., 2013). These initial changes can be compensated for enabling the brain to continue to function normally. However, with the increase in neuronal damage subtle cognitive decline initiates, and as the disease progresses the damage increases and patients show strong cognitive decline, memory loss, confusion, and ultimately basic bodily functions become impaired (Alzheimer's Association, 2016).

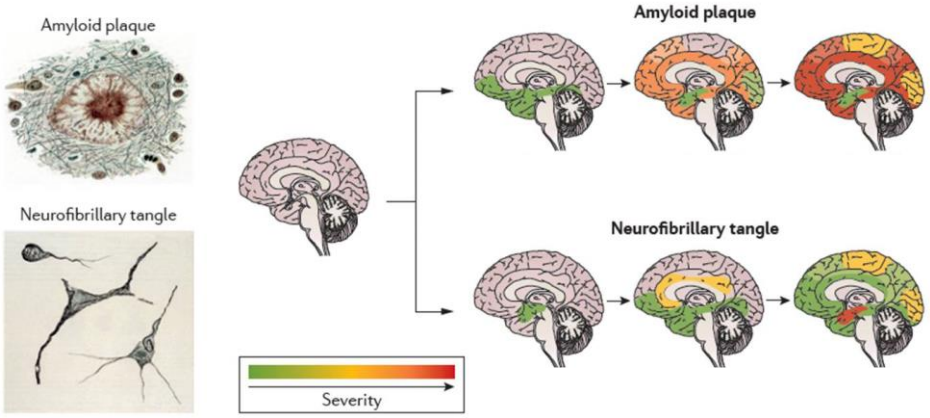

Figure 20. The pathological hallmarks and evolution of Alzheimer's disease. Amyloid plaques and neurofibrillary tangles spread through the brain as the disease progresses. Modified from: Masters CL et al., 2015.

Most AD cases belong to the sporadic type, which is correlated with age and characterized by late onset, usually after 65, accounting for about 95% of all cases (Hoyer S, 2004). The remaining 5% of cases involve the familiar form, usually diagnosed in mid-life and associated with dominant inherited mutations in genes encoding for APP and for presenilins 1 and 2 (PSEN1 and PSEN2) (Goate A et al., 1991; Schellenberg GD et al., 1992; Levy-Lahad E et al., 1995). From a clinical point of view, the two forms of AD are comparable, including the rate of disease progression and many biomarker profiles.

With the exception of familiar cases, AD results from the interaction of multiple risk factors. Age is the main risk factor for sporadic AD: as age increases, the risk of developing AD grows. However, AD is obviously not a normal part of ageing; advanced age is not sufficient to cause AD, and other factors, both environmental and genetic, have a role in determining it (Ballard C et al., 2011). People with a first-degree relative with AD have a higher probability of developing AD, and the risk is even higher for individuals who have more than one first-degree relative with AD (Lautenschlager NT et al., 1996; Loy CT et al., 2014).

Several genetic variants with a role in the risk of developing AD have been identified. Inherit apolipoprotein E (APOE) ε4 allele is one of the major genetic risk factors; the risk of developing AD is three times more likely with one allele and twelve times more likely with two alleles (Farrer LA et al., 1997; Saunders AM, 2000). In fact, the APOE ε4 allele impairs Aβ clearance from the brain and is associated with an increase in the accumulation

of Aβ peptides in the brain at earlier ages (Zlokovic BV, 2013). Furthermore, the ApoE ε4 allele decreases the age of onset of AD by approximately 8 years in APOE ε4 heterozygote patients and 15 years in APOE ε4 homozygote subjects (Corder EH et al., 1993; Tanzi RE, 2012). However, the relative risk associated with other identified variants is lower, usually between 1.2 and 1.5 (Ballard C et al., 2011).

2.3.1. Pathogenesis

Over the years, several theories have been presented to explain AD pathogenesis. However, it is difficult to assess their validity since not all the events observed in AD pathogenesis can be clearly defined as primary or secondary events.

The oldest theory is the cholinergic hypothesis, based on investigations made before 1980. A reduction in choline acetyltransferase activity and acetylcholinesterase at the cerebral cortex level was observed in AD patients (Davies P and Maloney AJ, 1976). Furthermore, a significant loss of acetylcholine in AD brains was reported (Bowen DM and Davison AN, 1980). The alteration in the number of nicotinic and muscarinic acetylcholine receptors in the presynaptic cholinergic terminals was linked to the decline of cognitive functions (Whitehouse PJ et al., 1988; Nordberg A et al., 1992). Furthermore, Aβ peptide can inhibit cholinergic neurotransmission *in vitro* (Kar S et al., 1998). Based on the hypothesis that degeneration in cholinergic neurotransmission in the cerebral cortex and in other areas may contribute to the impairment of cognitive function in AD patients, different therapeutic approaches to improve cholinergic neurotransmission have been developed, including cholinesterase inhibitors, choline precursor, and postsynaptic and presynaptic cholinergic stimulation with a muscarinic and nicotinic agonist (Lleó A et al., 2006; Contestabile A, 2011). Beneficial effects on cognitive, functional and behavioural symptoms were noticed with the use of cholinesterase inhibitors (Rogers SL et al., 1998; Tariot PN et al., 2000), and several of these drugs have been approved by the FDA for the treatment of mild to moderate AD: donepezil, rivastigmine and galantamine (Giacobini E, 2002). However, long-term administration of cholinesterase inhibitors to patients with mild cognitive impairment (MCI) has failed to reduce the risk or to delay the onset of AD (Raschetti R et al., 2007; Contestabile A, 2011). It is probable that the cholinergic dysfunction in AD is only a part of a multisystem degeneration and not the primary cause of the disease.

In recent years, evidence suggests the involvement of the dopaminergic system in AD: deficits in dopaminergic signalling have been related to memory dysfunction both in AD patients and in the animal models (Tanaka Y et al., 2003; Moreno-Castilla P et al., 2016). Furthermore, a recent work highlights the fact that, in a mouse model, apoptotic processes in the ventral tegmental area cause a progressive degeneration of dopaminergic neurons at a very early stage, before Aβ deposition and the formation of NFTs (Nobili A et al., 2017). A

decrease in dopamine outflow in the hippocampus may contribute to synaptic plasticity dysfunction and memory deficits (Nobili A et al., 2017).
However, the amyloid cascade hypothesis has been the major pathogenic mechanism considered; the accumulation of Aβ peptide followed by the deposition of NFTs leads to synaptic and neuronal dysfunction and loss (Figure 21).

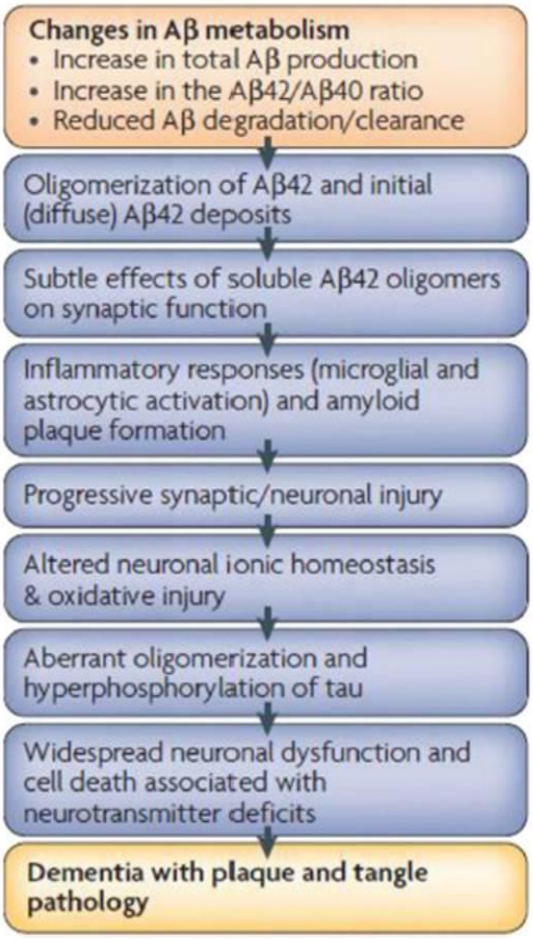

Figure 21. Diagram of the amyloid cascade hypothesis. From Barage SH and Sonawane KD, 2015.

According to the amyloid cascade hypothesis, the aberrant processing APP and/or the altered clearance of Aβ peptide represent the initiating events in the pathogenesis of AD (Hardy J and Selkoe DJ, 2002). The cleavage of APP can follow two pathways (Figure 22).

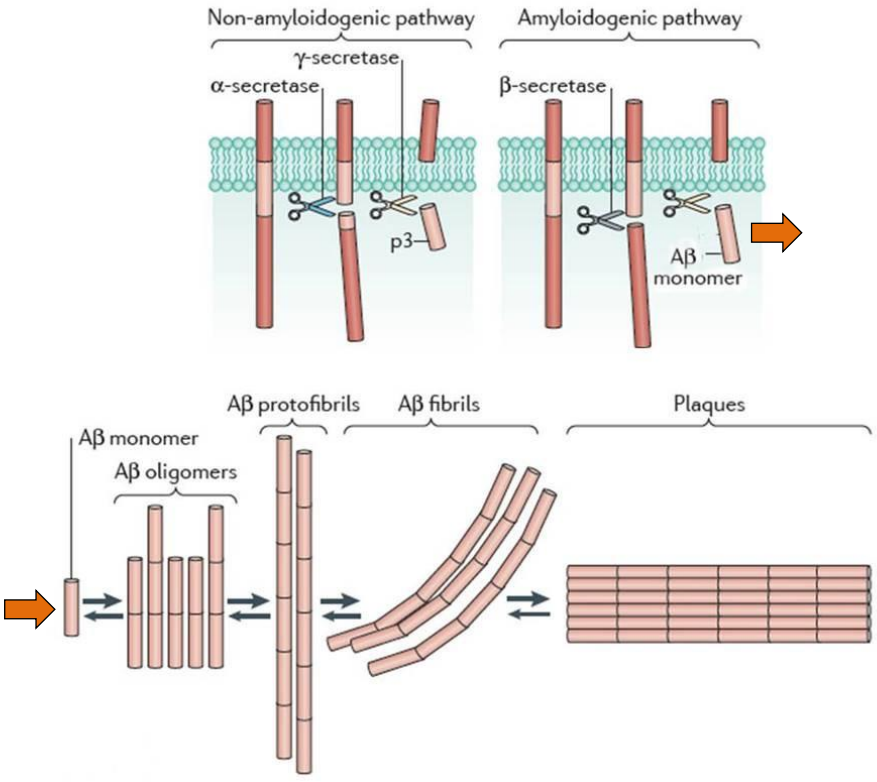

Figure 22. The non-amyloidogenic and the amyloidogenic pathways for APP cleavage. Cleavage of APP in the non-amyloidogenic pathway is physiological: APP is cleaved first by α-secretase and then by γ-secretase. In the amyloidogenic pathway, γ-secretase cleavage of APP is preceded by β-secretase cleavage, resulting in the release of Aβ peptides into the extracellular compartment. Aβ monomers can form oligomers or other aggregates. The arrow thickness indicates the likelihood of conversion of Aβ species. Modified from: Heppner FL et al., 2015.

The non-amyloidogenic pathway is physiological and APP is cleaved by α-secretase and then by γ-secretase; however, in the amyloidogenic pathway, which is pathological, α-secretase is substituted by β-secretase (Haass C et al., 2012). The γ-secretase can act on several cleavage sites to produce peptides of different size: Aβ38, Aβ40 and Aβ42 (Gu Y et al., 2001). These peptides display a different degree of aggregation, with the Aβ42 having the higher propensity to aggregate (Suzuki N e a., 1994). Furthermore, the Aβ42 peptide is

neurotoxic (Mucke L and Selkoe DJ, 2012). Aβ peptides can accumulate in distinct forms: monomers, oligomers, protofibrils and fibrils, forming senile plaques in the extracellular compartment (Mucke L and Selkoe DJ, 2012). Impaired Aβ clearance through the interstitial fluid (ISF) and the CSF can aggravate the process of accumulation of Aβ peptide (Mawuenyega KG et al., 2010; Hong S et al., 2011).

Early evidence supports the amyloid cascade hypothesis, including observations from animal models. High levels of human mutant APP in transgenic mice results in Aβ deposition, gliosis and synaptic loss (Games D et al., 1995). Moreover, genetic studies provide evidence for this hypothesis: in familiar AD, autosomal dominant mutations in APP, PSEN1 and PSEN2 genes lead to abnormal Aβ peptide production (Goate A et al., 1991; Schellenberg GD et al., 1992; Levy-Lahad E et al., 1995).

However, the amyloid cascade hypothesis cannot completely explain the neuronal damage observed in AD patients. It has been observed that amyloid deposition and NFTs can be found in cognitively normal elderly subjects (Aizenstein HJ et al., 2008). Furthermore, Aβ accumulation does not correlate completely with neuronal loss and cognitive dysfunction (Serrano-Pozo A et al., 2013). Moreover, treatments against Aβ, although efficient in reducing Aβ fragments, do not stop AD progression (Salloway S et al., 2009). This evidence suggests that other factors besides Aβ deposition and neurofibrillary tangles contribute to the neuronal damage typical in AD.

Aβ deposition and plaque formation can activate both astrocytes and microglia (Hu J et al., 1998; Jin JJ et al., 2008). For this reason, in the last decade more attention to neuroinflammation has been paid; the inflammatory response is driven by microglia and increases with the progression of the disease. Nowadays, it is thought that neuroinflammation contributes to AD pathology, exacerbating the disease progression (Sudduth TL et al., 2013).

2.3.2. Neuroinflammation in Alzheimer's disease: inflammatory hypothesis

Some evidence suggests a role of the innate immune system in the pathogenesis of AD. Neuroinflammation seems to be an important contributor to neurodegeneration; in several neurologic and neurodegenerative diseases, neurodegeneration is associated with the presence of an inflammatory process, but the precise role of inflammation is controversial: it can be a possible cause of the disease, a consequence, and a beneficial response (Wyss-Coray T et al., 2006).

Early studies showed increased levels of pro-inflammatory cytokines: TNF-α and IL-6 have been found at the level of the brain and in the serum of AD patients (Fillit H et al., 1991; Strauss S et al., 1992). Furthermore, activated microglia surround amyloid plaques, and the presence of reactive astrocytes has been observed in the brain of AD patients (Perlmutter

LS et al., 1990; Pike CJ et al., 1994). Initially the inflammation was thought to be a consequence of Aβ deposition, since Aβ aggregates could activate microglia, astrocytes and the complement system cascade, leading to the release of pro-inflammatory cytokines. This chronic activation contributes heavily to neuronal damage. This hypothesis is also supported by recent experimental evidences from the AD (PS1V97L-Tg) mouse model. At 6 months a significant increase in Aβ was observed, associated with memory dysfunction and tau phosphorylation (Wang W et al., 2016). Furthermore, PS1V97L-Tg mice can be differentiated from WT littermates in terms of the detection of increased plasma levels of pro-inflammatory cytokines.

However, some evidence suggests an earlier role for neuroinflammation in AD, not only as a consequence of Aβ deposition; several studies correlate clinical symptoms that precede AD, such as MCI, with the presence of inflammatory changes. Increased plasma levels of C-reactive protein, α1-antichymotrypsin and IL-6 were found before the clinical onset of dementia and AD (Engelhart MJ et al., 2004). Furthermore, in patients with MCI and at risk of developing AD, increased levels of the pro-inflammatory cytokine TNF-α and decreased production of the anti-inflammatory cytokine TGF-β were found in the CSF (Tarkowski E et al., 2003).

The possible key-role of inflammation in AD is also supported by a meta-analysis, which suggests that non-steroidal anti-inflammatory drugs might decrease the risk of developing AD (McGeer PL et al., 1996). Other studies show that the use of anti-inflammatory drugs in patients with symptomatic AD or MCI did not result in a beneficial effect (Aisen PS et al., 2003; Thal LJ et al., 2005). However, the treatment of asymptomatic individuals with anti-inflammatory drugs reduces the incidence of AD (Breitner JC et al., 2011), a fact that supports the beneficial role of anti-inflammatory treatment only when administered in the early, asymptomatic phase of the disorder.

Recently, it has been noticed that systemic immune challenge in WT mice leads to the development of an AD-like pathology with the formation of Aβ plaques, tau aggregation, microglia activation and reactive gliosis (Krstic D et al., 2012). This observation suggests that the immune reaction can precede AD and may drive AD pathogenesis independently of Aβ accumulation, thus exacerbating the disease. Furthermore, acute and chronic inflammation are associated with increased cognitive decline in AD patients (Holmes C et al., 2009).

Positron emission tomography imaging studies revealed that in AD patients the cognitive status is inversely correlated with microglia activation but not with amyloid load (Yokokura M et al., 2011).

Moreover, genetics supports the early involvement of neuroinflammation in AD pathology. The association between the presence of AD and several mutations in genes involved in the immune system function provide evidence beyond the purely descriptive level: mutations in TREM2 (Guerreiro R et al., 2013), the myeloid cell surface antigen CD33 (Bradshaw EM

et al., 2013), and CR1 (Lambert JC et al., 2009) have been described. The discovery of risk variants in genes involved in the immune system function have shed a new light on the findings of increased pro-inflammatory cytokine levels in AD patients in the prodromal forms of the disease as well (Tarkowski E et al., 2003; Brosseron F et al., 2014).

A study of the possible involvement in AD risk of variants of genes encoding for inflammatory mediators has led to the identification of SNPs in the MCH class III, whose genotypic frequencies deviate between AD patients and healthy controls. In particular, variants of RAGE and TNF-α genes have been related to AD susceptibility (Maggioli E et al., 2013). Furthermore, the frequency of TTGAA haplotype (given by -374 T/A and -429 T/C polymorphism of RAGE, and -238 G/A, -308 G/A and -857 G/A polymorphisms of TNF-α) decreases significantly in AD patients compared with healthy controls. The implication for the disease of this haplotype points to a possible involvement of the entire HLA class III region in the variation of AD risk; a fact confirmed by another study in which a larger MHC class III haplotype was investigated, considering also HSP70 genes (Boiocchi C et al., 2015). The haplotypes TTGATAGG and TTGATGGG (given by -429 T/C RAGE, -374 T/A RAGE, +190 G/C HSP70-1, +1267 A/G HSP70-2, +2437 T/C HSP70-HOM, -238 G/A TNF, -307 GA TNF and -857 G/A TNF polymorphisms) were more frequent in AD patients than in healthy controls. The authors suggested that patients with these haplotypes may have greater Aβ aggregation, increased oxidative stress and higher expression of RAGE, HSP70 and TNF. Patients with a greater expression of these genes may be more prone to progression and amplification of the inflammatory process.

2.3.3. Etiology

AD can be classified into two types: familiar and sporadic. These two forms share the same symptoms and progression but usually appear at different ages, and their etiology is different. The familiar cases are related to genetic mutations in specific genes involved in the production of Aβ: APP, PSEN1 and PSEN2 (Goate A et al., 1991; Schellenberg GD et al., 1992; Levy-Lahad E et al., 1995).

The most frequent form, the sporadic one, has a more complex etiology: it is a multifactorial disease like the majority of neurodegenerative diseases. A third of the risk of developing AD is due to common genetic variants (Gandhi S and Wood NW, 2010); environmental factors and epigenetic alteration also contribute to susceptibility to the disease (Lunnon K and Mill J, 2013).

Potentially modifiable risk factors for AD have been determined: diabetes mellitus, mid-life hypertension, mid-life obesity, physical inactivity, depression and smoking (Norton S et al., 2014). However, these factors require further evaluation to assess their role. Furthermore, a modification in lifestyle, including diet, exercise and cognitive training, has been shown to

have beneficial effects on the cognitive outcome (Ngandu T et al., 2015).
The initial evidence of an involvement of epigenetics in the development of AD was from studies on twins; even monozygotic twins have discordant AD outcomes (Mastroeni D et al., 2009). In AD, robust changes in DNA methylation patterns in specific genes have been identified (Roubroeks JAY et al., 2017). However, it is not clear whether these changes are a cause or only a consequence of the disorder.

2.3.3.1. Genetic factors: the importance of genes involved in the immune function

GWAS have identified several genes that can contribute to the risk of developing sporadic AD. These genes are linked to the Aβ cascade and tau pathology and can be divided into three groups involved in lipid metabolism, synaptic functioning and immune response (Giri M et al., 2016). Although GWAS is a powerful method to identify risk genes, it is not easy to understand how variants directly contribute to AD pathogenesis; it is also necessary to consider that identified variants are sometimes in linkage disequilibrium with others that are more important (Cuyvers E and Sleegers K, 2016). Thanks to further investigations, several advances have been made in identifying functional variants for AD risk that affect gene function, regulation and splicing (Cuyvers E and Sleegers K, 2016) (Figure 23).

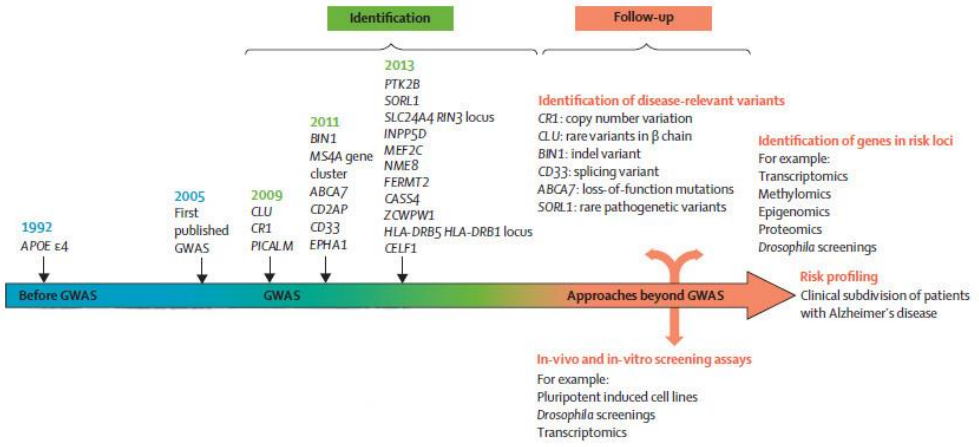

Figure 23. Progress in gene identification by GWAS for AD risk. The functional follow-up consists in the study of the risk variants identified to understand their relevance. Modified from Cuyvers E and Sleegers K, 2016.

Some genes are involved in the lipid and cholesterol metabolism. This is consistent with the observation that high cholesterol levels in mid-life increases the risk of developing AD (Solomon A et al., 2009). As mentioned above, APOE ε4 allele is the stronger genetic risk factor for sporadic AD, augmenting the risk of developing AD threefold with one allele to twelvefold with two alleles (Farrer LA et al., 1993; Saunders AM, 2000). This gene, located on chromosome 19q13.2, codes for the major protein that carries cholesterol to the brain and is involved in cholesterol metabolism and lipid transport; it also controls inflammation, synaptic function, neurogenesis, and the generation and trafficking of APP and Aβ (Hauser PS et al., 2011; Rebeck GW, 2017). Several studies have shown that ApoE isoforms can influence Aβ clearance, aggregation and deposition in a different way (Bales KR et al., 2009; Castellano JM et al., 2011). APOE ε4 is less efficient in Aβ clearance compared with ε2 and ε3 (Bales KR et al., 2009). Consistent with this fact, patients with APOE ε4 allele show increased amyloid levels (Ramanan VK et al., 2014); furthermore, subjects with this allele have elevated hippocampal atrophy (Lu PH et al., 2011). Moreover, APOE ε4 contributes to AD with an Aβ-independent mechanism; this isoform promotes Aβ-induced inflammatory response in neuronal cells in AD, whereas APOE ε2 suppresses it (Dorey E et al., 2017). APOE is also linked to tau protein. CSF levels of tau and phosphorylated-tau have been observed to increase in patients homozygous for APOE ε4 (Han MR et al., 2010). Also in this case, the APOE ε2 allele is associated with a decrease in AD pathology, since it is related to a decrease in tau and phosphorylated-tau (Chiang GC et al., 2010).

Several SNPs located in other genes involved in lipid metabolism, such as ABCA7 (ATP binding cassette subfamily A member 7), CLU (clusterin), and SORL1 (sortilin related receptor 1), were found to be linked to the risk of developing AD (Harold D et al., 2009; Lambert JC et al., 2009; Reitz C et al., 2013; Wang Z et al., 2016).

In recent GWASs, several SNPs in genes involved in the regulation of the endocytosis have been related to the risk of developing AD, such as BIN1 (bridging integrator 1), CD2AP (CD2 associated protein), and PICALM (phosphatidylinositol binding clathrin assembly protein). These genes are involved in APP trafficking, synaptic transmission, and the response to neuronal damage (Harold D et al., 2009; Hollingworth P et al., 2011; Chapuis J et al., 2013; Lambert JC et al., 2013).

Variants in genes involved in the immune response are also associated with AD risk, highlighting the primary role of inflammation in the pathogenesis of the disease. In particular, several variants associated with AD are located in genes that are important for the innate immune system, supporting the link between neuroinflammation and the disorder.

CR1 was one of the first susceptibility genes identified for AD (Lambert JC et al., 2009). This gene, located on chromosome 1q32.2, encodes a transmembrane glycoprotein found mainly on the membrane of erythrocytes, leukocytes and dendritic cells; this glycoproten is

involved in complement system regulation (Wilson JG et al., 1987). CR1 on phagocytes facilitates the uptake and the removal of immune complexes and is involved in immune regulation (Khera R and Das N, 2009). An intragenic CNV leads to the formation of a longer isoform (CR1-S: slow migration) which has more C3b and C4b binding sites than does the shorter isoform (CR1-F: fast migration), a fact that might inhibit complement activation. The association between CR1-S form and AD has been identified (Szigeti K et al., 2013). Furthermore, SNPs in CR1 are associated with AD: SNPs rs3818361 and rs6656401 are associated with increased risk of AD (Lambert JC et al., 2009), and SNP rs1408077 is associated with plaque load in the brain of patients (Kok EH et al., 2011).
SNPs related to AD have also been identified in other genes important for the immune system functioning such as the genes for CD33 (a transmembrane protein expressed on myeloid cells and microglia) and TREM2 (triggering receptor on myeloid cells 2) (Tateno H et al., 2007; Jonsson T et al., 2013; Guerreiro R et al., 2013; Jin SC et al., 2014). Mutations in TLRs may have a role in the clearance of Aβ deposits. A TLR9 polymorphism (rs187084) may modify AD risk in Han Chinese population (Wang YL et al., 2013).

2.3.4. The complement system

2.3.4.1. The complement system: a player in the innate immune system

The complement system consists of more than 50 serum and membrane proteins which are extremely important for the innate immune system in its response to pathogens and endogenous danger signals. It contributes to several homeostatic processes such as lipid metabolism, tissue modelling and maintenance, and angiogenesis; furthermore, it is involved in acute and chronic pathologies (Ricklin D et al., 2010). Genes coding for complement proteins map on several chromosomes (1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 16, 19, 20, 21 and X); only a few chromosomes do not contain genes for complement (Mayilyan KR, 2012). The genes located on chromosome 6, C2, Bf, C4A and C4B, cluster together in the MHC class III region (Mayilyan KR, 2012).
The complement system can be activated by PAMPs and DAMPs and involves a series of proteolytic reactions that can follow three different pathways: classical, lectin and alternative, each converging to the cleavage of the inactive C3 protein into the functional fragments C3a, an inflammatory mediator, and C3b, an opsonin that can bind the cell surface. In this way, all three pathways converge in a common terminal pathway that finally leads to the osmotic lysis of the pathogen (Figure 24).
In addition, a new activation pathway has emerged recently: the extrinsic pathway, which suggests a potential crosstalk between complement and coagulation pathways (Markiewski MM et al., 2007). This pathway is driven by serine proteases of the coagulation system,

such as thrombin, which can directly cleave the C5 (Huber-Lang M et al., 2006). Furthermore, evidence suggests that proteases such as thrombin, human coagulation factor Xa, plasmin and tissue plasminogen activator may be able to cleave C3 (Amara U et al., 2010; Zhao XJ et al., 2017).

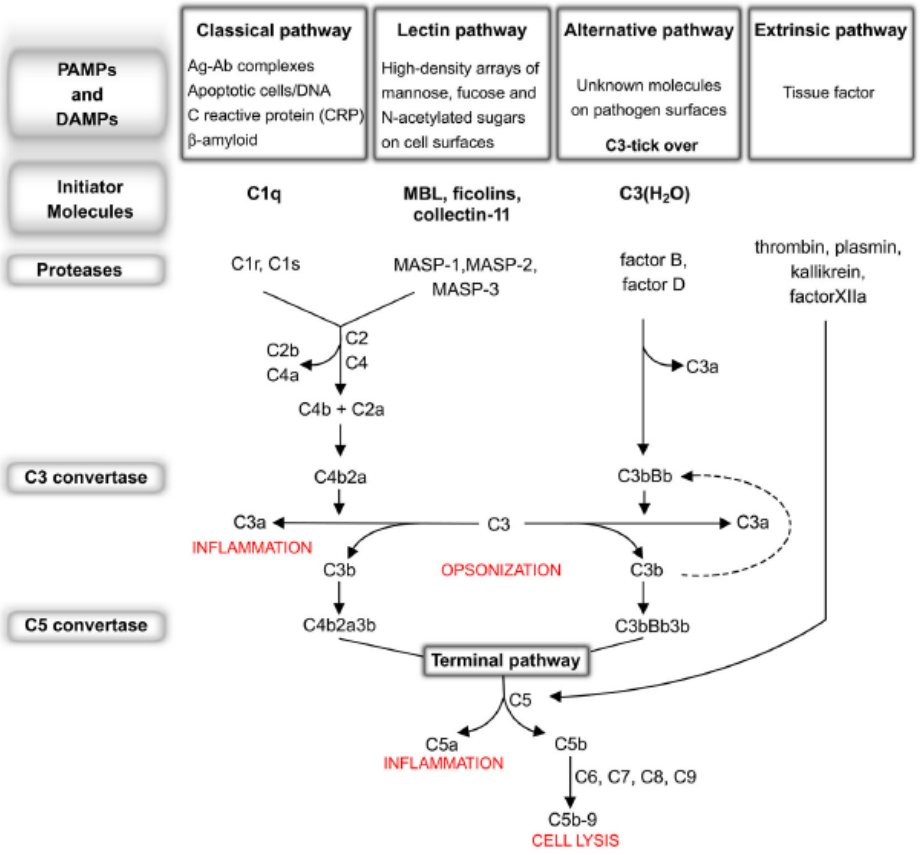

Figure 24. Overview of the complement system cascade. In the classical pathway, C1q recognizes antigen-antibody complexes of specific molecules that activate the complement system. In the lectin pathway, MBL, ficolins and collectin are the initiators. The activation of the alternative pathway is driven by the spontaneous hydrolysis of circulating C3. In the recently characterized extrinsic pathway, proteolytic enzymes of the coagulation pathway cleave C5. With the exception of the extrinsic pathway, the pathways converge to the terminal pathway with the formation of the C5 convertase. From Orsini F et al., 2014.

The **classical pathway** is activated by the binding of a complex described as a "bouquet of flowers" composed of several chains of three types, called A, B and C, which form six globular target recognition domains. C1q is able to bind different target molecules, including IgG and IgM, already bound to the surface antigens, PAMPs such as bacterial porins and LPS, and molecules exposed on the surface of dying cells (Albertí S et al., 1996; Nauta AJ et al., 2002; Roumenina LT et al., 2008). Once C1q binds the target, a conformational change allows the induction of the auto-activation of C1r, a serine protease, which cleaves and activates C1s (Wallis R et al., 2010). In this way, C1s can interact with C4 and C2, cleaving these complement components and allowing the formation of the C3 convertase in the proximity of the C1 complex-binding site (Gaboriaud C et al., 2014). C4 is cleaved in the bioactive form C4b and in a small fragment C4a. C4b is able to bind the antigen-antibody complex or the adjacent surface of the cell (Ziccardi RJ, 1981). C2 is cleaved in C2a, which remains bound to C4b, thus forming the C3 convertase C4b2a, whereas the smaller fragment C2b is released into the circulation (Ziccardi RJ, 1981; Krishnan V et al., 2009).

The activation of the **lectin pathway** is possible thanks to pattern recognition receptors, including the mannose binding lectin (MBL), collectins and ficolins, which bind carbohydrates on the surface of bacteria, viruses and dying cells (Kjaer TR et al., 2013). When the ligands are clustered on the surface to form a specific pattern, the complex can engage several carbohydrate recognition domains or fibrinogen-like domains for collectins and ficolins. MBL-associated serine proteases associated with the recognition molecules are activated and cleave C4 and C2, leading to the formation of the C3 convertase C4b2a (Dahl MR et al., 2001).

In the **alternative pathway**, the C3 is spontaneously hydrolyzed to the bioactive form C3(H_2O) which undergoes a structural change that allows the exposition of novel binding sites necessary to recruit the Factor B (FB). The C3(H_2O)-bound FB is then cleaved by the serine protease Factor D (FD) into Ba and Bb; Bb remains associated, thus allowing the formation of the fluid phase C3 convertase C3(H_2O)Bb, which is able to cleave native C3 molecules into C3a and C3b (Isenman DE et al., 1981; Nishida N et al., 2006). C3b binds covalently to surfaces containing hydroxyl groups, but not all these groups equally attract C3b; only the particular combination of sugars on the pathogen surface can determine the efficacy of complement activation (Sahu A et al., 1994). The bound C3Bb can associate again with FB, allowing its cleavage to form the convertase of the alternative pathway C3bBb (Milder FJ et al., 2007).

After the formation of the C3 convertase, the three pathways converge into a common **terminal pathway**: the C3 convertase (C4b2a for the classical and lectine pathways, and C3bBb for the alternative pathway) cleaves C3 into C3a and C3b, allowing the formation of a new enzymatic complex, the C5 convertase. This complex cleaves C5 into the bioactive fragments C5a and C5b. C5b recruits several complement components (C6, C7, C8 and

C9), leading to the formation of the membrane attack complex (MAC) pore (Bubeck D, 2014). C5b interacts with C6, and this complex recruits C7; C5b-7 is lipophilic and binds to the cell membrane (Preissner KT et al., 1985). C8 can penetrate the lipid bilayer, and up to 18 C9 molecules can be recruited to form a tubular channel (Bhakdi S and Tranum-Jensen J, 1991), creating a functional MAC which can directly lyse metabolically inert cells and Gram-negative bacteria (Koski CL et al., 1983; Bhakdi S et al., 1987).

Metabolically active cells are more resistant to the lysis induced by the complement system. Multiple MACs have to be inserted in the membrane (Morgan BP, 1989), leading to increased Ca^{2+} influx and signal transduction that contribute to cell death (Morgan BP and Campbell AK, 1985). In this way, the complement system is important for the clearance of apoptotic cells and the elimination of pathogens.

The elimination of apoptotic cells occurs thanks to membrane changes that allow the recognition of cells; furthermore, apoptotic cells decrease the expression of complement regulators (Verbovetski I et al., 2002). The proteins of the complement system are also able, thanks to the interaction with specific receptors, to recruit phagocytes that remove cell debris, thus preventing an immune response toward self-antigens (Baudino L et al., 2014).

C3 generated by the spontaneous activation of the alternative pathway can bind to the pathogens surface; furthermore, the pathogen molecules can activate both the classical and the lectin pathways. In this way, the classical and the lectin pathways are critical for pathogen recognition and the initiation of the complement cascade; but the alternative pathway assures more than 80% of the terminal complement activity.

The activation of the complement system not only leads to the formation of the MAC but also to opsonisation. Opsonisation is important for pathogen elimination: the deposition of complement fragments on the pathogen surface allows for its recognition by phagocytes and for the recruitment of adaptive immune system cells that express specific receptors for complement components (van Lookeren Campagne M et al., 2007; He JQ et al., 2008). This cascade amplifies the opsonisation of the pathogen, leading to its elimination (Lachmann PJ, 2009), due in part to the increased generation of complement anaphylatoxins. These small molecules (C3a and C5a), constantly released during complement activation, support the inflammation and activation of cells that express anaphylatoxin receptors (Klos A et al., 2009), leading to the recruitment and activation of immune cells such as macrophages, eosinophils and neutrophils (Murakami Y et al., 1993; Elsner J et al., 1994; Ehrengruber MU et al., 1994).

2.3.4.2. Activation of the complement system in Alzheimer's disease: an unclear role

Under physiological conditions in the CNS, microglia, astrocytes, oligodendrocytes and neurons can directly synthetize, at low levels, several components of the complement

system, such as C1q and C3, together with complement regulators and receptors (Stevens B et al., 2007; Woodruff TM et al., 2010), which are involved in brain development and in the maintenance of its homeostasis. The production of complement components increases in the presence of cellular damage (Woodruff TM et al., 2010).

The activation of the complement system in the CNS has been demonstrated under acute neuroinflammatory conditions and also in neurodegenerative diseases, conditions in which the complement can also damage the neurons, probably because of their low basal expression of membrane-associated complement regulators (Singhrao SK et al., 2000).

During acute brain injury, such as brain trauma or stroke, in addition to complement proteins produced locally by the CNS cells, complement proteins circulating in the blood together with immune system cells can also invade the brain parenchyma (Stahel PF et al., 2001). The complement system contributes in this way to the removal of cell debris; however, when its activation is excessive it may affect neuronal and glial integrity (Leinhase I et al., 2006). On the other hand, in neurodegenerative disorders, the BBB injury usually occurs at late stages of the disease, and the uncontrolled local synthesis and activation of complement components are critical contributors to neuronal damage (Phuan PW et al. 2013).

Regarding AD, since the early 1980s, the presence of complement components has been reported. Early studies showed the presence of C1q, C3, C3b and C4 in the senile plaques (Eikelenboom P and Stam FC, 1982) and revealed that NFTs are immunopositive for C1q, C3, C4 and C5b-9 (McGeer PL et al., 1989). The latter observation has also been confirmed by a more recent analysis: tau such as Aβ peptide is a potent activator of the classical complement pathway (Shen Y et al., 2001). Furthermore, more recent *post mortem* analysis of the brain of AD patients revealed an increase in complement components (Zanjani H et al., 2005), and several studies showed that Aβ peptides activate the classical and the alternative pathways of the complement system *in vitro* (Jiang H et al., 1994; Bradt BM et al., 1998).

Complement components were observed at the CSF level and different patterns of complement factors expression have been reported at different stages of the disease. CSF levels of complement components, such as C1q, C3 and C4, are higher in AD patients than in healthy controls, in particular during the early stages of the disease (Wang Y et al., 2011; Daborg J et al., 2012). On the other hand, terminal complement components (C9 and C5b-9) have been reported only in severe AD (Loeffler DA et al., 2008).

Although the presence of complement components in AD brains has been well investigated, the role played by the complement system is not clear, since studies have mainly focussed on the classical pathway. It seems that complement activation can have a protective role during the early stages of the disease, probably contributing to Aβ clearance. However, during the more chronic phases of AD, complement activation seems to have a negative role, contributing to neurotoxicity with subsequent aggravation of the inflammatory

response (Alexander JJ et al., 2008).

Several studies have revealed a protective role for C1q, which seems to confer neuroprotection against neuronal death induced by fibrillary and oligomeric Aβ in cortical neurons both *in vitro* and in the animal models (Pisalyaput K and Tenner AJ, 2008; Benoit ME et al., 2013). Using mouse models of AD, at early stages of the disease C1q seems to induce the activation of the transcription factor CREB (cAMP response element-binding protein), increasing the expression of neuroprotective genes (Benoit ME et al., 2013). However, at later stages C1q seems to have a detrimental effect on neuronal integrity, enhancing the inflammatory process, as suggested by experiments on mice lacking in C1q. These mice have shown a reduction in the activation of microglia surrounding senile plaques and improved neuronal integrity (Fonseca MI et al., 2004).

C3 may play a role in tissue homeostasis, decreasing pathology in Aβ plaques. Elevated C3 levels were associated with reduced Aβ accumulation in transgenic AD mice (Wyss-Coray T et al., 2002). Furthermore, the inhibition of C3 activation leads to increased Aβ deposition and the degeneration of neurons, suggesting that the complement system may protect against Aβ-induced neurotoxicity, probably promoting Aβ clearance (Wyss-Coray T et al., 2002). Consistent with these findings, AD transgenic mice deficient in C3 show an age-associated increase in Aβ deposition and neuronal loss; furthermore, their microglia show an activation towards the immunosuppressive alternative phenotype M2, which promotes the resolution of inflammation and tissue repair, suggesting C3 may have a role in the modulation of the microglia phenotype (Maier M et al., 2008).

Regarding the terminal pathway, its components seem to play a detrimental role during AD progression. During Aβ accumulation in senile plaques, increased levels of C5a receptors have been observed; furthermore, C5a receptors colocalize with NFTs (Fonseca MI et al., 2013). Transgenic AD mice showed an age- and disease-associated increase of C5a receptors on microglia located in proximity to Aβ plaques (Ager RR et al., 2010). Furthermore, the use of C5 receptors antagonist in transgenic AD mice induced a decrease in microglia activation and in the burden of Aβ plaques, which correlated with an increase in cognitive performance (Fonseca MI et al., 2009).

It is important to consider that complement regulator levels normally decrease with ageing, a fact that may result in the reduction of protection against the lytic effect of the MAC for neurons in the plaque area (Yang LB et al., 2000).

2.3.4.3. Complement component 4

The forth serum complement component (C4) protein is a betaglobulin that is essential for the propagation of the classical pathway of complement system. When it is cleaved, C4b is a subunit of the C3 and the C5 convertases, allowing for the activation of these molecules

and the formation of anaphylotoxines.

C4 exists in two isotypes: C4A and C4B, which have 99% sequence identity. However, they show some differences in hemolytic activity, serological reactivity, and affinity to antigens and immune complexes (Isenman DE and Young JR, 1984; Law SK et al., 1984; Dodds AW et al., 1996).

C4 is the most polymorphic protein of the complement system and is synthesized as a single-chain precursor molecule of 200 kDa, which is subsequently processed in three chains, linked by disulphide bounds (Hall RE and Colten HR, 1977; Janatova J, 1986). Furthermore, the mature C4 protein is formed after a series of post-translational modifications such as sulfation and glycosylation (Fey G et al., 1980; Karp DR, 1983).

The C4 gene is in the MHC class III region and included in a module of four consecutive genes, called RCCX, which contains the genes RP for the serine/threonine kinase, C4, CYP21 for the steroid 21-hydroxilase, and TNX for tenascin X. These four genes constitute a module and are always duplicated together. When duplicated in a bimodular structure, an additional functional C4 gene is created, whereas the other duplicated genes usually are non-functional (Yang Z et al., 1999) (Figure 25).

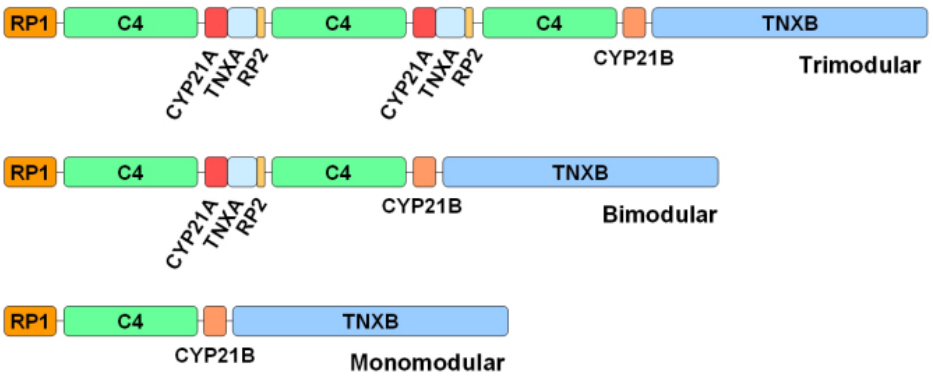

Figure 25. Modular variations of human RCCX module in the MHC class III region. RP1: Ser/Thr protein kinase; C4: Complement Component 4 (each C4 gene may code either the C4A or the C4B protein); CYP21: Steroid 21-hydroxylase; TNX: tenascin-X. From Szilagyi A et al., 2006.

C4A and C4B encode the two isoforms and present CNVs, size variations, and nucleotide polymorphisms (Schneider PM et al., 1986; Dangel AW et al., 1994). C4A varies in copy number from 0 to 5, while C4B genes from 0 to 4. The most common copy number counts for C4 in a European-derived diploid genome are 4 copies: 2 copies of C4A and 2 of C4B (Yang Y et al., 2007). The presence of C4 null alleles, C4AQ0 and C4BQ0 is possible; the

homozygous C4A null state is seen in approximately 1% of Europeans, the homozygous C4B null state is observed in 2–10%, and heterozygous C4A or C4B null alleles occur in approximately 45–56% (Yang Y et al., 2007). Complete C4 homozygous deficiency is extremely rare, and only a few cases have been reported (Wu YL et al., 2009).
In addition, C4A and C4B genes show size variations, with a long gene of 20.6 kb and a short gene of 14.2 kb. This variation is due to the integration of the human endogenous retrovirus HERV-K(C4) of 6.36 kb in intron 9, which lengthens the gene without changing the protein sequence (Dangel AW et al., 1994).

2.3.4.4. Complement C3b/C4b receptor: CR1

The complement system participates in the opsonisation by targeting pathogen structures and cell debris. In this way, C3 fragments on pathogens or dying cells can be recognized by receptors on the surface of phagocytes.
The complement C3b/C4b receptor 1 called CR1 is one of these receptors: it is a glycoprotein that plays a central role in immune complex clearance. It is formed by four main structural domains: a signal peptide, an extracellular domain, a transmembrane domain and a cytoplasmic domain (Klickstein LB et al., 1987).
It is expressed on monocytes, macrophages, neutrophils and erythrocytes, and it is able to bind C3b and also C4b fragments (van Lookeren Campagne M et al., 2007). CR1 on the surface of erythrocytes is important for the clearance of soluble immune complexes: they are transported to the liver and the spleen, where they are cleared by macrophages and removed from the circulation (Taylor RP et al., 1997). CR1 on phagocytes facilitates the uptake of immune complexes and CR1 on B and T lymphocytes is involved in immune regulation (Erdei A et al., 2009). CR1 interacts with the C3 and C5 convertases of the classical and alternative pathways, thus inhibiting their activation (Krych-Goldberg M and Atkinson JP, 2001).
CR1 gene maps on chromosome 1q32.2 and is polymorphic. There are four allotypes which differ for the extracellular domain, which is composed of multiple short consensus repeat (SCRs). Seven SCRs are grouped in long homologous repeats (LHRs) (Klickstein LB et al., 1987) (Figure 26). The number of LHR regions differ for the isoforms, so they have a different size. CR1*3 (CR1-C) has 3 LHRs (160 kDa), CR1*1 (CR1-A) has 4 LHRs (190 kDa), CR1*2 (CR1-B) has 5 LHRs (220 kDa) and CR1*4 (CR1-D) has 6 LHRs (250 kDa). The CR1*1 and CR1*2 isoforms are the most common in the population (83% and 15%, respectively) (Krych-Goldberg M and Atkinson JP, 2001).
CR1 seems to play a role in AD pathogenesis by modulating the clearance of Aβ via C3b-mediated adherence to erythrocytes (Rogers J et al., 2006); furthermore, CR1 is expressed on microglia, contributing directly the Aβ clearance in the brain (Crehan H et al., 2013).

However, activated microglia show increased CR1 expression, that correlate with increased intracellular superoxide anion generation and the secretion of TNF-α and IL-1β, thus having a detrimental effect on neurons (Crehan H et al., 2013).

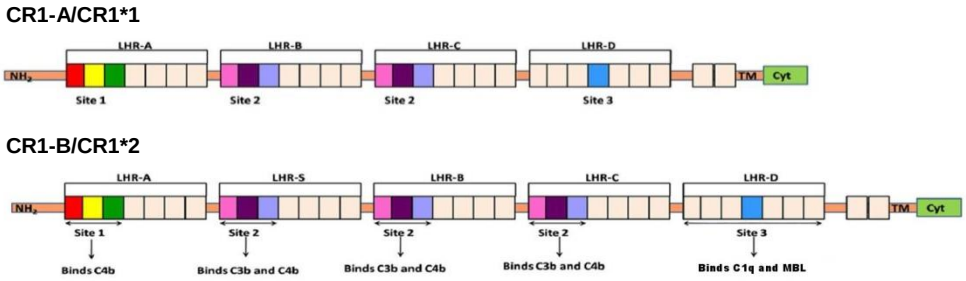

Figure 26. Structure of CR1-A and CR1-B isoforms. They contains 30 and 37 short consensus repeats (SCR) grouped into four or five long homologous repeats (LHRs), respectively. Modified from Zhu XC et al., 2015.

GWAS have identified SNPs in CR1 gene as risk factors for AD (Lambert JC et al., 2009; Corneveaux JJ et al., 2010), but it is unclear their influence AD pathogenesis. Many studies on several SNPs in CR1 in distinct populations show their association with AD (Schjeide BM et al., 2011; Chen LH et al., 2012), but the association is not found in studies on other populations (Chung SJ et al., 2013).

Among the several SNPs in CR1 studied in AD, the CR1 rs6656401 polymorphism causes a A to G transition, located in a non-coding region; it is associated with the inclusion of a fifth LHR domain, creating the CR1*2 isoform (Lambert JC et al., 2009). The CR1 rs4844609 polymorphism causes a transvertion from A to T and is located in the coding region of the gene, leading to an amino acid substitution from Ser to Thr (Keenan BT et al., 2012). This SNP is in a region of the molecule that is implicated in the binding to C1q, MBL and ficolins (Jacquet M et al., 2013). C1q, C3b and C4b are present in AD plaques (Eikelenboom P and Stam FC, 1982) and the CR1 rs4844609 polymorphism, together with the CR1 rs6656401 polymorphism, may have an impact in the clearance of Aβ peptides. The CR1 rs2274567 polymorphism causes a substitution from A to G that leads to an amino acid change from His to Arg (Ma XY et al., 2014).

3. Studying Multiple Sclerosis

3.1. Aims of the work

MS is a chronic disimmune disease of the CNS characterized by demyelination and neurodegeneration. Neurodegeneration is thought to be the culmination of different events, including oxidative stress, energy deficiency, ionic imbalance, and the failure of regenerative and neuroprotective mechanisms. The etiology of this disease is complex: both genetic and environmental factors play a significant role in determining the risk of developing MS.

Our work on MS focuses mainly on two projects: one regarding potential genetic risk factors in the MHC class III region, and the other concerning the study of the interplay between genetic and environmental factors, in particular vitamin D.

Our research group has been interested for many years in genes located in the MHC class III (chromosome 6p21.3). Our attention is directed to polymorphic variants that may be involved in autoimmune diseases and disorders with an inflammatory component. HSPA1A (or HSP70-1), HSPA1B (or HSP70-2) and HSPA1L (or HSP70-HOM) are three polymorphic genes which map on the MHC class III region and code for Hsp70s, which are highly conserved molecular chaperones. Some of them are expressed in normal physiological conditions, while the amount of the others increases in response to a variety of stress stimuli, including oxidative stress. The role of Hsp70s in MS pathogenesis is not clear: they may have a neuroprotective role, but when they are secreted in the extracellular environment, they can act as pro-inflammatory cytokine and participate in neuroinflammation.

In 2014, our research group found that HSP70-2 rs1061581 polymorphism is related to the risk of developing MS: the G allele frequency is increased in MS patients compared to healthy controls. Based on this consideration, another HSP70 gene will be analysed in this study: HSPA1L, which encodes the constitutively expressed Hsp70-Hom. On the other hand, we want to continue the analysis of the possible role of the stress inducible Hsp70-2 in MS, considering its role in the response to oxidative stress.

In detail, the aims of the study are to:

- investigate the association of HSP70-HOM rs2227956 polymorphism with MS risk and severity and elucidate its involvement in the regulation of Hsp70-Hom protein expression levels. The HSP70-HOM rs2227956 polymorphism, also known as HSP70-HOM +2437 T/C, is localized in the coding region of the HSPA1L gene and causes a Met to Thr amino acid substitution at position 493, located in the peptide-

binding domain region. This substitution seems to be associated with the variation in the peptide binding specificity, thus affecting the HSP70-Hom biologic function.
A case-control study will be conducted to compare allelic and genotypic frequencies between MS patients and healthy subjects, in order to establish a possible association of the polymorphism with MS risk and severity (considering the multiple sclerosis severity score, MSSS). Since HSPA1B and HSPA1L are in linkage disequilibrium, we will focus on multilocus analysis to test whether it is possible to identify combined genetic predictors of MS risk. Subsequently, Hsp70-Hom protein levels will be considered in order to understand whether the HSP70-HOM rs2227956 polymorphism may influence the protein expression. Furthermore, a statistical analysis will be performed to assess a possible association between Hsp70-Hom protein levels and MS severity.

- investigate *ex vivo* the influence of oxidative stress on PBMCs from MS patients and healthy controls. PBMCs will be treated with hydrogen peroxide; their mitochondrial activity, Hsp70-2 protein expression and the production of intracellular ROS will be assessed at different time points. In addition, mitochondrial activity and Hsp70-2 proteins levels will be related to the previously studied HSP70-2 rs1061581 polymorphism, also known as HSP70-2 +1267 A/G. This polymorphism is localized in the coding region of the HSPA1B gene and causes a synonymous mutation that seems to influence the process of RNA translation; therefore, it may vary the response to oxidative stress.

Considering the complex interplay between genetic and environmental factors in determining MS risk, we will direct our attention to vitamin D and its receptor, VDR.
Vitamin D has immunomodulatory effects and its deficiency is a risk factor for the development of MS. Vitamin D exerts its effects by binding to the VDR, which can recognize and bind to VDREs, upregulating or downregulating the transcription of target genes. Although the VDR gene is not located in the MHC class III, it attracts our attention because it is polymorphic; it is located on chromosome 12q13.1 and genetic variations within this gene could alter the response to vitamin D.
The aim is to investigate the possible role of VDR polymorphisms in MS. Specifically, allelic and genotypic frequencies of VDR rs731236 (TaqI T/C) and VDR rs433408 (HpyCH4V G/A) polymorphisms will be assessed, comparing MS patients and healthy controls. The first polymorphism is located in the exon 9 of the VDR gene and causes a substitution from T to C, which results in a silent codon change. The second polymorphism, VDR rs4334089, is located in the 5'UTR. Furthermore, total, cytoplasmic and nuclear VDR protein expression will be analysed to understand whether the two VDR polymorphisms could influence VDR protein expression and/or its distribution in the cell. In addition, serum vitamin D levels [$25(OH)D_3$] will be determined in MS patients to assess a possible

association with VDR protein levels.
Understanding the possible influence of the polymorphisms of VDR in MS and the potential implications with vitamin D levels could provide valuable information to shape resource and healthcare planning as well as individual therapeutic intervention.

3.2. Materials and methods

3.2.1.Subjects and ethics statement

Patients with a diagnosis of MS according to the 2010 revised McDonald Criteria were recruited from the MS Centre of the IRCCS National Neurological Institute "C. Mondino" (Pavia, Italy). The neurological disability of MS patients was quantify by the Expanded Disability Status Scale (EDSS) and the clinical impact of the disease was calculated applying the Multiple Sclerosis Severity Score (MSSS), which relates scores on EDSS to disease duration.
Healthy controls matched for ethnicity and age, were selected from healthy subjects assessed to be free from any kind of disorders, whether physical or mental, at the time of blood sampling. Healthy subject were recruited by the IRCCS National Neurological Institute "C. Mondino" (Pavia, Italy).
The study has been approved by the Ethics Committee of each institution and has been conducted in accordance with the principles set out in the World Medical Association Declaration of Helsinki. All patients and controls signed informed consent.

3.2.2.Separation of blood components

PBMCs from MS patients and healthy controls were separated by the other blood component by density gradient centrifugation, using Ficoll (Histopaque, d=1077 at room temperature, Sigma Aldrich). Blood was stratified over the Ficoll and centrifugated at 450 g for 30 minutes. During the centrifugation, different blood components migrate through gradient forming distinct phases: lower layer with erythrocytes, Ficoll layer, leukocyte ring, upper layer with plasma and platelets.
A portion of the plasma layer was collected and stored frozen at -80°C.
Lymphocytes were harvested from the ring above Ficoll and washed with PBS (Phosphate Buffered Saline). The samples were centrifuged at 300 g for 10 minutes. Cells were counted using a Burker chamber and by staining with Trypan blue. Cells for extracting proteins were stored at -80°C as dry pellet, another amount was suspended in RPMI for the cell culture.

3.2.3.Evaluation of vitamin D plasma levels

Vitamin D plasma levels were evaluated by using the Elecsys® Vitamin D total assay on the cobas e601 immunoanalyzer (Roche Diagnostics).

3.2.4.Cell cultures and H_2O_2 treatments

3.2.4.1. PBMC cultures

Isolated PBMCs are primary culture; in absence of mitogens they are not able to proliferate and have a limited survival. PBMCs were cultivated in RPMI 1640 medium (EuroClone), supplemented with 10% fetal bovine serum, 1% L-glutammine, penicillin (100 U/ml) and streptomycin (100 μg/ml). PBMCs were incubated at 37 °C in 5% CO_2 atmosphere.

3.2.4.2. MTT assay and H_2O_2 treatments

The MTT assay is a colorimetric assay used to assess cell viability or to measure cell proliferation. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a yellow light sensitive salt, when solubilized in PBS, it is able to enter into the cells. Mitochondrial succinate dehydrogenase cleave the tetrazolium ring, forming the insoluble purple formazan. The amount of formazan generated is directly proportional to the mitochondrial activity of the cells examined. The formazan must be solubilized to produce a homogeneous solution suitable for measurement of optical density.

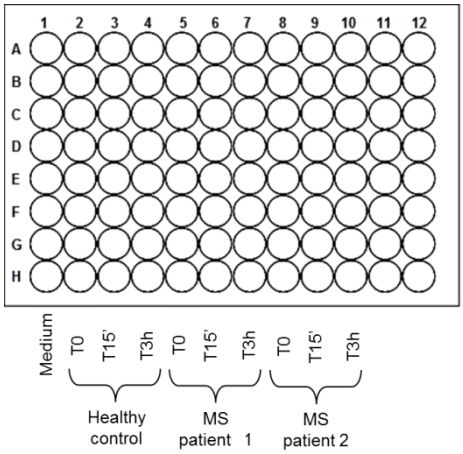

Figure 27. Scheme for the treatment with H_2O_2 for the MTT assay

In this study the MTT assay was used to assess PBMCs viability after H_2O_2 treatments. Briefly, PBMCs were seeded into 96 well plates at a density of 5.0 x 10^4 cells/well in 100 µl of RPMI 1640 medium and incubated at 37 °C in 5% CO_2 atmosphere. A scheme of the subdivision of the wells is reported in figure 27. Normalization was done on the medium. Cells were treated with 10 µM H_2O_2 (Sigma-Aldrich) for different time exposures: 15 minutes and 3 hours. Immediately after removing the medium with H_2O_2, 10 µl of MTT [solubilized in PBS (1 mg: 1 ml)] was added to each well.
After 4 h at 37 °C, formazan crystals were solubilized with 100 µl of lysis buffer (20% sodium dodecyl sulfate (SDS) in 50% dimethylformamide), at 37 °C overnight. Absorbance values were measured at 595 nm in a microplate reader (SynergyHT, BioTek Instruments, Inc.).

3.2.4.3. H_2O_2 treatments for the evaluation of Hsp70-2 protein expression

To assess the Hsp70 protein levels, PBMCs were seeded into 6 well plates at a density of 3.0 x 10^6 cells/well in 1 ml of RPMI 1640 medium and incubated at 37 °C in 5% CO_2 atmosphere.
Cells were treated with 10 µM H_2O_2 (Figure 28). After the oxidative treatment, the medium with H_2O_2 was removed, the cells washed with 1 ml of PBS. PBMCs were resuspended in PBS and collected using a cell scraper. PBMCs were then washed, and the cellular pellet was stored at -80 °C for subsequent Western blotting analysis.

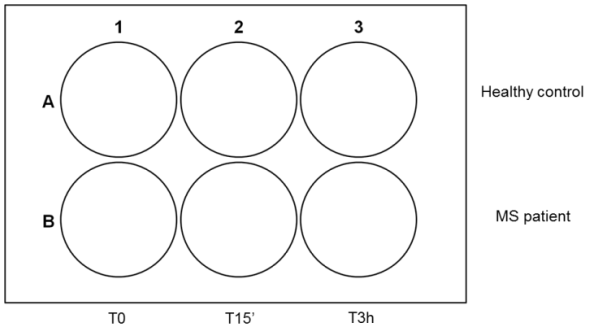

Figure 28. Scheme for the treatment with H_2O_2 to evaluate Hsp70-2 protein levels.

3.2.5. Cell fractionation

Nuclear and cytoplasmic fractions of PBMC from MS patients and healthy controls were prepared using the Nuclear Extract Kit (Active Motif) following the manufacturer's

instructions, and stored for subsequent Western blot analysis on VDR protein.
Briefly, cellular pellets, obtained after the separation of blood component, were gently resuspended in a Hypotonic buffer by pipetting. Cells were allowed to swell by incubating for 15 min on ice; then a detergent solution was added and samples were vortexed for 10 seconds at the highest setting. The suspension were centrifuged for 30 sec at 14000 x g at 4°C. The supernatant, that constitute the cytoplasmic fraction, was transferred and stored at -80°C. The pellet was used for nuclear fraction collection: it was resuspended in Complete Lysis Buffer by pipetting and then vortexed 10 sec at the highest setting. The suspension was incubated for 30 min on ice on a rocking platform at 150 rpm. The suspension was vortexed for 30 sec at the highest setting and centrifuged for 10 min at 14000 x at 4°C. The supernatant, representing the nuclear fraction, was transferred and store at -80°C.

3.2.6.Protein expression analysis

3.2.6.1. Homogenization of the samples

To evaluate Hsp70-Hom and total VDR protein expression, PBMCs isolated from peripheral blood of MS patients and healthy controls and stored as dry pellet at -80°C were used. The cells were homogenized in a buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 50 mM 2-mercaptoethanol, 0.32 mM sucrose, with the addition of a protease inhibitor cocktail at the dilution suggested by the manufacturer (Roche Molecular Biochemicals), by using a Teflon/glass homogenizer and sonicating twice for 10 s.

3.2.6.2. Protein content estimation

The protein content of each sample of nuclear, cytoplasmic and whole-cell fractions was measured via Bradford's method using bovine serum albumin (BSA; Sigma Aldrich) as standard. 96 wells plates were used and each sample was analysed in triplicate. Dilutions of the samples were prepared using the lysis buffer as diluent. BSA was used to produce a standard curve (0; 0.5 µg/ml; 1 µg/ml; 2 µg/ml; 4 µg/ml; 8 µg/ml; 16 µg/ml). Each well was filled with 200 µl of a solution formed by mixing the diluted sample and Bradford 1X. Plates were read at 595 nm with a spectrophotometer. Interpolation of the absorbance and standard known concentrations were used to calculate the concentration of each sample.

3.2.6.3. Preparation of the samples for SDS-electrophoresis

To evaluate Hsp70-Hom protein expression, equal amounts of extracted proteins were diluted in a protein gel loading solution 2X containing 0.125 M Tris HCl (pH 6.8), 20% glycerol, 4% SDS, 0.05% bromophenol blue, 10% mM 2-mercaptoethanol and boiled for 5 min, at 95°C to denature the proteins.
To evaluate Hsp70-2 protein expression cells treated *in vitro* were used. PBMCs were resuspended directly in 25 µl of 1X SDS protein gel loading solution, sonicated and boiled for 5 min, at 95°C.

3.2.6.4. Electrophoresis and western blot

SDS-polyacrylamide gel electrophoresis is based on the denaturation of proteins by the anionic detergent SDS, which binds proteins, giving to them a negative charge. In this way, the proteins run towards the anode and are separated on base of their molecular weight.
Gels were casted mounting glasses for gel electrophoresis and 1.5 mm spacers, using the relative support (Biorad). Resolving gels were prepared at 12% of polyacrylamide content, using Tris HCl 1.5 M pH 8.8, 0.01% SDS, 0.05% ammonium persulfate (APS), 0.05% N,N,N',N'-tetrametiletilendiammine (TEMED) and poured into the gap between the outer and the inner glass. Gels were left to polymerise for 45-60 minutes at room temperature After polymerization, the stacking gel was added (5% polyacrylamide, Tris HCl 1.5 M pH 6.8, 0.01% SDS, 0.01% APS, 0.01% TEMED).
Gels were mounting into the running apparatus (Biorad) and samples were loaded into the gel wells. A pre-stained protein ladder (Amersham Pharmacia Biotech Rainbow Markers, Amersham) was loaded as well. Gels were running at 120 mV in running buffer containing 0.025 M TRIS-HCl, 0.2 M glycine and 3.4 mM SDS. Resolved proteins were transferred to nitrocellulose membrane, previously conditioning in the transfer buffer containing 0.025 M TRIS-HCl, 0.2 M glycine and 20% methanol. A gel sandwich was prepared in a cassette (Biorad) by layering foam pad, filter paper, gel, membrane, filter paper and foam pad. The cassette was placed into its module and tank, filled with chilled transfer buffer. Transfer was performed at 4°C for 1 hour at 250 mA. After the transfer, the membranes were stained with red Ponceau to assess the correct loading of the proteins. The membranes were washed in TBS-T buffer pH 7.5 containing 10 mM TrisHCl, 100 mM NaCl and 0,1% Tween20 and then blocked in 6% milk in TBS-T buffer for 2 hours to avoid non-specific antibodies binding. Membranes were incubated with the primary antibodies at 4°C overnight. Mouse monoclonal anti-Hsp70-Hom antibody (Enzo Lifescience) was diluted at 1:1000, rabbit monoclonal anti-Hsp70-2 antibody (Enzo Lifescience) was diluted 1:750, mouse monoclonal anti-VDR antibody (Santa Cruz Biotechnology) was diluted 1:750, and mouse

monoclonal antibody anti-α-tubulin (Sigma-Aldrich) was diluted at 1:1000 in 6% milk in TBS-T buffer. Membranes were washed four times for 10 minutes with TBS-T buffer to remove the unbound antibody and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at RT. Anti-mouse and anti-rabbit antibodies (Sigma-Aldrich) were diluted 1:3000 in 6% milk in TBS-T buffer. The unbound antibody was removed by washing the membranes three times for 10 minutes with TBS-T buffer. Membranes were treated with enhance chemiluminescence (ECL) liquid (Biorad) for 2 minutes and the excess of liquid was removed to detect immune-reactive bands on autoradiographic films (Amersham Biosciences) after 1 to 10 minutes of exposition under dark conditions. The chemiluminescent reaction is based on the H_2O_2-dependent oxidative activity of HRP on luminol, the intensity of subsequent emission of light is proportional to the quantity of HRP, so to the quantity of the protein of interest. Films were developed using developer and fixer liquids (Kodak) and acquired by scanning. Bands intensity was analysed on the densitometric values obtained using the V1.62 NIH Image software.

3.2.7. Genetic analysis

3.2.7.1. DNA extraction from whole blood

Whole blood was collected by venepuncture, in Vacutainer tubes containing ethylenedinitrilotetraacetic acid (EDTA). Human genomic DNA was obtained from 200 µl of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN) following the manufacturer's protocol. The concentration and purity of DNA was determined by spectrophotometric analysis. Obtained DNA were stored at -20°C.

3.2.7.2. Gene polymorphism analysis with PCR-RFLP

In order to establish alleles and genotypes for HSP70-Hom rs2227956, HSP70-2 rs1061581 and VDR rs731236 polymorphisms, a Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) was performed.
The DNA sequence of interest was amplified by PCR. The amplification of a specific genomic region is possible thanks to the use of two specific primers. The reaction proceed in a series of three repeated cycles: denaturation of the DNA, annealing of primers to the single-stranded DNA templates and extension.
To analyse HSP70-Hom rs2227956, HSP70-2 rs1061581 and VDR rs731236 polymorphisms, PCR amplifications were carried out in a total volume of 25 µl containing: 1 µl of genomic DNA (final concentration 20 ng/µl), 0.1 µl of Taq polymerase (Eurobio)

(final concentration 0.02 U/µl), 2.5 µl PCR Buffer 10X (Eurobio), 0.75 µlMgCl2 (Eurobio) (final concentration 1.5 mM), 2.5 µl dNTPs (final concentration 2 mM each), 1 µl of each specific forward and reverse primers (final concentration 4 ng/µl) (Table 2) and 16.15 µl of sterile H_2O, using the thermal cycle I-Cycler (Biorad).

Gene	Primers	Size
HSP70-HOM	F: 5'-GGACAAGTCTGAGAAGGTACAG-3' R: 5'-GTAACTTAGATTCAGGTCTGG-3'	878 bp
HSP70-2	F: 5'-CATCGACTTCTACACGTCCA-3' R: 5'-CAAAGTCCTTGAGTCCCCAAC-3'	1117 bp
VDR	F: 5'-GTCACTGGAGGGCTTTGG-3' R: 5'-GCTGCACTCAGGCTGGAA-3'	381 bp

Table 2. Sequence of primers and size of fragments obtained following the PCR.

The following amplification conditions were applied for HSP70-Hom rs2227956 and HSP70-2 rs1061581 polymorphisms: initial incubation at 95 °C for 5 min, followed by 35 cycles each of 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min and 30 s; final extension was carried out at 72 °C for 7 min.

To analyse VDR rs731236 polymorphism the following amplification conditions were applied: initial incubation at 95 °C for 5 min, followed by 30 cycles each of 95 °C for 30 s, 59.5 °C for 30 s, 72 °C for 30 s; final extension was carried out at 72 °C for 7 min.

To verify the correct amplification of the genomic region of interest, 2% agarose gel electrophoresis was performed. The agarose in crystals (Eurobio) was melted in 1X TRIS-Boric acid-EDTA (TBE) electrophoresis buffer pH 8.3. Subsequently, ethidium bromide (Pharmacia Biotech), a DNA intercalating agent, was added to the solution to a final concentration of 0.5 mg/ml. The solution was poured in the specific tank and once solidified, the gel was placed in an electrophoretic apparatus and covered with 1X TBE buffer. Subsequently, 10 µl of sample of amplified DNA and a DNA ladder (Gene Ruler DNA Ladder; Fermentas) were loaded into the wells. The gels were run at constant voltage (5V/cm), for 2 hours to obtain the migration of the DNA polyanion toward the anode. At the end of the race, the bands are displayed on a UV transilluminator (λ = 312 nm). At this wavelength, the ethidium bromide emits radiation in the visible allowing the detection of the bands: 878 bp for HSP70-Hom rs2227956 polymorphism, 1117 bp for HSP70-2 rs1061581 polymorphism, 381 bp for VDR rs731236 polymorphism (Table 2).

Genotypes for HSP70-HOM rs2227956, HSP70-2 rs1061581 and VDR rs731236 polymorphisms were determined by digestion with an appropriate restriction enzyme, through the PCR-RFLP. This method is based on the fact that a single nucleotide change in the recognition sequence of restriction enzymes alters the pattern of "cuts" operated in

DNA, and therefore the restriction enzyme is no able to cut the DNA.

For HSP70-HOM rs2227956 polymorphism, PCR products were then treated with NcoI (New England Biolabs) as restriction enzyme following this protocol: 15 µl PCR product, 3.5 µl Nuclease Enzyme (NE) Buffer 10X, 0.5 µl NcoI (final concentration: 0.2 U/µl), 0.35 µl BSA and 5.65 µl H_2O.

The same protocol was applied for HSP70-2 rs1061581 polymorphism, but using PstI (New England Biolabs) as restriction enzyme.

For VDR rs731236, PCR products were then treated with TaqI (New England Biolabs) as restriction enzyme following the protocol: 15 µl PCR product, 3.5 µl NE Buffer 10X, 0.5 µl TaqI (final concentration: 0.2 U/µl) and 6.0 µl H_2O.

The reaction was then carried out for HSP70-HOM rs2227956 and HSP70-2 rs1061581 polymorphisms at 37°C, for VDR rs731236 at 65°C, for 16 hours and the restriction fragments were then separated on a 3% agarose gel (Table 3). Finally, the gel was photographed using a Kodak DC290 digital camera (Figure 29).

Gene	SNP	Enzyme	Fragments (bp)
HSP70-HOM	rs222795	Ncol	878 (C) 551 (T) 327
HSP70-2	rs1061581	Pstl	1117 (A) 936 (G) 181
VDR	rs731236	TaqI	381 (T) 203 (C) 178

Table 3. Restriction enzymes and restriction fragments of the investigated polymorphisms.

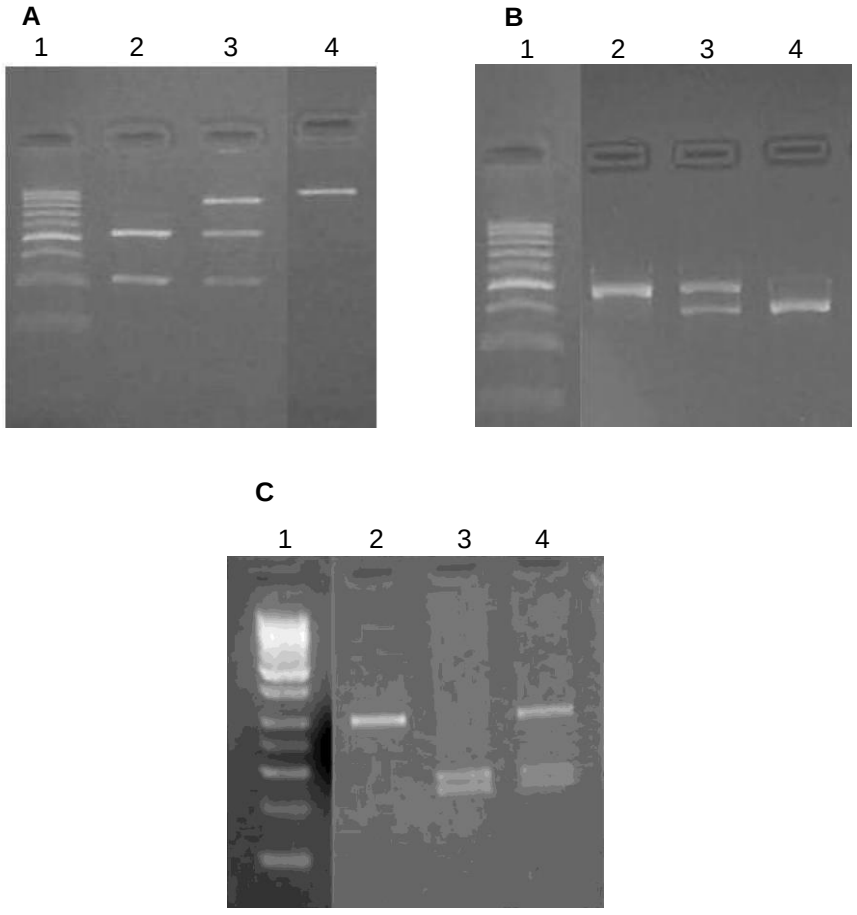

Figure 29. Representative images of agarose gels for the genotyping by PCR-RFLP. A| Genotyping of the HSP70-HOM rs2227956 polymorphism. Lane 1: DNA ladder; lane 2: homozygous genotype TT; lanes 3: heterozygous genotype TC; lane 4: homozygous genotype CC. **B|** Genotyping of the HSP70-2 rs1061581 polymorphism. Lane 1: DNA ladder; lane 2: homozygous genotype AA; lanes 3: heterozygous genotype AG; lane 4: homozygous genotype GG. **C|** Genotyping of the VDR rs731236 polymorphism. Lane 1: DNA ladder; lane 2: homozygous genotype TT; lanes 3: homozygous genotype CC; lane 4: heterozygous genotype TC.

3.2.7.3. Real-Time PCR

Alleles and genotypes for VDR rs4334089 polymorphism were assessed by Real-Time PCR.

The analysis of a SNP using Real-Time PCR takes advantage of two TaqMan probes. TaqMan probes are single strand oligonucleotide, a fluorophore is covalently attached to the 5'-end of the probe and a quencher at the 3'-end. The quencher molecule suppresses the fluorescence emitted by the fluorophore. During the extension phase of the amplification, the probe binds to a specific target sequence and is degraded by the hydrolysis of the nuclease activity of the Taq polymerase. In this way, the quenching effect is abolished and the fluorescent signal is detectable. Sequence-specific forward and reverse primers allow DNA amplification and allele-specific probes, which hybridize to the SNP polymorphic site, permit the discrimination of the two alleles. The two alleles are labelled with different fluorophores: VIC-labeled probe detects allele 1 sequence and FAM-labeled probe detects allele 2 sequence. During the reaction, the fluorescence signal of each dye is detected and measured by specific software and the genotypes are assigned. Results are reported as a scatter plot of the allele 1 (VIC dye) versus allele 2 (FAM dye), in which each dot represent a single sample (Figure 30).

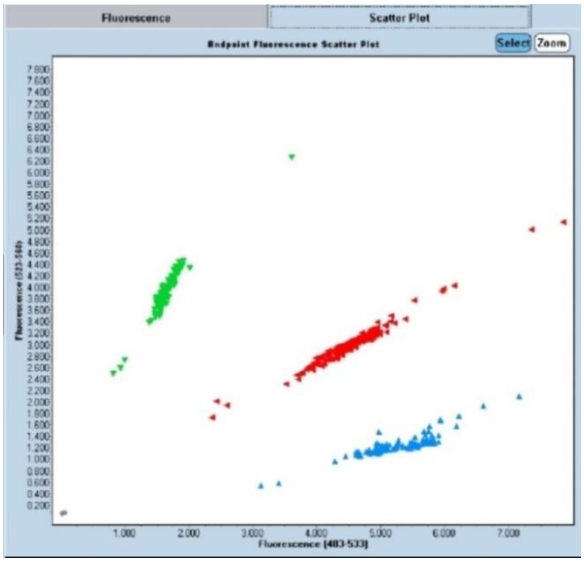

Figure 30. Representative result of qPCR based on the custom allele discrimination Taqman assay. The results for the VDR rs4334089 polymorphism are shown. Clusters show genotypes: green for homozygous GG (minor) genotype; red for heterozygous AG genotype and blue for homozygous AA (wild type) genotype.

The VDR rs4334089 was analysed by Real-Time PCR using the specific assay C___2880798_10 (Applied Biosystem). 1 µl of DNA were aliquoted into each well of a 384-well plate (final concentration: 10 ng/µl); the reaction was carried out in a final volume of 5 µl adding: 2.5 µl of 2X Master mix Probe (Biorad), 0.0625 µl µl of assay and 2.4375 µl of sterile H_2O. The SNP detection was carried out in the LightCycler 480 (Roche), following the thermal cycling conditions: one denaturation cycle of 10 min at 96°C, followed by 45 cycles each of 92°C for 15 s and 60°C for 90. Fluorescence was measured by the dedicated software (RealPlex 2.0) and the genotype was determined (Figure 30).

3.2.8. Statistical analysis

Genotypic and allele frequencies of the analysed polymorphisms (HSP70-HOM rs2227956, VDR rs731236 and VDR rs4334089) were calculated in MS patients and healthy controls.
Allelic frequencies in controls were examined to detect any significant deviation from the Hardy–Weinberg Equilibrium using a goodness of fit χ^2 test.
An unconditional logistic regression analysis, adjusted by sex and age, were performed to assess the association between the analysed polymorphisms and MS; adjusted Odds Ratios (OR) with 95% confidence intervals (95% CI) were derived and used as measure of effect.
For the HSP70-HOM rs2227956 polymorphism an additive allelic model and a genotypic model were fitted to estimate the C allele risk, the heterozygous TC versus wild type TT risk and the homozygous CC versus wild type TT genotype risk.
For the VDR rs731236 polymorphism a genotypic model was fitted to estimate the heterozygous TC versus wild type TT risk and the homozygous CC versus wild type TT genotype risk.
For the VDR rs4334089 polymorphism a genotypic model was fitted to estimate the heterozygous GA versus wild type GG risk and the homozygous AA versus wild type GG genotype risk.
For the VDR rs731236 and VDR rs4334089 polymorphisms a dominant model was applied to estimate the allelic risk, considering at least one minor allele: C allele for VDR rs731236 and A allele for VDR rs4334089.
The Likelihood Ratio (LR) based omnibus association test was used to analyse whether the overall HSP70-2 and HSP70-HOM multilocus variation influences MS presence and severity and whether a single locus had an independent effect.
A linear regression model was use to analyse the association between HSP70-HOM polymorphism and MS severity, and between HSP70-HOM polymorphism and the quantitative expression of Hsp70-Hom protein in MS patients and healthy controls. The direction of the regression coefficient represents the effect of the risk genotype (CC or TC) versus the reference genotype (TT) (i.e. a negative regression coefficient means that the risk

allele decreases MSSS mean or Hsp70-Hom protein level mean); sex and age were included as covariates.

The same type of analysis was applied to analyse the association between VDR rs731236 and VDR rs4334089 polymorphisms and the total, cytoplasmic and nuclear VDR protein expression, respectively. The direction of the regression coefficient represents the effect of the risk genotypes (TC and CC for VDR rs731236; GA or AA for VDR rs4334089) versus the reference genotype (TT for VDR rs731236; GG for VDR rs4334089) (i.e. a negative regression coefficient means that the risk allele decreases VDR protein level mean).

Furthermore, a linear regression model was used to test whether plasma vitamin D levels affect VDR protein expression. The direction of the coefficient represents the effect of the vitamin D levels (i.e. a negative regression coefficient means that the increase in vitamin D levels decreases VDR protein expression); sex, age and MS severity were included as covariates. Separated models were fitted to study protein expression in the overall sample and in cases/controls samples separately.

The Wilcoxon rank-sum test was performed to compare the Hsp70-Hom protein expression levels between low and high MS impact patients.

For data on MTT, Hsp70-2 protein expression and ROS levels, normality distribution of endpoint variables was tested by the Shapiro-Wilk test and by the skewness and kurtosis values compared to their standard error and homogeneity of variance using Levene test.

For the MTT and ROS endpoint variable a mixed model parametric analysis of variance (ANOVA) was used with the aim of verifying the effect of groups (MS cases vs healthy controls), time period (T0, T15', T3h) and their interaction; the effect of genotype categories (AA, AG, GG), time period (T0, T15', T3h) and their interaction was evaluated for MTT only. Post hoc Student t test with Bonferroni correction for multiple comparisons was used to compare mean pairs when differences were significant.

For Hsp70-2 protein levels of PBMCs endpoint variables a non-parametric approach was chosen, using Friedman test for the evaluation of the effect of the time of exposure (T0, T15', T3h) and Mann-Whitney and Kruskal-Wallis tests for the comparison of the two groups (MS cases vs healthy controls) and the genotype categories (AA, AG, GG) respectively.

All the statistical analyses were performed using Plink 1.07 and Stata 14 statistical software (Stata Corporation, College Station, TX, USA).

3.3. Results

3.3.1. Role of Hsp70-Hom in multiple sclerosis

3.3.1.1. HSP70-HOM rs2227956 polymorphism

The SNP Hsp70-HOM rs2227956 was analysed in order to find a possible relation with MS onset and/or disease progression.
The genotype of 191 MS patients and 365 healthy controls, displayed full clinical and demographic information, was determined. Statistical analysis were adjusted by age and gender as possible MS risk confounders (MS patients were older compared to healthy controls, 44.1 ± 10.7 vs 35.7 ± 8.6 years, $p < 0.001$; MS female to male ratio was higher compared to the group of controls 1.81 vs 1.31, $p < 0.05$). Genotypic frequencies in the control group did not deviate from the Hardy–Weinberg equilibrium ($p > 0.05$).
TT genotype and T allele were considered respectively as the reference genotype and the reference allele in the analysis. Comparing the group of MS patients with those of healthy subjects, HSP70-HOM gene polymorphism resulted significantly associated with MS (Table 4).

	MS patients	Healthy controls	Adjusted OR (95% CI)	p value ♣
	N=191	**N=365**		
CC	29 (15%)	10 (3%)	6.71 (3.11-14.43)	**<0.0001**
TC	52 (27%)	84 (23%)	1.53 (1.00-2.35)	0.046
TT	110 (58%)	271 (74%)	1	
C	110 (29%)	104 (14%)	2.13 (1.60-2.86)	**<0.0001**
T	272 (71%)	626 (86%)	1	

Table 4. Alleles and genotypes frequencies of HSP70-HOM rs2227956 polymorphism and their association with MS assessed using logistic models corrected by age and gender.
TT genotype was considered the reference genotype in the genotypic model.
T was considered the reference allele in the additive allelic model.
♣ Bonferroni corrected threshold p-value is 0.01 for the genotypic model and 0.0125 for the allelic model.

Regarding the genotypic model, the CC genotype increased the risk of developing MS of almost seven-fold compared to the TT wild-type genotype (OR = 6.71, $p < 0.0001$, Bonferroni threshold applied to the multivariable model: p=0.01), whereas the TC genotype conferred only a moderate risk (OR = 1.53, $p = 0.046$). With regard to the allelic model, the

C allele increased of two-fold the risk of developing the disease (OR = 2.13, p < 0.0001) (Table 4).

Also the disease severity was considered in the analysis: MS severity, measured by MSSS, tended to be distributed differently among MS patient depending on their HSP70-HOM rs2227956 polymorphism genotype. CC patients exhibited an increased MSSS on average of 1.22 compared to TT patients (95% CI: 0.22–2.21, p=0.017), whereas MSSS did not significantly increase in TC heterozygous patients (β = 0.09, 95% CI: −0.70–0.89, p=0.81) (Figure 31).

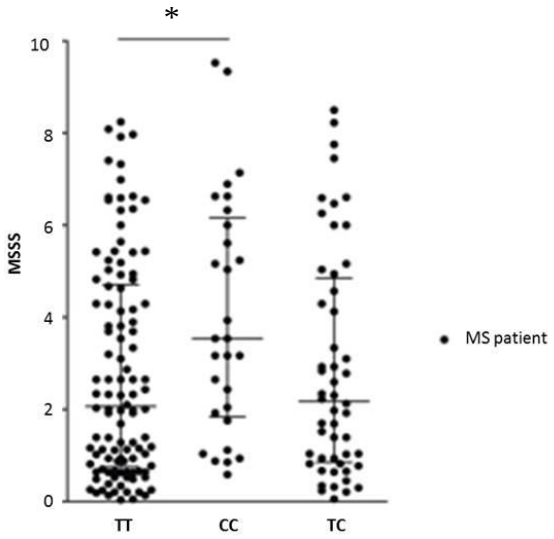

Figure 31. MSSS distribution by HSP70-HOM rs2227956 genotype. MS patients (black dots) are grouped by Hsp70-Hom rs2227956 genotype. 25th percentile, median and 75th percentile are reported on the dot blot (black bars). MSSS is significantly increased in CC carriers, compared to TT carriers.

3.3.1.2. Combined effect of HSP70-2 and HSP70-HOM

In this work, HSP70-HOM rs2227956 polymorphism was found to be associated with MS. Furthermore, our research group previously find an association between HSP70-2 rs1061581 polymorphism and MS, with the G allele as risk allele (Boiocchi C et al, 2014). Considering that the variants of the two polymorphisms are in partial linkage disequilibrium (D′ = 0.2), a multilocus analysis was perform to test whether it was possible to identify combined genetic predictors of MS risk.

A significant increased frequency of the combination of the GC risk alleles of the HSP70-2

rs1061581 and the HSP70-HOM rs2227956 polymorphisms, respectively was found in MS patients compared to healthy controls (OR: 3.49, $p < 0.0001$) (Table 5). On the other hand, MS patients showed a significant decreased frequency of the combination of HSP70-2 rs1061581 and the HSP70-HOM rs2227956 polymorphisms wild-type AT alleles compared to healthy controls (OR: 0,57, $p < 0.0001$) (Table 5).

HSP70-2 rs1061581	HSP70-HOM rs2227956	MS patients	Healthy controls	Adjusted OR (95% CI)	p value
		N=191	N=365		
G	**C**	14%	5%	3.49 (2.17-5.6)	**<0.0001**
A	**C**	15%	9%	1.87 (1.27-2.75)	0.000152
G	**T**	26%	28%	0.93 (0.706-1.22)	0.582
A	**T**	45%	59%	0.57 (0.44-0.73)	**<0.0001**

Table 5. HSP70-2 and HSP70-HOM multimarker analysis. The overall variation of the alleles formed by the HSP70-2 rs1061581 and HSP70-HOM rs2227956 polymorphisms influences MS risk (omnibus association LR test $p < 0.0001$).
The AT combination is considered the reference.

After adjusting for the global effect of the alleles variation in the combined loci, HSP70-HOM exhibited an independent effect on MS risk (conditional LR test $p < 0.0001$), whereas HSP70-2 was only borderline significant (conditional LR test $p = 0.059$).

3.3.1.3. Hsp70-Hom protein expression

To evaluate Hsp70-Hom protein expression, proteins levels were quantified by Western blot, using PMBCs from a subgroup of 47 MS patients and 29 healthy controls, randomly selected (Figure 32).
Hsp70-Hom protein expression did not significantly vary between MS patients and healthy controls or within HSP70-Hom genotypes (Table 6; Figure 33 A).
However, Hsp70-Hom protein levels ware significantly linked to MS severity. We arbitrary chose a cut–off value of an MSSS of 3: MS patients with a MSSS < 3 were considered affected by a mild form of disease, MSSS ≥ 3 reflected patients with a moderate to severe form of MS. Patients with a mild form of MS (MSSS score < 3; 55% of patients) showed a lower level of the median protein levels, compared to that of the other MS patients (MSSS

≥ 3; 45% of patients) (676.1 and 1134, respectively, p=0.038). We noticed that none of the patients with Hsp70-Hom protein levels below the 25th percentile had a high value of MSSS (high disease severity) (Figure 33 B).

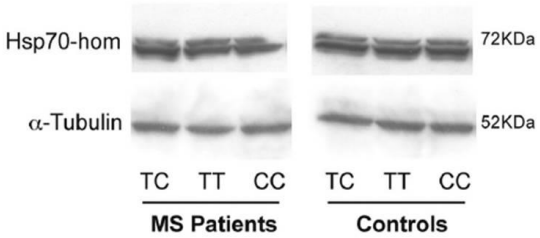

Figure 32. Representative Western blot images of Hsp70-Hom and α-tubulin protein content in PBMCs from MS patients and controls. They are subdivided by the HSP70-HOM rs2227956 genotype (TC, TT, CC). α-tubulin was used to normalize the data.

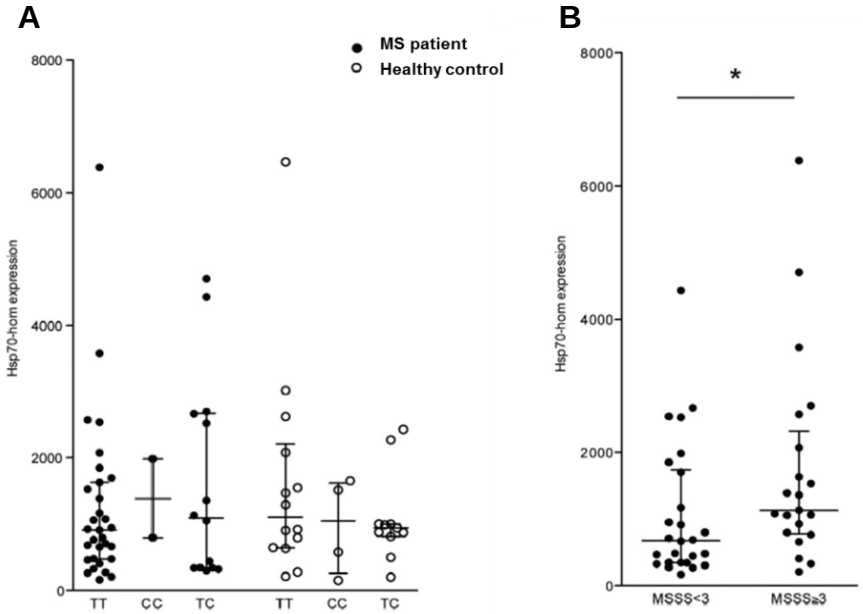

Figure 33. Hsp70-Hom protein expression. A| MS patients (black dots) and controls (white dots) are grouped by HSP70-HOM rs2227956 genotype; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significantly difference between MS patients and healthy controls or within HSP70-HOM genotypes are observe ($p > 0.05$). **B|** MS patients (black dots) are grouped by MS severity (MSSS), 25th percentile, median, 75th percentile are reported on the dot plot (black bars). Hsp70-Hom protein levels are significantly reduced in MS patients with low MS severity (MSSS < 3), compared to MS patients with higher disease severity (MSSS ≥ 3).

	β-coefficient	Standard error	p-value
HSP70-hom genotypes			
CT vs TT	397.32	663.9	0.49
CC vs TT	452.87	657	0.55
Multiple sclerosis yes *vs* no	-209.18	657.04	0.49
Sex (female *vs* male)	-78.42	415.94	0.62
Age (in years)	77.84	349.98	0.82

Table 6. Hsp70-Hom protein expression levels. β-coefficient and standard error of analysed factors are derived from a linear regression model and are adjusted by age and sex. TT genotype is considered the reference genotype.

3.3.2.Role of Hsp70-2 and oxidative stress in multiple sclerosis

3.3.2.1. Subjects analysed

To understand the influence of oxidative stress on PBMC, mitochondrial activity, Hsp70-2 protein levels and production of intracellular ROS were assessed using PBMCs from a total of 77 MS patients and 49 healthy controls displaying full clinical and demographic information. Of every subject HSP70-2 rs1061581 polymorphism genotype was determined. Genotypic frequencies of HSP70-2 rs1061581 polymorphism in the control group did not deviate from the Hardy-Weinberg equilibrium ($p > 0.05$).
Among MS patients, 23% were males and the mean age at the time of samples collection was 48.20±11.73 years. Among the healthy donors, males represented the bigger proportion (58.33%, $p < 0.001$), whereas the mean age at the time of samples collection was quite similar to MS cases (48.00±13.30, $p > 0.01$).

3.3.2.2. MTT levels

To investigate the influence of oxidative stress on PBMCs, we analyzed the mitochondrial activity of PBMCs *in vitro*.
Concentration/response curves to H_2O_2 of PBMCs cultures from 49 MS patients and 46 healthy controls were assessed (Figure 34).

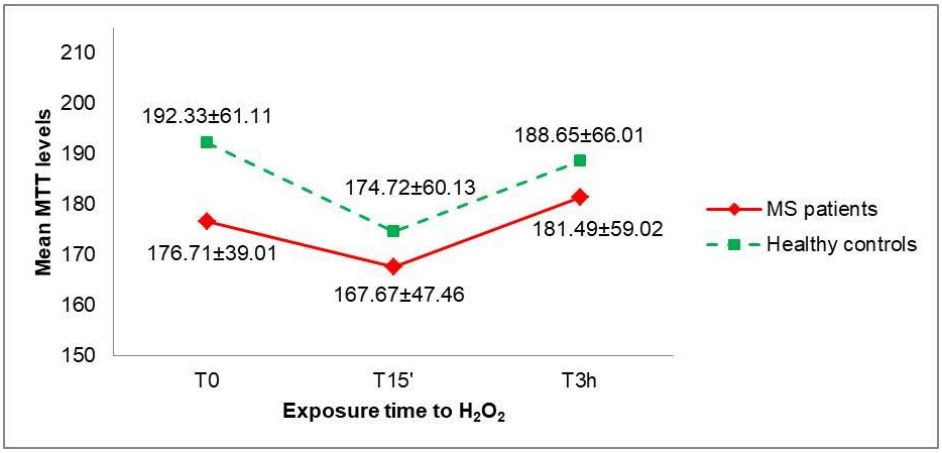

Figure 34. MTT levels in response to H_2O_2 of PBMC cultures from MS patients (red) and healthy controls (green). Mean values and standard deviations are reported.

The relationship among MTT levels, exposure time and group of subjects was studied using a parametric approach.
MTT levels of MS cases were not significantly different compared to healthy controls, but the parametric analysis of variance showed a significant MTT variability over time points for both groups (Table 7 and Figure 34).

Variability source	f	df	p value
Exposure time	8.88	1.74;161.54*	**<0.001**
Group of subjects	0.87	1;93	0.354
Interaction	0.87	1.74; 161.54*	0.408

Table 7. ANOVA mixed model for the relationship between MTT levels, exposure time to H_2O_2 and group of subjects. f is the F-test statistics value, df are the degrees of freedom combination.
* Due to the violation of sphericity the Huynh-Feldt correction was applied.

Considering the whole sample (MS patients and healthy controls together), the *post hoc* analysis for multiple comparisons among exposure time levels shows that MTT basal levels decreased after 15 minutes from the oxidative stress ($t_{Bonferroni}$=4.72, df=94, p<0.001). In detail, PBMCs from MS patients reduced their mitochondrial activity of 9.04 units, compared to the basal levels, and PBMCs from healthy controls had a reduction of 17.61 units. Interestingly, after 3 hours from the oxidative stimulus, basal levels were almost restored ($t_{Bonferroni}$=3.41, df=94, p=0.003): no significant difference was found between

basal level and 3 hours of oxidative stress.

Since the importance of HSP70-2 rs1061581 polymorphism for MS risk, we decided to analyze whether it could influence PBMCs response to oxidative stress. For this reason, our data were stratified by the HSP70-2 rs1061581 genotype. Among MS cases, 37% (18 subjects) were AA, 35% (17 subjects) were AG and 28% (14 subjects) were GG. Among healthy controls, 44% (20 subjects) were AA, 47% (21 subjects) were AG and 9% (4 subjects) were GG; 1 subject had a missing genotype.

The parametric analysis of variance showed a significant MTT variability over time points that was independent of the HSP70-2 rs1061581 genotype; no interaction effects was detected between time and genotype (Table 8 and Figure 35).

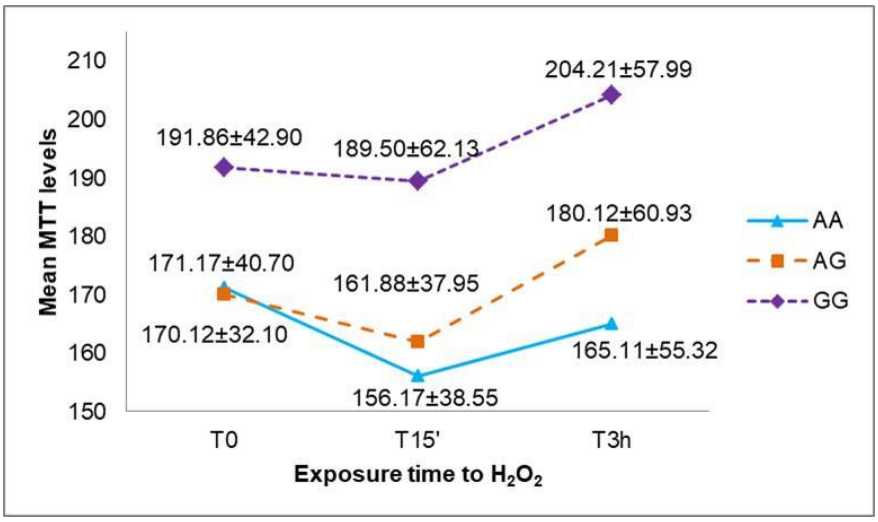

Figure 35. MTT levels in response to H_2O_2 of PBMC cultures from subjects (MS patients and healthy controls together) with different HSP70-2 rs1061581 genotype, AA (blu), AG (orange) and GG (purple). Mean values and standard deviations of MTT levels are reported.

Specifically, MTT levels of GG subjects were slightly higher, compared to MTT levels of AG and AA individuals at each time point, but the difference was not statistically significant. *Post hoc* analysis for the multiple comparisons among exposure time levels shows that, for each genotype, MTT levels decreased considering 15 minutes of oxidative stimulus, compared to the basal levels ($t_{Bonferroni}$=2.72, df=48, p=0.028). In detail, the reduction was of 15.00, 8.24, 2.36 units for AA, AG and GG individuals, respectively. The mitochondrial activity increased at 3 hours ($t_{Bonferroni}$=2.36, df=48, p=0.067), particularly for GG and AG patients that showed a borderline significant increase of 14.71 and 18.24

units, respectively. AA patients had a less pronounced mitochondrial activity increment, compared to AG and GG patients, with a smaller increment of 8.94 units.

Variability source	f	df	P value
Exposure time	3.87	1.54;71.09*	0.035
Genotype	2.12	2;46	0.132
Interaction	0.73	3.09; 71.09*	0.729

Table 8. ANOVA mixed model for the relationship between MTT levels, exposure time and HSP70-2 rs1061581 genotype. f is the F-test statistics value, df are the degrees of freedom combination.
* Due to the violation of sphericity the Huynh-Feldt correction was applied.

3.3.2.3. Hsp70-2 protein levels

Hsp70-2 protein expression was evaluated following H_2O_2 treatments on PBMCs from 77 MS patients and 49 healthy donors. Protein expression was quantified by Western Blotting (Figure 36).

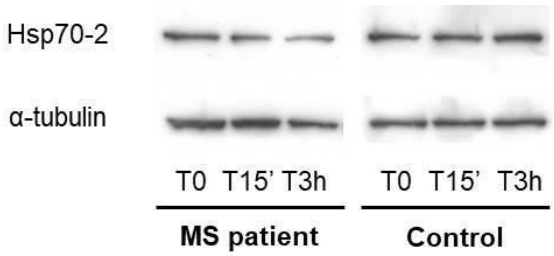

Figure 36. Representative Western blot images of Hsp70-2 and α-tubulin protein content in PBMC from MS patients and controls. They are subdivided by exposure time to H_2O_2 (T0, T15', T3h). α-tubulin was used to normalize the data.

The relationship among Hsp70-2 protein expression, exposure time, and group of subjects was studied using a non-parametric approach and the mean Hsp70-2 protein levels were visualized on a curve on the base of time exposure to H_2O_2 (Figure 37).
The exposure time to H_2O_2 did not change significantly Hsp70-2 protein expression in either MS patients (Friedman's chi-square=1.63, df=2, exact p=0.462) or healthy controls (Friedman's chi-square=1.11, df=2, exact p=0.596). Furthermore there was no significant

difference between the two groups at each level of exposure time to oxidative treatment (Mann Whitney U: at T0=1800, exact p=0.81; at T15'=1639.5, exact p=0.26, at T3h=1677.5, exact p=0.74); although a slightly increase is noticed after 15 min of oxidative stress.

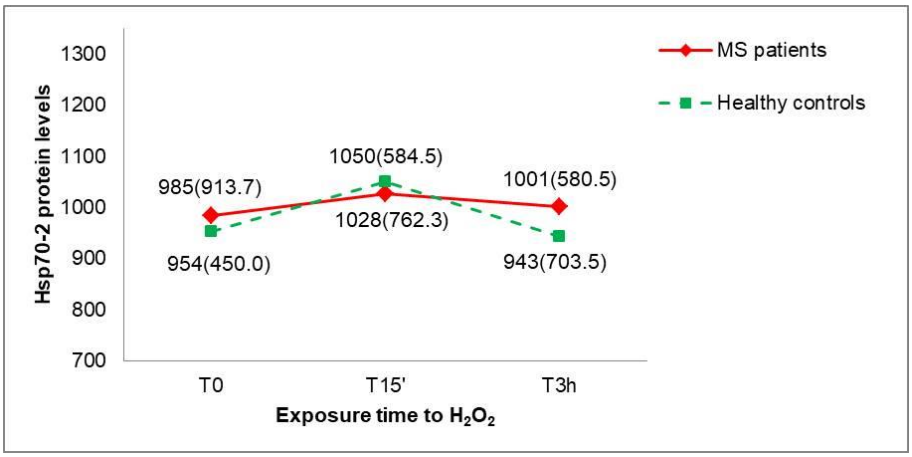

Figure 37. Hsp70-2 protein levels in response to H_2O_2 of PBMC cultures from MS patients subjects (red) and healthy subjects (green). Median values (interquartile range) of Hsp70-2 protein expression are reported.

Also in this case the HSP70-2 polymorphism was considered, stratifying the analyses by the HSP70-2 rs1061581 genotype in each group of subjects (Figure 38 A and 38 B). Among MS cases, 32% (26 subjects) were AA, 46% (38 subjects) were AG and 22% (18 subjects) were GG, whereas, among healthy controls, 46% (23 subjects) were AA, 44% (22 subjects) were AG and 10% (5 subjects) were GG.

In healthy subjects no significant effect of exposure time to H_2O_2 in any genotype category was found (AA: Friedman's chi-square=2.1, df=2, exact p=0.37; AG: Friedman's chi-square=0.42, df=2, exact p=0.83; GG: Friedman's chi-square=2.8, df=2, exact p=0.37). We did not find significant differences among genotypes after different H_2O_2 exposure times (Kruskal-Wallis chi square: at T0=4.01, p=0.13; at T15'=2.26, p=0.32, at T3h=0.91, p=0.63).

Also MS patients did not show significant effects related to H_2O_2 in any genotype category (AA: Friedman's chi-square=1.53, df=2, exact p=0.51; AG: Friedman's chi-square=3.85, df=2, exact p=0.15; GG: Friedman's chi-square=1.73, df=2, exact p=0.46). No significant differences among genotypes was found at different time-points (Kruskal-Wallis chi square: at T0=0.26, p=0.88; at T15'=4.99, p=0.08, at T3h=1.92, p=0.38).

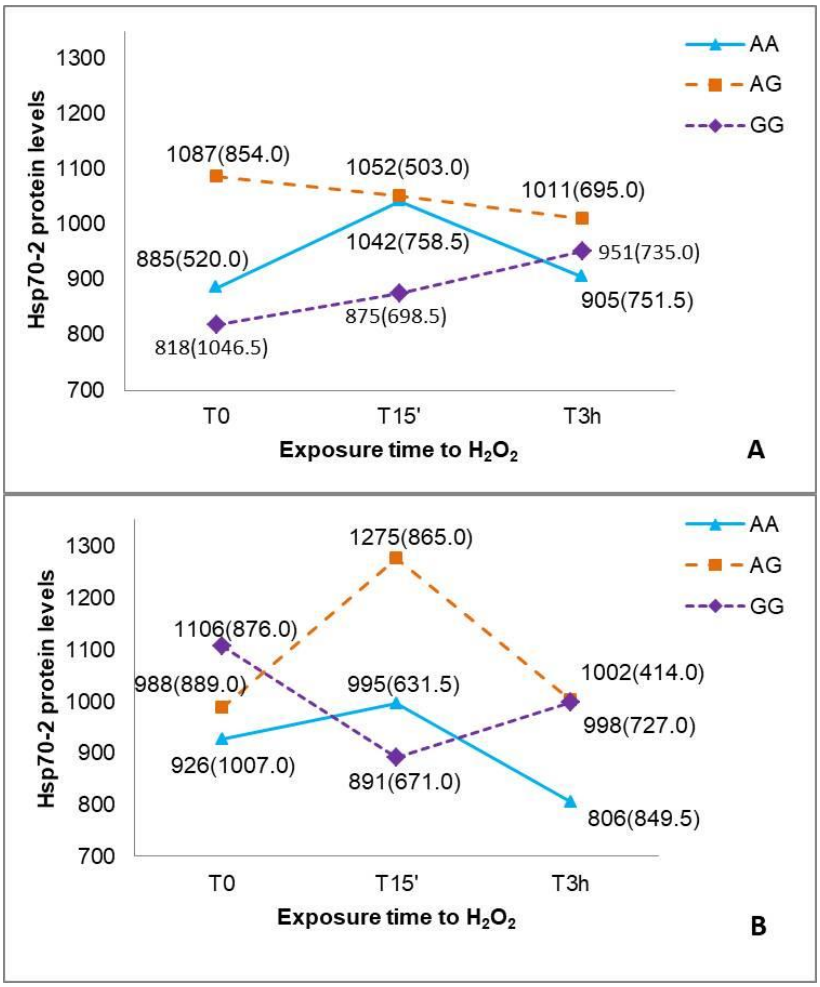

Figure 38. Hsp70-2 protein levels in response to H_2O_2 of PBMC cultures with different HSP70-2 rs1061581 genotype, AA (blu), AG (orange) and GG (purple), A| in the group of healthy controls and B| in MS patients. Median values (interquartile range) of Hsp70-2 protein expression are reported.

3.3.2.4. ROS formation

The formation of intracellular ROS was measured on PBMCs from 32 MS patients and 22 healthy controls after treatment with H_2O_2 and the data were analyzed with a parametric

analysis of variance.

An important variability of ROS levels over time points was reported, while ROS levels of MS patients were not significantly different compared to healthy controls. Furthermore, there was no interaction effect between exposure time and group of subjects (Table 9 and Figure 39).

Variability source	f	df	P value
Exposure time	3.23	2;104	**0.043**
Group of subjects	0.25	1;52	0.62
Interaction	0.58	2;104	0.56

Table 9. ANOVA mixed model for the relationship between ROS levels, exposure time and group of subjects. f is the F-test statistics value, df are the degrees of freedom combination.

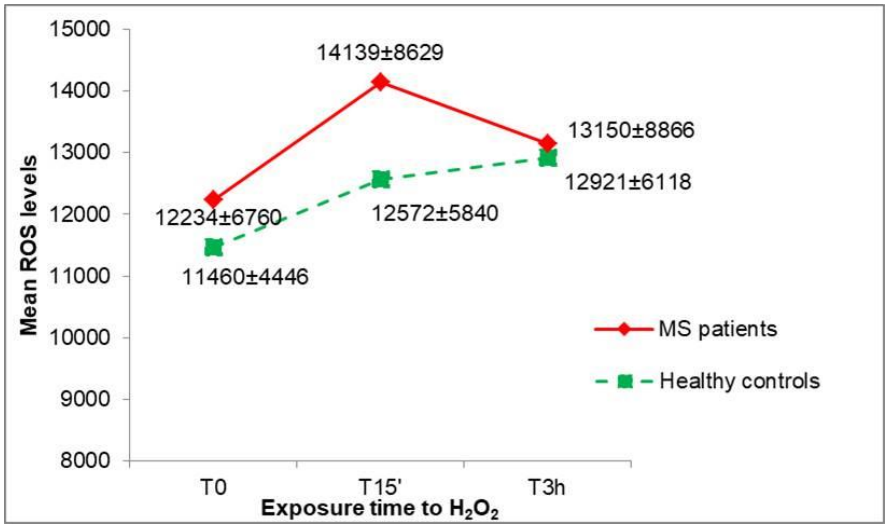

Figure 39. ROS levels in response to H_2O_2 of PBMC cultures from MS patients subjects (red) and healthy subjects (green). Mean values and standard deviations of ROS levels are reported.

Considering the whole sample (MS patients and healthy controls together), *post hoc* analysis for multiple comparisons among exposure time levels showed that ROS basal levels increased after 15 minutes of the oxidative stress ($t_{Bonferroni}$=2.52,df=53, p=0.044). In detail, PBMCs from MS patients incremented their ROS levels of 1905 units, compared to basal levels, and PBMCs from healthy controls had an increment of 1112 units. The

differences between T15' and T3h and between T0 and T3h did not result statistically significant.

3.3.3. VDR polymorphisms and vitamin D levels in multiple sclerosis

3.3.3.1. VDR polymorphisms

The VDR SNPs rs731236 and rs4334089 were analysed in order to find a possible relation with MS onset.

The genotype of 186 MS patients and 263 healthy controls displaying full clinical and demographic information was determined. Statistical analysis were adjusted by age and gender as possible MS risk confounders (MS patients were older compared to healthy controls, 45±11 vs 37±9 years, $p< 0.001$; MS female to male ratio was slightly higher compared to the group of controls but not significant). Genotypic frequencies in the control group did not deviate from the Hardy–Weinberg equilibrium ($p > 0.05$).

For the VDR rs731236 polymorphism, the TT genotype was considered as the reference genotype in the analysis (Table 10), a dominant model was also applied to consider the effect of "at least one C allele" (Table 11).

VDR rs731236 (Taq-I)	MS patients	Healthy controls	Adjusted OR (95% CI)	p value
	N=185	**N=242**		
CC	20 (11%)	24 (10%)	1.13 (0.73-1.76)	0.58
TC	94 (51%)	119 (49%)	1.06 (0.52-2.17)	0.87
TT	71 (38%)	99 (41%)	1	

Table 10. Genotype frequencies of VDR rs731236 polymorphism and their association with MS assessed using logistic models corrected by age and gender.
TT genotype was considered the reference genotype in the genotypic model.

VDR rs731236 (Taq-I)	MS patients	Healthy controls	Adjusted OR (95% CI)	p value
	N=185	**N=242**		
C	134 (36%)	167 (35%)	1.12 (0.73-1.71)	0.60
T	236 (64%)	317 (65%)		

Table 11. Alleles frequencies of VDR rs731236 polymorphism and their association with MS assessed using a dominant model.
The OR was calculated using a dominant model: at least one minor allele (C) vs absence of C allele.

For the VDR rs4334089 the reference genotype was the GG genotype (Table 12); a dominant model was applied to consider the effect of "at least one A allele" (Table 13).
Comparing the group of MS patients with those of healthy subjects, for both the analysed polymorphism, no significant association with MS was found.

VDR rs4334089 (HpyCH4V)	MS patients	Healthy controls	Adjusted OR (95% CI)	p value
	N=186	**N=263**		
AA	12 (6%)	23 (9%)	0.98 (0.64-1.51)	0.92
AG	74 (40%)	96 (36%)	0.66 (0.29-1.50)	0.33
GG	100 (54%)	144 (55%)	1	

Table 12. Genotype frequencies of VDR rs4334089 polymorphism and their association with MS assessed using logistic models corrected by age and gender.
GG genotype was considered the reference genotype in the genotypic model.

VDR rs4334089 (HpyCH4V)	MS patients	Healthy controls	Adjusted OR (95% CI)	p value
	N=186	**N=263**		
A	98 (26%)	142 (27%)	0.92 (0.61-1.39)	0.68
G	274 (74%)	384 (73%)		

Table 13. Alleles frequencies of VDR rs4334089 polymorphism and their association with MS assessed using a dominant model.
The OR was calculated using a dominant model: at least one minor allele (A) vs absence of A allele.

3.3.3.2. Vitamin D receptor protein expression

3.3.3.2.1. Total VDR protein expression

To evaluate the total VDR protein expression, protein quantification was performed, using Western blot on PMBCs from 111 MS patients and 55 healthy controls (Figure 40 A).
Total VDR protein expression did not significantly vary between MS patients and healthy controls: MS patients had a mean VDR protein expression of 1128 ± 579 arbitrary units and healthy controls showed a mean VDR protein expression of 1099 ± 341 arbitrary units ($p > 0.05$) (Figure 40 B).
In order to assess whether VDR rs731236 polymorphism is associated with an altered VDR protein expression, data of total VDR protein expression were associated with the different

genotypes, using a linear regression model adjusted by the presence of MS (Table 14). No statistically significant difference emerged between the VDR rs731236 polymorphism and the total expression levels of VDR protein ($p > 0.05$) (Table 14).

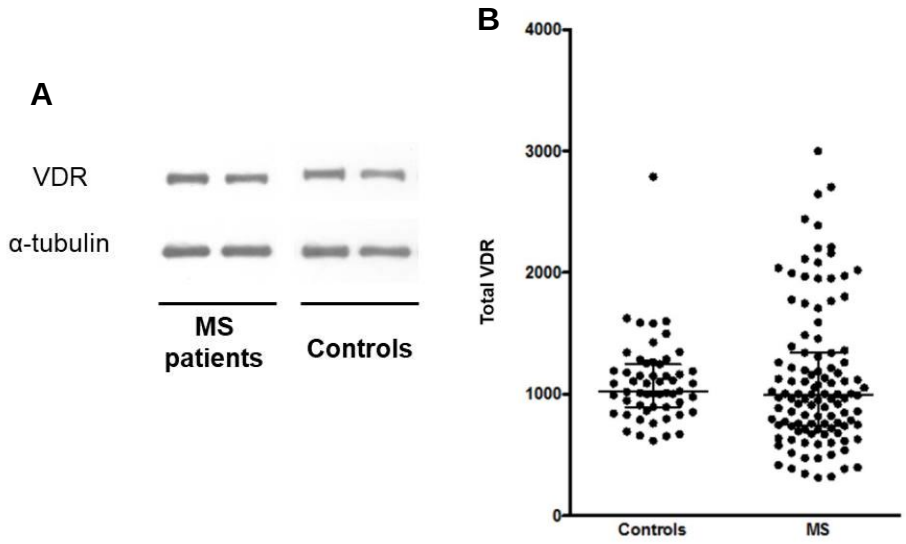

Figure 40. A| Representative Western blot images of total VDR and α-tubulin protein content in PBMC from MS patients and controls. α-tubulin was used to normalize the data. **B| Total VDR protein expression.** MS patients and controls (black dots) are grouped; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significantly difference between MS patients and healthy controls are observed ($p > 0.05$).

	β-coefficient	**Standard error**	**p-value**
VDR rs731236 (Taq-I) genotypes			
TC vs TT	-11.76	90.41	0.897
CC vs TT	-98.31	118.78	0.409
Multiple sclerosis yes *vs* no	33.22	85.21	0.697

Table 14. Total VDR protein expression levels related to VDR rs731236 polymorphism. β-coefficient and standard error of analysed factors are derived from a linear regression model. TT genotype is considered the reference genotype.

The same type of analysis was performed for the total VDR protein expression levels and to the second polymorphism studied: VDR rs4334089. No statistically significant differences between the rs4334089 polymorphism and the total expression levels of VDR were found ($p > 0.05$) (Table 15).

	β-coefficient	**Standard error**	**p value**
VDR rs4334089 (HpyCH4V) genotypes			
GA vs GG	-121.62	97.25	0.213
AA vs GG	12.84	130.51	0.922
Multiple sclerosis yes *vs* no	42.78	102.91	0.678

Table 15. Total VDR protein expression levels related to VDR rs4334089 polymorphism. β-coefficient and standard error of analysed factors are derived from a linear regression model. GG genotype is considered the reference genotype.

3.3.3.2.2. Cytoplasmic and nuclear VDR expression

PBMCs from a subgroup of 72 MS patients and 30 healthy controls were used; the cytoplasmic and nuclear fractions were separated and Western blot was performed to assess VDR protein expression levels in the cytoplasm and in the nucleus, respectively (Figure 41).

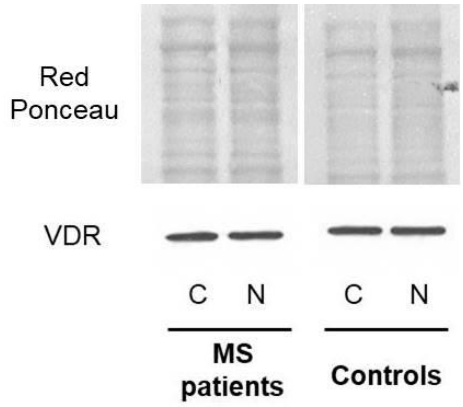

Figure 41. Representative Western blot images VDR protein content in PBMC from MS patients and controls. They are subdivided in cytoplasmic (C) and nuclear (N) fraction. Red ponceau was used to normalize the data.

No statistically significant differences between MS patients and healthy controls were observed both for the VDR expression in the cytosol and in the nucleus. PBMCs from MS patients showed a mean cytosolic protein expression of 1033 ± 524 arbitrary units, whereas PBMCs from healthy donors had a mean protein expression of 1017 ± 437 arbitrary units ($p > 0.05$) (Figure 42 A). Regarding the VDR protein levels in the nucleus, PBMCs from MS patients had a mean protein expression of 1216 ± 465 arbitrary units and those from healthy subjects showed a mean protein expression of 1165 ± 323 arbitrary units ($p > 0.05$) (Figure 42 B).

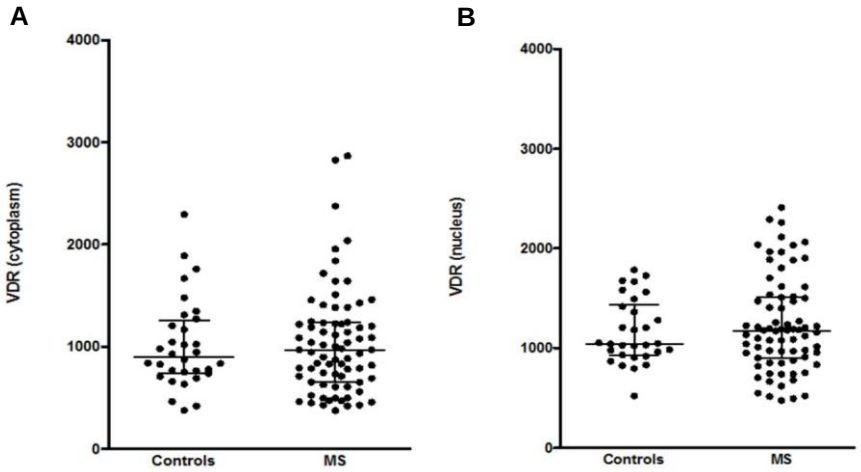

Figure 42. A| Cytoplasmic and B| nuclear VDR protein expression. MS patients and controls (black dots) are grouped; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significantly difference between MS patients and healthy controls are observed ($p > 0.05$).

To test whether the two studied polymorphisms may influence the VDR protein localization inside the cell, a linear regression model adjusted by presence of MS has been performed, considering the VDR protein expression levels in the cytoplasm and in the nucleus, respectively. From the analysis, no statistically significant differences emerged between the VDR rs731236 polymorphism and the cytoplasmic and nuclear expression levels of VDR protein ($p > 0.05$) (Table 16). Also for the VDR rs4334089 polymorphism, no statistically significant differences were found ($p > 0.05$) (Table 17).

Cytosol	β-coefficient	Standard error	p-value
VDR rs731236 (Taq-I) genotypes			
TC vs TT	127.22	108.79	0.245
CC vs TT	179.97	163.37	0.273
Multiple sclerosis yes *vs* no	24.38	106.00	0.819
Nucleus			
VDR rs731236 (Taq-I) genotypes			
TC vs TT	-77.35	95.10	0.418
CC vs TT	-40.25	146.10	0.783
Multiple sclerosis yes *vs* no	54.09	93.89	0.566

Table 16. Cytoplasmic and nuclear VDR protein expression levels related to VDR rs731236 polymorphism. β-coefficient and standard error of analysed factors are derived from a linear regression model. TT genotype is considered the reference genotype.

Cytosol	β-coefficient	Standard error	p-value
VDR rs4334089 (HpyCH4V) genotypes			
GA vs GG	26.72	114.42	0.816
AA vs GG	-282.43	192.88	0.147
Multiple sclerosis yes *vs* no	113.49	139.89	0.420
Nucleus			
VDR rs4334089 (HpyCH4V) genotypes			
GA vs GG	-40.14	104.76	0.703
AA vs GG	-235.84	176.06	0.184
Multiple sclerosis yes *vs* no	32.71	134.24	0.808

Table 17. Cytoplasmic and nuclear VDR protein expression levels related to VDR rs4334089 polymorphism. β-coefficient and standard error of analysed factors are derived from a linear regression model. GG genotype is considered the reference genotype.

3.3.3.3. VDR expression and vitamin D plasma levels

Since the importance of vitamin D levels in the susceptibility to MS, the plasma levels of $25(OH)D_3$ of a subgroup of 89 MS patients, randomly selected, were determined. The mean age of this subgroup was 45.52 ± 11.26 and the MSSS was 3.19 ± 2.47.
MS patients showed a mean $25(OH)D_3$ levels of 21.82 ± 13.61 ng/ml.

To understand a possible relation between total VDR protein expression levels and $25(OH)D_3$ plasma levels, a linear regression model, considering also the clinical parameters, were analysed. The analysis revealed that the $25(OH)D_3$ plasma levels were significantly able to modulate the total VDR protein expression ($p = 0.023$) (Table 18). We noticed an inverse relation between $25(OH)D_3$ plasma levels and VDR protein expression: the increase of 1 unit of vitamin D significantly decreases the total VDR protein expression by 10 units.

Total VDR	β-coefficient	Standard error	p-value
$25(OH)D_3$ levels	-10.68	4.60	**0.023**
Sex (female *vs* male)	-63.68	126.83	0.617
Age (in years)	-1.88	5.69	0.742
MSSS	24.81	130.99	0.850

Table 18. Total VDR protein expression levels related to $25(OH)D_3$ plasma levels in MS patients. β-coefficient and standard error of analysed factors are derived from a linear regression model.
$p < 0.05$ is considered statistically significant.

A similar model was applied in order to verify whether the $25(OH)D_3$ plasma levels could influence the VDR expression in the cytosol and in the nucleus, respectively. No significant results were reported (Table 19 and 20). $25(OH)D_3$ plasma levels seems only to influence the total VDR expression.

Cytosolic VDR	β-coefficient	Standard error	p-value
$25(OH)D_3$ levels	4.30	4.46	0.339
Sex (female *vs* male)	87.93	123.02	0.477
Age (in years)	-10.68	6.14	0.087
MSSS	-69.85	127.87	0.587

Table 19. Cytosolic VDR protein expression levels related to $25(OH)D_3$ plasma levels in MS patients. β-coefficient and standard error of analysed factors are derived from a linear regression model.

Nuclear VDR	β-coefficient	Standard error	p-value
25(OH)D_3 levels	5.30	4.24	0.216
Sex (female *vs* male)	-98.07	120.96	0.421
Age (in years)	5.55	5.95	0.355
MSSS	28.70	124.30	0.818

Table 20. Nuclear VDR protein expression levels related to 25(OH)D_3 plasma levels in MS patients. β-coefficient and standard error of analysed factors are derived from a linear regression model.

3.4. Discussion

Up to now, MS etiology has not been well understood. Genetic, environmental and epigenetic factors are involved in MS onset. Genetic factors account for about 30% of MS risk, and a vast number of allelic variants relatively common within the population probably have a cumulative effect, contributing only a small portion to the risk. The environmental factors are important, as underlined by migration studies: environmental exposure during childhood and adolescence are of essential importance for disease risk (Hammond SR et al., 2000; Cabre P, 2007).

This neurodegenerative disease is characterized by an intense inflammatory component: the immune attack plays a central role, with T lymphocytes driving the autoimmune attack against CNS peptides, in particular of the myelin (McFarland HF and Martin R, 2007). Activated T lymphocytes can break the BBB and infiltrate inside the CNS. T lymphocytes initiate the characteristic auto-inflammatory processes of MS pathogenesis, which involves macrophages, microglia activation, B cells and the production of auto-antibodies, thus leading to demyelination, neuro-axonal injury and oligodendrocytes loss (Yong VW and Marks S, 2010). With myelin destruction, the correct transmission of the nerve impulse is impaired and the symptoms, which depend on the location of the area of demyelination, arise. With the progression of the disease, additional amplification mechanisms of tissue injury become increasingly important; mitochondrial damages in brain cells have the potential to amplify oxidative injury within the brains of MS patients (Lassmann H et al., 2014).

Considerable evidence indicates that oxidative stress plays an important role in the pathogenesis of MS. The excess of ROS and RNS is mainly generated by immune cells, particularly the activated microglia, and has been implicated as a mediator of demyelination

and axonal damage (Dheen ST et al., 2007). In MS plaques, increased free radical levels and alterations of antioxidant expression were observed (van Horssen J et al., 2011). Activated mononuclear cells and microglia produce vast amounts of ROS and NO·, contributing to establish an oxidative environment in the CNS (Lu F et al., 2000). In turn, ROS can further induce the inflammatory response within the brain and damage mitochondria (Lassmann H et al., 2014).

Numerous factors participate in the pathogenesis of MS, and Hsp70s are assumed to be involved (Mansilla MJ et al., 2012). However, the exact role of these highly conserved molecular chaperones in MS is not clear. Hsp70s are essential to guarantee cell survival in physiological conditions and under stress factors. This protective role seems to be central for neuroprotection, as demonstrated in several neurodegenerative diseases, such as AD (Hoshino T et al., 2011) and PD (Gifondorwa DJ et al., 2012). On the other hand, Hsp70s can trigger both the innate and adaptive immune system (Asea A et al., 2002; Becker T et al., 2002), complicating the analysis of their role in a neurodegenerative and disimmune diseases like MS.

Considering the importance of genetic risk factors for MS, we decided to investigate genetic variants that may be involved in disease onset and progression. One of the main interests of our research for many years has been the MHC class III region, which contains several polymorphic genes related to immune system function. Interestingly, three genes code for Hsp70s: HSPA1A (or HSP70-2) HSPA1B (HSP70-2) and HSPA1L (HSP70-HOM). Taking advantage of this consideration, we decided to investigate more closely the role of Hsp70s in MS, considering the importance of genetic variants in their genes. Furthermore, our group previously published a work on the stress-inducible Hsp70-2: the HSP70-2 rs1061581 polymorphism revealed a significant association between the G allele and the presence of MS (Boiocchi C et al., 2014). Our idea was to continue analysing genetic variants in HSP genes on MHC class III region and to further investigate the role of Hsp70-2 in MS. For this reason, the HSPA1L gene, which encodes the constitutively expressed Hsp70-Hom protein, was considered by analysing the HSP70-HOM rs2227956 polymorphism. On the other hand, the analysis of the possible role of Hsp70-2 in MS was continued, considering the importance of oxidative stress for disease pathogenesis.

The HSP70-HOM rs2227956 polymorphism, located in the coding region of the HSPA1L gene, which encodes the constitutively expressed Hsp70-Hom protein, was considered in order to analyse the possible role of the polymorphisms in MS. The C allele is more frequent in MS patients compared to healthy controls, and it confers a twofold increased risk of developing the disease (OR = 2.13, 95% CI: 1.60–2.86, $p < 0.0001$). Furthermore, comparing the genotypic frequencies of MS patients and healthy controls, MS risk is significantly higher for the homozygous CC, leading to an increased risk of about seven times (OR = 6.71, 95% CI: 3.11–14.43, $p < 0.0001$).

MS severity was taken into account; it tends to be distributed differently among patients depending on their HSP70-HOM genotype. CC patients exhibited an increased MSSS of 1.22 on average compared to TT carriers (95% CI: 0.22–2.21, p=0.017).

Our findings are in contrast with previous studies in the literature that did not detect any significant correlation. Niino and colleagues analysed the influence of HSP70-HOM +2437 T/C polymorphism on MS risk, severity and progression in the Japanese population (Niino M et al., 2001). In this study, no significant differences in the genotypic distribution analysed was observed. Moreover, a work that analysed 59 Canadian MS patients did not detect any significant correlation between the HSP70-HOM polymorphism and MS (Ramachandran S and Bell RB, 1995). The different results can be due, respectively, to ethnical differences and the small number of MS Canadian patients involved in the study.

In addition to analysing the distribution of the HSP70-HOM polymorphism, a multilocus analysis was performed, including the HSP70-2 rs1061581 and HSP70-HOM rs2227956 polymorphisms. The analysis shows that the combination of the two risk alleles, the G of the HSP70-2 rs1061581 polymorphism and the C for the HSP70-HOM rs2227956 polymorphism, increases MS risk by most than three times (OR=3.49, 95% CI: 2.17-5.6, p<0.0001) and shows that HSP70-HOM rs2227956 polymorphism affects MS risk independently of HSP70-2 rs1061581.

Although it is clear that the HSP70-HOM rs2227956 polymorphism is related to MS risk, it is difficult to understand its biological consequences, due to the lack of functional studies. The T to C substitution leads to a changing in the amino acid residue in position 493 of the HSP70-Hom protein: from Met to Thr (Milner CM and Campbell RD, 1992). This substitution seems to affect the substrate specificity and chaperone activity, but its biological consequences can only be speculated on; it may affect the functional efficiency of peptide-binding specificity, leading to an accumulation of misfolded proteins in neurons and glia. Such an accumulation may lead to primary cytodegeneration and to the subsequent release of antigens, thus causing the autoimmune response in predisposed individuals. However, it is essential to consider that Hsp70-Hom is not only important for its chaperoning ability: it also induces anti-apoptotic mechanisms in neurons and glia, suggesting that HSP70-HOM polymorphism may also influence this mechanism, increasing MS risk. Furthermore, Hsp70s can trigger both innate and adaptive responses. Hsp70s can act as an adjuvant by binding immunogenic peptides, thus inducing T cell response. These peptides are internalized into the cells and subsequently presented via MHC molecules (Li Z et al., 2002). The alteration of Hsp70 expression and/or a perturbation of their functions might affect MS pathogenesis, thereby exacerbating the immune response or eliciting the presentation of autoantigens.

Hsp70-Hom protein levels was also investigated. From the obtained data we can deduce that the protein expression does not correlate with the HSP70-HOM rs2227956 genotype in either MS patients or healthy controls. The lack of correlation between HSP70-HOM

rs2227956 polymorphism and protein expression in PBMCs from MS patients and healthy subjects may be because Hsp70-Hom have a low but constitutive RNA expression (Milner CM and Campbell RD, 1990). Similarly, data on Hsp70-1 and Hsp70-2 expression shows that PBMCs from MS patients and healthy controls produce the same baseline levels of these two stress-inducible proteins (Cwiklinska H et al., 2010). Only under stress conditions (heat shock and LPS stimulation) an overexpression of Hsp70-1 and Hsp70-2 in PBMCs from MS patients has been reported. However, the exact mechanisms and pathways involved in Hsp70-Hom regulation and expression are not clear. Fourie and colleagues reported that the Hsp70-Hom protein is relatively more expressed compared to Hsp70-1 and Hsp70-2, in response to IFN-γ (Fourie AM et al., 2001). Further experiments are needed to understand the exact regulation of Hsp70-Hom.

Our work reports a direct and significant relationship between Hsp70-Hom protein expression and MS severity. MS patients with mild disease severity display low levels of Hsp70-Hom, whereas patients with a moderate to severe form of MS have higher protein expression. However, it is not clear whether the severity of the disease is the cause or the effect of Hsp70-Hom expression. This may be because Hsp70 proteins play a role in immune-modulation. Lower Hsp70-Hom protein levels seem to be related to a mild inflammatory process and thus to a better disease progression. These results are supported by data from EAE mice: hsp70.1$^{-/-}$ mice, in contrast to hsp70-sufficient mice, presented a milder form of EAE (Mycko MP et al., 2008).

These results indicate that Hsp70-Hom plays a more relevant role in promoting immune system activation and an effective T lymphocytes response against myelin antigens compared to its neuroprotective role. However, the underlying mechanisms involved in the negative outcome are not clear, and additional studies will be important in explaining the exact role of Hsp70-Hom and its possible application as a biomarker and/or therapeutic target in MS therapy.

At the same time, we decided to continue studying the stress-inducible Hsp70-2 protein, based also on the positive results from our previous published work, in which the association of the HSP70-2 rs1061581 polymorphism with the presence of MS was reported, where the G allele was the risk allele. This protein is involved in the response to oxidative stress. Hsp70-2 protein expression is induced by several types of stress, including oxidative stress, and it plays a role in cell protection. In the pathogenesis of MS, oxidative stress contributes to disease pathogenesis (Ohl K et al., 2016). For this reason, the use of anti-oxidative drugs is promising, as demonstrated by the introduction of DMF in MS therapy; this drug can induce the production of antioxidant enzymes and play a neuroprotective role combined with its anti-inflammatory effects (Pistono C et al., 2017).

We decided to analyse Hsp70-2 in the pathogenesis of MS and its involvement in the response to oxidative stress. The effect of oxidative stress on PBMCs from MS patients and

healthy controls, using 10 µM H_2O_2 as an oxidative *stimulus*, was analysed by assessing mitochondrial activity. PBMCs from both MS patients and healthy subjects have similar behaviour, showing a significant variation in the mitochondrial activity when different exposure times to oxidative stress are considered. After 15 minutes of oxidative stress, the mitochondrial activity first decreases and then is restored after 3 hours from the exposure to H_2O_2. No significant difference between the two groups was found, suggesting that both PBMCs from MS patients and healthy controls initially suffer in the presence of an oxidative challenge, which reduces their mitochondrial activity. However, they can restore the physiologic levels after 3 hours, probably because they are able to contrast the damage induced by oxidative stress. The fact that PBMCs can adapt to an oxidative challenge is consistent with the observation that similar treatments with H_2O_2 at increasing concentrations of 50 µM, 100 µM and 250 µM for 2 hours did not show any cytotoxic effect. Only 2 hours of exposure to H_2O_2 500 µM significantly reduced cell viability (Emamgholipour S et al., 2016).

We then speculated that Hsp70-2 could be involved in the response to oxidative stress; therefore, the expression of Hsp70-2 in PBMCs from MS patients and healthy donors was analysed. Under stress conditions Hsp70-2 expression can vary really quickly; Hsp70-2 levels may increase within one hour after heath shock (Maloyan A et al., 1999; Doberentz E et al., 2017). However, in our case the exposure time to H_2O_2 does not significantly change the protein expression, either in PBMCs from MS patients or in the healthy controls, thus confirming that PBMCs respond in a similar way to the presence of an oxidative challenge, independently of the presence of the disease.

To better understand the role of the stress inducible Hsp70-2 protein in the response to an oxidative *stimulus*, the HSP70-2 rs1061581 polymorphism was investigated to verify whether the presence of a variant may influence the response to oxidative stress, given that this specific polymorphism has been associated with autoimmune diseases (Zouari Bouassida K et al., 2004; Wang YP et al., 2015). For this reason, our data regarding the mitochondrial activity and the Hsp70-2 protein expression levels were stratified by the HSP70-2 rs1061581 genotype. The correlation of MTT and Hsp70-2 protein levels with the HSP70-2 rs1061581 genotype does not show any significant difference. This polymorphism does not influence the response to oxidative stress, although it seems to be important in the susceptibility to MS (Boiocchi C et al., 2014).

The data suggests that Hsp70-2 does not appear to play a key role in cell survival in the presence of an oxidative challenge in PBMCs, although it is essential for the proper folding of the proteins and has anti-apoptotic functions (Beere HM, 2004); other compensatory cellular mechanisms are probably involved in such a response.

The intracellular ROS levels were then investigated. In PBMCs from both MS patients and healthy subjects, a significant increase after 15 minutes of oxidative stress was observed. Consistently with our findings, rat PBMCs treated with H_2O_2 for 1 hour showed an increase

in intracellular superoxide radicals (Chiu HY et al., 2009). ROS increment after 15 minutes of oxidative stress may be correlated to the initial decreased viability observed. Although ROS levels remain unaltered after 3 hours, compared to the basal levels and to 15 minutes of oxidative *stimulus*, mitochondrial activity is enhanced, suggesting that PBMCs can trigger a compensatory response to counter oxidative stress. A putative mechanism of response to an oxidative *stimulus* is the activation of Nrf2. This transcription factor modulates the expression of genes involved in detoxification pathways and in the defence against oxidative stress, such as superoxide dismutase, catalase, sulfaredoxin, thioredoxin, glutathione peroxidase and glutathione reductase (Joshi G and Johnson JA 2012). The involvement of the anti-oxidant enzymes at different levels may be assumed; for instance, in human PBMCs an increment in catalase activity after oxidative stress has been reported, as opposed to a reduced activity of the manganese-dependent superoxide dismutase, MnSOD (Emamgholipour S et al., 2016).

Assessing the role of Hsp70-2 in the pathogenesis of MS is not easy since this protein can act, on the one hand, as a neuroprotective element, and on the other may also promote a pro-inflammatory response. In conclusion, these results highlight that PBMCs, regardless of the genotype and the presence of MS, are able to adapt under oxidative stress conditions. Further studies regarding oxidative stress in MS will be important in order to improve MS treatments. Consistently, the reduction of oxidative stress has a positive influence on the patients. In this regard, our results indicate that Hsp70-2 does not seem to play a key role in the protective reaction against oxidative stress. Nevertheless, considering that MS patients benefit from anti-oxidative drugs, such as DMF, further studies are needed to identify which specific proteins significantly contribute to this defensive response in order to improve MS treatments.

The results on Hsp70-2 and Hsp70-Hom highlight the complex role of the Hsps in MS pathogenesis. Although genetic variants in their genes are important for MS susceptibility, it is difficult to assess their exact role, which can be neuroprotective and also pro-inflammatory. A further level of difficulty derives from the fact that we work on PBMCs. Although these cells are central in MS pathogenesis, they do not give us a complete indication of what happens during MS pathogenesis, where the CNS is the site of the disease. However, understanding the effects of genetic variants in MS risk and progression is important in understanding the factors that lead to these disorders. Furthermore, trying to better assess the role of Hsp70s may be important in understanding better the pathogenic mechanisms of MS, which are useful in suggesting new treatments.

Our attention was also focused on vitamin D. Low levels of vitamin D constitute an important environmental risk factor for developing MS. The biological actions of vitamin D are mediated by the VDR (gene on chromosome 12q13.11). The interaction between $1,25(OH)_2D_3$ and the receptor induces the heterodimerization with RXR and the

translocation of the complex into the nucleus (Prüfer K et al., 2000). Here, VDR binds to specific DNA sequences, called VDRE, regulating the transcription of target genes (Pike JW et al., 2012).

The observation that a VDRE is located in the promoter of HLA-DRB1*1501 underlines the involvement of vitamin D in MS susceptibility; VDRE is highly conserved in the major MS associated haplotype HLA-DRB1*15, but not among non-MS associated haplotypes (Ramagopalan SV et al., 2009). The regulatory sequences of NF-kB, a transcription factor important for pro-inflammatory response, also contain VDREs (Yu XP et al., 1995). These observations highlight the ability of vitamin D to modulate immune-related genes, thus influencing immune function.

Polymorphisms in the VDR gene might influence MS susceptibility, thus altering the action of vitamin D on immune cells. Not only inadequate vitamin D intake but also impaired vitamin D signalling may contribute to the onset and progression of the disease.

Given the importance of the interaction between environmental and genetic factors, our attention was focused on the VDR, through which vitamin D has its effects. The VDR gene is polymorphic, and several studies have analysed the role of different VDR gene SNPs in MS, although their results are conflicting (García-Martín E et al., 2013; Tizaoui K et al., 2015).

In our analysis, two SNPs of the VDR gene were considered: rs731236 (Taq-I) and rs4334089 (HpyCH4V). MS patients and healthy controls do not show any significant difference either in allelic or genotypic frequencies for the two polymorphisms.

The absence of an association between the VDR rs731236 polymorphism and MS is supported by other studies (Steckley JL et al., 2000; Čierny D et al., 2016); however, other researchers suggest an involvement of this polymorphism in MS (Cox MB et al., 2012; Abdollahzadeh R et al., 2016). A possible reason for the contrasting results could be a lack of statistical power due to the sample size. For example, the study by Cox and colleagues, which found a weak trend towards increased risk of MS for the VDR rs731236 CC genotype, involved 727 MS patients, 1,153 trio families (MS patients and both parents) and 604 healthy controls (Cox MB et al., 2012). However, a recent study by Abdollahzadeh and collaborators on the Iranian population, which included only 160 patients and 150 healthy controls, found an association between MS and the VDR rs731236 CC genotype (Abdollahzadeh R et al., 2016). Interestingly, a Canadian family-based study involving a larger sample size (1,364 MS patients and 1,661 healthy controls, first-degree relatives) also found no association between VDR rs731236 polymorphism and MS risk (Orton SM et al., 2011). The differences are probably also due to the fact that different populations were considered, with genetic differences between the studied groups. In this regard, another study on the Italian population did not find any difference between MS patients and healthy controls for the VDR rs731236 polymorphism (Agnello L et al., 2016). Understanding the real consequences of the VDR rs731236 polymorphism is not easy

because, although it is on exon 9, the T to C transition is a synonymous change, and so the amino acid does not change and the protein structure is not impaired.

Regarding the VDR rs4334089 polymorphism, at present no studies correlate this polymorphism to MS. In this case as well, finding no significant differences may be due to the sample sizes. There is also the possibility that the VDR rs4334089 polymorphism, which is located in the 5' UTR, may not affect the transcript. To our knowledge, our study is the first to analyse the VDR rs4334089 polymorphism in MS pathogenesis. The VDR rs4334089 polymorphism has been studied in PD (Lv Z et al., 2013; Lin CH et al., 2014). For now, we suggest that VDR rs4334089 has no association with the development of neurodegenerative diseases, such as MS and PD, though further and larger investigations are required.

To investigate whether an altered expression of VDR could be associated with MS risk, the total VDR protein expression was analysed. VDR protein levels do not change between MS patients and healthy controls. Moreover, total VDR protein levels do not correlate with VDR rs731236 and VDR rs4334089 polymorphisms, both in MS patients and in healthy controls. The lack of correlation between the two polymorphisms and protein expression in PBMCs from MS patients and healthy subjects may be because the two polymorphisms do not influence RNA expression; VDR rs731236 polymorphism causes a synonym mutation in exon 9 and the VDR rs4334089 polymorphism is located in the 5' UTR. They probably do not affect transcript stability or translational modulation, and thus may not vary protein levels.

Consistently with our results, a 2011 study reported no changes in VDR protein expression in PBMCs from MS patients according to the VDR rs731236 genotype (Agliardi C et al., 2011). The authors described an alteration in VDR protein expression only in PBMCs stimulated with the MBP: the cells carrying the TT or CT genotypes have significantly higher VDR protein levels compared to CC genotype cells.

The bioactive $1\alpha,25(OH)_2D_3$ exerts its effects thanks to the association with VDR; in the absence of $1\alpha,25(OH)_2D_3$, VDR shuttles between the nucleus and the cytoplasm. The binding with $1\alpha,25(OH)_2D_3$ stabilized the VDR/RXR heterodimer, which moves to the nucleus, where it binds to the VDREs (Prüfer K and Barsony J, 2002). We wondered whether a variation in the levels of VDR protein in the nucleus or the cytoplasm could affect its availability for the ligand $1\alpha,25(OH)_2D_3$, thus influencing indirectly the immunomodulatory effect of vitamin D. For this reason, we also analysed the expression of VDR in the cytosol and in the nucleus. No statistically significant differences were obtained between MS patients and healthy controls, either in the cytosol or nucleus.

In this case as well, the data was analysed in relation to the two studied polymorphisms: VDR rs731236 and VDR rs4334089, to analyse whether their variants may influence VDR localization. No statistically significant differences in the levels of expression of the VDR protein in the cytoplasm and nucleus were associated with VDR rs731236 and VDR

rs4334089.

The data suggests that VDR rs731236 and VDR rs4334089 may not influence MS risk and VDR total, cytoplasmic and nucleic protein expression. It is possible that, in this context, genetic variation does not have a great impact on the risk of developing MS; rather, it is the environment that mainly determines this risk. Vitamin D is related to several autoimmune diseases, and have immunomodulatory functions. Studies on animal models support the potential protective effect of vitamin D in autoimmune disorders (Lemire JM et al., 1992; Casteels K et al., 1998), and the efficiency of high-dose vitamin D supplementation in patients with autoimmune diseases has been tested (Muris AH et al., 2016; Buondonno I et al., 2017).

Given the important role of low levels of vitamin D for MS risk and the potential of vitamin D supplementation in MS therapy, the possible implication of vitamin D levels on VDR protein expression was investigated, with a particular focus on MS patients.

Our patients have a mean $25(OH)D_3$ plasma levels of 21.82 ± 13.61 ng/ml. This is consistent with the fact that low $25(OH)D_3$ levels, around 20 ng/ml, are usually observed in MS patients already at the beginning of the disease, such as in CIS or during the first relapses in RRMS patients (Ascherio A et al., 2014; Behrens JR et al., 2016).

$25(OH)D_3$ plasma levels can modulate the total VDR protein expression; the increase of 1 unit of vitamin D significantly decreases the total VDR protein expression by 10 units.

It is known that in some cell types, VDR expression is modulated by the presence of its own ligand $1\alpha,25(OH)_2D_3$. The typical response to $1\alpha,25(OH)_2D_3$ is up-regulation of VDR expression. This can be due to the fact that the VDR gene contains VDREs; in this way, $1\alpha,25(OH)_2D_3$ induces VDR gene transcription (Zella LA et al., 2010). Moreover, some studies suggest that the VDR up-regulation can also be due to the stabilization of the VDR itself (Peleg S and Nguyen CV et al., 2010; Zella LA et al., 2010). $1\alpha,25(OH)_2D_3$ up-regulates VDR expression in PBMCs following their activation (Yu XP et al., 1991). A more recent study analysed the effect of $1\alpha,25(OH)_2D_3$ on VDR expression in purified human CD4+ T cells activated *in vitro*; $1\alpha,25(OH)_2D_3$ up-regulates VDR protein expression approximately 2-fold by protecting the VDR against proteasomal degradation (Kongsbak M et al., 2014). However, a study published this year reported that in PBMCs from MS patients treated with vitamin D supplementation for 2 months the VDR mRNA expression decreases (Shirvani-Farsani Z et al., 2017). This result supports our findings that the increment in $25(OH)D_3$ plasma levels leads to decreased VDR expression.

The opposite results are likely because of the transience of the up-regulation of VDR expression. This is supported by Khan and colleagues: the incubation of rat ileum slices with $1\alpha,25(OH)_2D_3$ for 8 hours, but not after 12 hours, significantly induced VDR expression in a transient manner (Khan AA et al., 2010).

Further studies are needed to better understand the effects of vitamin D levels on VDR expression, considering that several complex mechanisms may affect gene regulation.

Understanding better the implications of VDR protein in MS may be important in reaching a more focused supplementation therapy.

4. Studying Alzheimer's disease

4.1. Aims of the work

In recent years, the importance of neuroinflammation in AD pathogenesis has increased in importance. In the context of neuroinflammation, the role of the complement system seems to be important, though it is not clear. A component frequently found in the autoptic parenchyma and in the CSF of AD patients is C4. C4 is encoded by two genes (C4A and C4B) which exhibit CNVs, and the different number of gene copies can influence C4 protein levels. The increased expression of C4 has been observed in AD patients in several studies.

To understand whether the increment in C4 protein in AD patients is a consequence or a cause involved in the inflammatory process, it is necessary to know better the pathogenesis of this complex disease.

For this reason, we are interested in investigating polymorphic genes for components of the complement system that may have an impact AD pathogenesis. We will focus our attention on C4A and C4B genes, located in the MHC class III, by determining the distribution of CNVs in AD patients compared with healthy controls, in order to analyse a possible genetic variation that can contribute to complement activation, thus playing a role in AD pathogenesis.

Our attention will be also directed on the C3b/C4b receptor called CR1. This receptor seems to be involved in Aβ clearance and several studies have identified many SNPs in its gene (chromosome 1q32.2) that may be associated to AD. Our attention will be focused on three SNPs: rs6656401, rs4844609 and rs2274567. The first is located in a non-coding region, but influence the isoform of CR1 protein produced. The CR1 rs4844609 and the rs2274567 polymorphisms are located in exon 37 and 30, respectively. A case-control study will be conducted, comparing allelic and genotypic frequencies between AD patients and healthy subjects, in order to establish a possible association of three SNPs with AD risk.

4.2. Materials and methods

4.2.1. Subjects and ethics statement

Patients with AD were enrolled by: 1) the Neurologic Science Department, IRCCS National Neurological Institute "C. Mondino" (Pavia, Italy); 2) the Department of Internal Medicine and Therapeutics, Section of Geriatrics and Gerontology, IDR "S. Margherita", University of Pavia (Pavia, Italy); 3) "Conte Franco Cella di Rivara Foundation" (Broni, Italy). Clinical presence of AD was assessed according to the NINCS-ADRDA diagnostic criteria were used (McKhann G et al., 1984). For patients with mild or moderate cognitive impairment [Mini Mental State Examination (MMSE) > 18], written informed consent was obtained from the patient and their families. For patients with severe cognitive impairment (MMSE ≤ 18), written consent was obtained only from the patients' relatives, who were informed about the study.

Healthy controls matched for ethnicity and age, were selected from healthy subjects assessed to be free from any kind of disorders, whether physical or mental, at the time of blood sampling. The healthy subjects analysed in this study were periodically followed-up. If a subject in the healthy control group presented a serious disease, such as diabetes, cardiovascular disorders, cancer, or neurological problems, he/she was removed from the analysis. Healthy subject were provided by the Immunogenetics Laboratory, Immunohematology and Transfusion Centre, Fondazione IRCCS, Policlinico San Matteo (Pavia, Italy). All controls signed informed consent.

The study has been approved by the Ethics Committee of each institution and has been conducted in accordance with the principles set out in the World Medical Association Declaration of Helsinki.

4.2.2. CNVs analysis

To evaluate the CNV of both the C4A and C4B genes, quantitative PCR (qPCR) was performed using TaqMan probes, based on the protocol previously described by Wu and collaborators (Wu YL et al., 2007). The qPCR technique allows detection and quantification of unique target sequences in DNA samples as the reaction progresses in real time.

Initially, samples with the most common C4A and C4B CNVs were used for assessment (Table 21). One sample was characterized thanks to the VII Complement Genetics Workshop of Mainz (Schneider PM et al., 1998).

C4A and C4B differ in five-base pairs on exon 26: to ensure specific amplification and the distinction between C4A and C4B, primers were selected based on published sequence. C4-

specific TaqMan probes (Eurofins Genomics, Ebersberg, Germany) were labelled with the fluorescent dye 6-FAM, while RP1 was used as an endogenous reference in multiplex reactions and labelled with the dye VIC. Each sample was analysed in triplicate and each reaction was carried out in a total volume of 20 µl, containing the forward and reverse primers (Table 22) for both targets and control amplicons (1µM), 100 nM of the target probe and the endogenous control probe (Table 23), 15 ng of test genomic DNA and 2X Master mix Probe master (Biorad). Real-time PCR was performed using the LightCycler 480 (Roche) with PCR cycles of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

To validate the results, a second qPCR test was performed. Copy number status was determined by TaqMan-based genomic qPCR using two TaqMan assays: Hs07226349_cn and Hs07226350_cn (Life Technologies), which are specifically designed for analysis of C4A and C4B. For this assay a 10 µl reaction mixture, containing 10 ng of genomic DNA, 2X Master mix Probe master (Biorad), C4A or C4B TaqMan probe, and RNaseP TaqMan probe, was used. Thermal cycling conditions consisted of 1 cycle of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

IHW Code	Copy number of C4A	Copy number of C4B
9020	2	0
WT51	4	0
9005	3	2
9017	1	2
9106	2	3
9023	0	2
9016	2	4
BS*	2	1

Table 21. DNA samples characterized for C4A and C4B CNV, obtained from the 2nd International Histocompatibility Working Group (www.ihvg.org) and * from VII Complement Genetics Workshop.

Gene	Primers	
C4A	**Forward C4F2**	**Reverse C4A32**
	5'-CCTTTGTGTTGAA GGTCCTGAGTT-3'	5'-TCCTGTCTAACACT GGACAGGGGT-3'
C4B	**Forward C4BF**	**Reverse C4BR2**
	5'-TGCAGGAGACATCT AACTGGCTTCT-3'	5'-CATGCTCCTATGTA TCACTGGAGAGA-3'
RP1	**Forward RP1E4F**	**Reverse RP1E4R**
	5'-GACCAAATGACA CAGACCTTTGG-3'	5'-GACTTTGGTTGG TTCCACAAGTC-3'

Table 22. Primers used for the amplification of C4A, C4B and the reference gene RP1.

Gene	Probe
C4A	**C4AB**
	VIC-CCAGGA GCA GGT AGG AGG CTC GC
C4B	**C4AB3**
	VIC-AGC AGG CTG ACG GC
RP1	**RP1**
	FAM-AGG GAC TCA GAAATC ACG T

Table 23. Probes used for the amplification of C4A, C4B and the reference gene RP1.

4.2.3.Real-Time PCR

Alleles and genotypes for CR1 rs6656401, CR1 rs4844609 and CR1 rs2274567 were assessed by Real-Time PCR.

The CR1 rs6656401, CR1 rs4844609 and CR1 rs2274567 were analysed by Real-Time PCR using the specific assay C__30033241_10, C__25598589_10 and C__12080027_10, respectively (Applied Biosystem). 1 µl of DNA were aliquoted into each well of a 384-well plate (final concentration: 10 ng/µl); the reaction was carried out in a final volume of 5 µl adding: 2.5 µl of 2X Master mix Probe (Biorad), 0.0625 µl µl of assay and 2.4375 µl of sterile H_2O. The SNP detection was carried out in the LightCycler 480 (Roche), following the thermal cycling conditions: one denaturation cycle of 10 min at 95°C, followed by 40 cycles each of 95°C for 15 s and 60°C for 1 min. Fluorescence was measured by the dedicated software (RealPlex 2.0) and the genotype was determined.

4.2.4.Statistical analysis

To analyse C4A and C4B CNVs a multivariate logistic regression model (with and without interaction) was fitted: AD presentation was the dependent variable and the number of C4A or C4B copies the independent variables. The OR and 95% CI were calculated using two copies as a reference value. A model selection was based on the likelihood-ratio test. Comparison of C4A and/or C4B copy numbers were performed with the non- parametric Mann-Whitney test, a p value of <0.05 was considered significant.

Genotype and allele frequencies of the analysed polymorphisms (CR1 rs4844609, CR1 rs6656401 and CR1 rs2274567) were calculated in AD patients and healthy controls.

Allelic frequencies in controls were examined to detect any significant deviation from the Hardy–Weinberg Equilibrium using a goodness of fit χ^2 test.

Allele and genotype frequencies were compared between AD patients and healthy controls using the χ^2 test, a p value of <0.05 was considered significant

All the statistical analyses were performed using Stata 14.1 statistical software (Stata Corporation, College Station, TX, USA).

4.3. Results

4.3.1. C4A and C4B copy number variations in Alzheimer's disease

C4A and C4B CNVs was determine in 191 AD patients and 120 healthy controls: the number of C4A and C4B gene copies for each individual was assessed, considering that also null genes are possible. CNV frequencies for both the genes of AD patients were compared to those of healthy subjects.

The analysis of C4A and C4B CNVs revealed a significant increase in copy number for both the genes in AD patients, compared to healthy subjects (Table 24 and Figure 43).

Since the likelihood-ratio test of the comparison of the two models with and without interaction terms was not significant, the model without interaction terms was considered. The analysis revealed that C4A and C4B were associated with AD following an independent fashion. In detail, AD patients with three copies of C4A or C4B were more prevalent, compared to healthy controls (OR = 5.78; $p < 0.001$ and OR = 6.66; $p < 0.001$ respectively).

	Healthy controls (N/%)	**AD patients (N/%)**
C4A copy number		
0	3 (1.2%)	0
1	40 (15.9%)	6 (4.2%)
2	160 (63.5%)	73 (51 %)
3	31(12.3%)	**33 (23.1%)***
4	18 (7.1%)	**31 (21.7)****
Total	252	143
C4B copy number		
0	12 (4.41%)	3 (1.9%)
1	95 (34.92%)	31 (20%)
2	113 (41.54%)	45 (29.03%)
3	52 (19.11%)	**76 (49.03%)*****
Total	272	155

Table 24. C4A and C4B copy numbers of healthy controls and AD patients.
*OR= 5.78; p< 0.001; C.I.= 2.61-12.82
** OR= 7.07; p< 0.001 C.I. 3.40-14.70
*** OR= 6.66; p <0.001 C.I.=2.94-15.06

In each subject, no more than three C4B copies were observed, but four C4A copies were

detected: we noticed that 31 AD patients had four C4A copies (21.7%), but only 18 healthy subjects presented four C4A copies (6.3%) and the difference were statistically significant (OR = 7.07; $p < 0.001$).

Also the median number of both C4A and C4B copies for each individual was calculated. AD patients showed a median number of three copies and healthy controls presented only two copies ($p<0.001$). Furthermore, a significant difference between AD patients and healthy subjects were reported considering the median total number of copies (C4A+C4B): AD patients showed five copies, whereas healthy controls had only four copies ($p<0.001$, Mann-Whitney test).

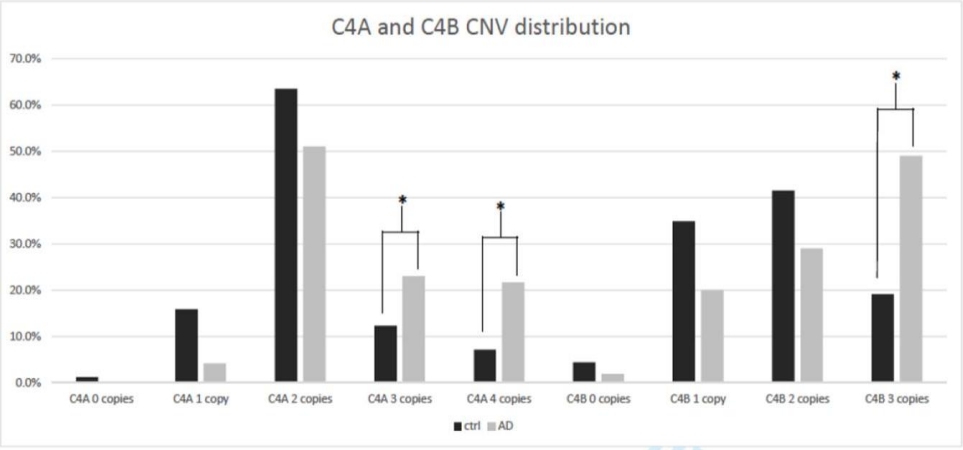

Figure 43. Bar graph showing the frequency of C4A and C4B gene copy number in AD patients and healthy controls. AD patients are represented by the black bars and healthy controls by the grey ones.

4.3.2.CR1 polymorphisms in Alzheimer's disease

The genotype for CR1 rs6656401, CR1 rs4844609 and CR1 rs2274567 polymorphisms were determine in 150 AD patients and 313 healthy controls. Genotypic and allelic frequencies of AD patients were compared to those of healthy subjects (Table 25).

Genotypic frequencies in the control group did not deviate from the Hardy–Weinberg equilibrium ($p > 0.05$).

Comparing the group of AD patients with those of healthy subjects, for all the three analysed polymorphisms, no significant association with AD was found ($p > 0.05$). Only considering the allelic frequencies of the SNP CR1 rs4844609 a difference between AD patients and healthy subjects was noticed, although it was not statistically significant

(p=0.068): the A allele was more frequent in AD patients.

rs6656401	Healthy controls	AD patients
	N=309	N=150
AA	8 (2.6%)	8 (5.3%)
AG	111 (35.9%)	50 (33.3%)
GG	190 (61.5%)	92 (61.3%)
A	127 (20.6%)	66 (22.0%)
G	491 (79.4%)	234 (78.0%)

rs4844609	Healthy controls	AD patients
	N=313	N=150
AA	1 (0.3%)	1 (0.6 %)
AT	6 (1.9%)	7 (4.7%)
TT	306 (97.8%)	142 (94.7%)
A	8 (1.3%)	9 (3.0%)
T	618 (98.7%)	291 (97.0%)

rs2274567	Healthy controls	AD patients
	N=304	N=149
AA	196 (64.5%)	99 (66.5%)
AG	93 (30.6%)	44 (29.5%)
GG	15 (4.9%)	6 (4.0%)
A	485 (79.8%)	242 (81.2%)
G	123 (20.2%)	56 (18.8%)

Table 25. Alleles and genotypes frequencies of the three SNPs in CR1 gene.

4.4. Discussion

AD is the most common type of age-related dementia, characterized by a progressive loss of cognitive abilities, severe neurodegeneration, and neuroinflammation. Aggregates of Aβ peptides in senile plaques and NFTs of hyper-phosphorylated tau are neuropathological hallmarks of AD (Krstic D and Knuesel I et al., 2013). The sporadic multifactorial form of AD accounts for the vast majority of cases, and risk factors implied in the alteration in innate immunity may play a role in disease etiology.

Our research group has been interested in the possible role of MHC class III genes in AD pathogenesis for several years (Maggioli E et al., 2013; Boiocchi C et al., 2015).

Some data in the literature suggests the possible role of the complement system in AD pathogenesis (Bennett S et al., 2012; Ma XY et al., 2014). Increased levels of complement components like C4 were observed both in the CSF and in the plasma of AD patients (Daborg J et al., 2012; Bennett S et al., 2012). Furthermore, the study by Daborg and collaborators reported that C4 expression increased according to the patients' age and disease stage (Daborg J et al., 2012).

The two genes, C4A and C4B, which encode this component of the complement system, map in MHC class III region. These two genes differ only for five nucleotides located in exon 26, and both are expressed. C4A and C4B are highly polymorphic genes with CNVs; gene duplications as well as null alleles, called C4AQ0 and C4BQ0, respectively, are possible (Castley AS and Martinez OP, 2012). An association has been reported between C4 genes copy number and C4 plasma protein levels: a linear correlation between C4 genes copy number and C4 plasma protein concentration was observed (Yang Y et al., 2007; Saxena K et al., 2009).

C4 CNVs are important in the susceptibility of autoimmune disorders. Low C4 copy numbers represent a risk factor for SLE (Yang Y et al., 2007; Wu YL et al., 2008) and the C4B deficiency has been reported in RA patients (Rigby WF et al., 2012). Furthermore, C4 in neuroinflammation is also linked to psychiatric disorders and the role of C4 has been established in schizophrenia.

The RNA expression of C4A and C4B increased proportionally with the copy number of C4A and C4B genes, respectively (Sekar A et al., 2016).

Based on this consideration, we decided to investigate the copy number of C4A and C4B genes, comparing AD patients with healthy subjects. Our analysis of the C4 CNVs reveals a significant increase in C4A and C4B copy number in AD patients in comparison with healthy donors.

The higher C4A and C4B copy number in AD patients can support the study of Yasojima and colleagues, which described doubled mRNA levels for C3 and C4 in post-mortem AD brains (Yasojima K et al., 1999). The correlation between a higher C4A and C4B copy

number and increased C4 production is consistent with the finding that in healthy subjects C4A and C4B plasma levels are genetically predetermined by the C4 gene copy number (Margery-Muir AA et al., 2014).
Interestingly, a study by our group showed that the presence of the C4BQ0 allele is less frequent in healthy elderly subjects than in younger individuals (Ricci G et al., 1996). A high percentage of healthy elderly people shows high C4B protein levels, suggesting a selection for individuals with a more efficient complement system. This result is consistent with a study performed in 2003, which showed an age-associated decrease in the frequency of the C4BQ0 allele in elderly people (Arason GJ et al., 2003). The null allele seems to be a negative selection factor for survival.
It is important to consider the possible involvement of linkage disequilibrium between C4 copy number variations and other HLA genes. However, in a 2010 study, only a slight correlation between C4 copy number and MHC class I and II variants was demonstrated (Fernando MM et al., 2010). Furthermore, Cleynen and collaborators also found a low level of positive linkage between C4 copy number and alleles of the classical MHC genes (Cleynen I et al., 2016).
This study reports an elevated number of C4 genes in AD patients than in healthy controls. Considering the limitations of this study, independent confirmation of our results in a larger cohort is necessary. The differences we observe should encourage further studies on markers of complement activation in AD.

Other components of the complement system may be involved in AD pathogenesis not only because of the importance of inflammation in this disorder, but also because it plays a role in normal neurodevelopment: complement system is involved in synapse formation and elimination (Stevens B et al., 2007). It seems to have a role in removing dysfunctional neurons and dendritic processes, contributing to sculpture the brain during the neurodevelopment. Alterations may contribute to neurological disorders (Nimgaonkar VL et al., 2017).
Our attention was focused on the polymorphic CR1 gene. Initially, the GERAD1 (Genetic and Environmental Risk in AD Consortium 1) study suggested the association of this gene with AD (Harold D et al., 2009) and in the last years independent large multicenter datasets and meta-analyses strongly support the role of several SNPs in CR1 in AD susceptibility (Jin C et al., 2012; Ma XY et al., 2014). Furthermore, CR1 has been related to several disorders characterized by an inflammatory component; for example, SNPs in this gene are associated with inflammation and with the risk of cardiovascular diseases (de Vries MA et al., 2017), and to SLE susceptibility (Panda AK et al., 2017).
Our attention was focused on three SNPs of the CR1 gene: rs6656401, rs4844609 and rs2274567. From our analysis, AD patients and healthy controls do not show any significant difference both in allelic and genotypic frequencies for the three

polymorphisms. However, other studies support a role of these SNPs in AD susceptibility.
In a GWAS performed in 2009 on 3,978 AD patients and 3,297 controls from Belgium, Finland, Italy and Spain, the SNP rs6656401 in the CR1 reached a genome-wide significance (Lambert JC et al., 2009). The association between the CR1 rs6656401 polymorphism and AD was also replicated in a case-control study on 462 patients and 350 healthy subjects from the Southern Chinese population, with the risk allele A conferring an OR of 1.97 (Chen LH et al., 2012). In our study, no significant association between the polymorphism and AD is reported; this contrasting result may be due to the lack of statistical power because of the smaller sample size. However, our finding supports the work by Klimkowicz-Mrowiec and collaborators on 253 AD patients and 240 healthy controls from Poland, in which no significant differences in the distribution of CR1 rs6656401 genotypes were observed (Klimkowicz-Mrowiec A, et al., 2013).
Also the CR1 rs4844609 polymorphism was associated to AD in GWAS (1,019 AD cases and 591 healthy controls) (Corneveaux JJ et al., 2010). Furthermore, a 2012 study associated the A allele to episodic memory decline, increasing the AD neuropathological feature (Keenan BT et al., 2012). Although it is possible that the SNP has an impact in the clearance of Aβ, the pathogenic mechanism is not clear and further analyses will be fundamental. Our result on the role of this polymorphism in AD is conflicting: no association between the CR1 rs4844609 polymorphism and AD is found. This result is supported by a 2013 study on a larger Flanders-Belgian cohort (1,276 AD patients and 1,128 healthy controls) (Van Cauwenberghe C et al., 2013). The authors reported no association between the CR1 rs4844609 polymorphism and AD susceptibility; in addition, the SNP does not seem to influence memory impairment and it is not associated with CSF $A\beta_{1-42}$ levels. The researchers supposed that CNVs in the CR1 gene may be the true functional risk factor for AD development.
Interestingly, in a study by Fonseca and colleagues, anti-CR1 antibodies directed against different epitopes of the receptor were used in order to localize CR1 in *post mortem* brains and to assess the relative binding affinities of the CR1 ligands (C1q and C3b) (Fonseca MI et al., 2016). They noticed that the amount of astrocyte staining varied among the samples, but the difference seems not to be associated with the SNPs rs4844609 and rs6656401 of CR1 gene. They suggested that it is unlikely that astrocyte CR1 expression levels or C1q or C3b binding activity were the cause of the association of CR1 variants with AD identified by GWAS. Their results further support the need of functional studies in order to determine if the variant-dictated number of CR1 expressed on red blood cells contributes to the clearance of peripheral Aβ or if other mechanisms are involved.
Finally, in our study the CR1 rs2274567 polymorphism was analysed. No significant association with AD was found. Our result is in contrast with the results by Ma and collaborators, which reported an association between the CR1 rs2274567 polymorphism and the presence of AD: the ATG haplotype, formed by the SNPs rs2274567, rs3737002,

and rs6691117, was associated with an increased risk for late onset AD (Ma XY et al., 2014).
The influence of these investigated polymorphisms on AD susceptibility and progression is probably weak, and a very large population is needed to reveal the association. Furthermore, multiple functional variants in the CR1 gene may be implicated in AD susceptibility, contributing together to the risk of developing the disease. It will be necessary to perform more in-depth genetic analysis of other functional variants to fully evaluate the possible association between CR1 and AD onset or progression. Studies on the biological mechanisms of CR1 in AD pathogenesis should be encouraged.

Future studies could clarify the exact role of complement activation in AD, because, at present, it is not clear whether it contributes to or is a defence against further neurodegeneration. It will be important to consider other genes for other complement components and analyse the possible interaction among their variants. A more comprehensive understanding of the complement system in AD will hopefully lead to the development of novel therapeutics for the prevention and treatment of this disorder.

5. Conclusions and perspectives

Neurodegenerative disorders are heterogeneous disorders, but a common characteristic is the presence of an inflammatory process alongside of neurodegeneration. Most neurodegenerative diseases are multifactorial: the interplay among genetic, environmental and epigenetic factors is essential in determining disease onset and the type of progression.
In the thesis, two multifactorial neurodegenerative disorders (MS and AD), in which the alteration of the immune system takes part to the pathogenic process, were studied. Their etiology and pathogenesis are not completely clear; the factors that contribute to disease onset and progression are difficult to detect. Furthermore, it is not always easy to assess the contribution of each single factor to the disease pathogenesis, because its effect may be obscured or confounded by other contributing factors.
Based on the importance of neuroinflammation in these neurodegenerative disorders, we were interested in investigating polymorphic genes that are involved in immunomodulation, to understand whether they have an impact on MS and AD risk and progression.
In the thesis, the attention was mainly focused on MS, a disimmune disease in which the auto-inflammatory process is one of the principal pathogenic factors and the alteration of the adaptive immune system plays a key role in the onset of the disease. Several other mechanisms contribute to neurodegeneration, such as oxidative stress, mitochondrial injury and the failure of regenerative mechanisms. Since the interest of our research group for the MHC class III genes (chromosome 6), the attention was focused on the polymorphic genes for Hsp70 proteins (HSPA1L and HSPA1B), because of their role in the protection against many type of stress and their involvement in immunoregulation. Our group has previously shown that a SNP (rs1061581) in the HSPA1B gene, which encodes the stress inducible chaperone called Hsp70-2, was related to the risk of developing MS: G allele is significantly associated to MS with an OR of 1.31 (Boiocchi C et al., 2014).
In this work, the rs2227956 polymorphism in the HLA1L gene, that encodes the constitutively expressed Hsp70-Hom, is significantly associated to MS: the C allele confers a twofold increased risk of developing MS (OR = 2.13). This polymorphism is not only related to MS risk, but also to its progression: the disease severity, measured by MSSS, distributes differently, depending on the HSP70-HOM genotype, with CC patients having an increased MSSS. These two polymorphic variants are in linkage disequilibrium and the combination of the two risk alleles increases MS risk by more than three times (OR = 3.49). Like for many other variants implicated in MS risk, these two polymorphisms contribute to small portions of the disease risk, because it is probably determined by cumulative effect of a great number of allelic variants. These positive results should encourage the inclusion of

polymorphisms of MHC class III genes in GWAS studies for MS genetic susceptibility, adding information on MS genetic risk.

However, it is not always clear how these polymorphisms could be associated the disease pathogenesis. Hsp70-Hom protein levels do not correlate with the HSP70-HOM rs2227956 genotype, but a direct significant relationship between Hsp70-Hom protein expression and MS severity has been reported, suggesting that Hsp70-Hom probably plays a role in promoting immune system activation.

Considering the importance of HSP70-2 rs1061581 polymorphism for MS susceptibility, the possible role of the Hsp70-2 protein in the response to oxidative stress was also investigated treating *in vitro* PBMCs from MS patients and healthy controls with H_2O_2. PBMCs suffer initially for the presence of oxidative stress, but then they are able to increase again their mitochondrial activity. However, no significant difference in mitochondrial activity, Hsp70-2 protein expression, and intracellular ROS production between MS patients and healthy subjects was found. Also, stratifying our data of the mitochondrial activity and the Hsp70-2 protein expression levels by the HSP70-2 rs1061581 genotype, no significant difference was noticed. The presence of an allelic variant, involved in the risk of developing MS, does not seem to influence the response to oxidative stress, probably because of the complex protective mechanisms that involve several molecules, including anti-oxidants enzymes.

Although the genetic risk factors are important, genetics explains only 30% of MS risk, with the environmental factors playing a fundamental role in determining MS susceptibility. For this reason, it is important to consider the possible interaction between genetic and environmental risk factors. In this regard, two polymorphisms of the vitamin D receptor (VDR) gene (chromosome 12q13.11) were analysed: VDR rs731236 (Taq-I) and rs4334089 (HpyCH4V). Vitamin D is involved in the correct regulation of the immune system and low levels of vitamin D are considered an important risk factor for developing MS. MS patients and healthy controls do not show significant differences in allelic and genotypic frequencies of the two analysed polymorphisms. However, controversial results about the association of the VDR rs731236 polymorphism with MS are present in literature, with both positive and negative findings. It should be stressed that the opposite results obtained in several studies can be due to the fact that different populations were considered; it is clear that differences in the genetic background between ethnic groups are presented. Regarding the VDR rs4334089 polymorphism, to our knowledge, our study is the first to analyse the VDR rs4334089 polymorphism in MS pathogenesis and larger investigations are required.

The two investigated polymorphisms are not related to a different VDR protein expression, considering the whole-cell, the cytosolic and the nuclear fractions. Furthermore, VDR protein levels do not change between MS patients and healthy controls. The lack of correlation between the two polymorphisms and protein expression in PBMCs from MS

patients and healthy subjects may be due to the fact that the two polymorphisms do not influence RNA expression. They probably do not affect transcript stability or translational modulation, and thus may not vary protein levels.

Our data suggests that VDR rs731236 and VDR rs4334089 may not influence MS risk and VDR protein expression in whole-cell, cytoplasmic and nuclear fractions. It is possible that, in this context, genetic variation does not have a great impact on the risk of developing MS; rather, it is the environment that mainly determines the risk. Based on our data, $25(OH)D_3$ plasma levels can modulate the total VDR protein expression; the increase of 1 unit of vitamin D significantly decreases the total VDR protein expression by 10 units. The typical response to $1\alpha,25(OH)_2D_3$ is up-regulation of VDR expression, but the transience of this process should be taken into account. This result highlights the importance to understand the VDR regulation in MS patients; it could be important for a more focused supplementation therapy. For this reason, further studies will be necessary to clearly understand the effects of vitamin D levels on VDR expression.

Furthermore, AD, where the action of the innate immune system is predominant and it is not completely clear whether the inflammation is a cause or a consequence of the neurodegeneration, was considered. Literature data support the role of the complement system in AD pathogenesis, although its effects are contradictory. Also in the context of AD, our attention was focus in analysing polymorphic genes involved in immunomodulation in order to understand whether they have an impact on disease risk. In this regard, we firstly focus the attention on two polymorphic genes located in the MHC class III: C4A and C4B. They encode two isoforms of the serum complement component 4, involved in the classical pathway. C4A and C4B are polymorphic genes, presenting CNVs, gene size variations, and nucleotide polymorphisms. Our analysis revealed a significant increase in C4A and C4B copy number in AD patients compared to healthy donors, a fact that may be related to the increased protein levels found in the CSF and plasma of AD patients, thus influencing neuroinflammation.

Also the gene for the complement C3b/C4b receptor 1 called CR1 (chromosome 1q32.2) was considered. CR1 binds to C3b and C4b and facilitates the uptake of immune complexes by phagocytes; furthermore, it is involved in the immunoregulation, inhibiting the activation of the C3 and C5 convertases. Three polymorphisms (rs6656401, rs4844609 and rs2274567) were analysed in order to understand whether they are involved in determining AD risk. Genotypic and allelic frequencies of the three polymorphisms do not show any significant difference between AD patients and healthy controls.

Although our data are not always positive, the results regarding the C4A and C4B genes support the involvement of complement genes in AD, highlighting the importance of neuroinflammation and supporting the “neuroinflammatory hypothesis”. It is important to consider that the influence of these investigated polymorphisms on AD risk may be weak, and a very large population is needed to reveal possible associations. Future studies,

considering also genes for other complement components, could clarify better the role of complement activation in AD.

It is not always easy to understand how genetic variants associated with neuroinflammation and neurodegenerative disorders affect disease onset and progression, a fact which reflects their complexity. Our observations may be important in better understanding the risk factors and the pathogenic mechanisms of neurodegenerative disorders. Although not always positive, the results may suggest in which direction new analysis should be directed. In his book “La battaglia navale”, Marco Malvaldi says about police investigations: “a field investigation is like a battleship. At the beginning you shoot randomly and don’t find anything. But it is fundamental that you remember where you shot, because the fact that you didn’t find anything is an important information. At a certain point, when you hit something without sinking it, you understand that you have to continue to shoot in the adjacent squares with a *criterion*. It is only a matter of time”. The police investigation can be compared to scientific research: in the complexity of neurodegenerative disorders, every result, even if negative, is important in guiding the research to understand the mechanisms, which can be pivotal for future therapeutic studies.

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List of original manuscripts

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- Zorzetto M, Datturi F, Divizia L, **Pistono C**, Campo I, De Silvestri A, Cuccia M, Ricevuti G. Complement C4A and C4B gene copy number study in Alzheimer's disease patients. Curr Alzheimer Res. 2017; 14:303-308.
- **Pistono C**, Osera C, Boiocchi C, Mallucci G, Cuccia M, Bergamaschi R, Pascale A. What is the best pharmacological oral treatment for Multiple Sclerosis? Be careful of immunogenetics. Pharmacol Res. 2017; 120:279-293. Review.
- **Pistono C**, Monti MC, Boiocchi C, Gigli Berzolari F, Osera C, Mallucci G, Cuccia M, Pascale A, Montomoli C, Bergamaschi R. Involvement of Hsp70-2 protein in oxidative stress: a study on multiple sclerosis. J Neuroimmunol. *Under review*.

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Heat shock protein 70-hom gene polymorphism and protein expression in multiple sclerosis

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ABSTRACT

Immune-mediated and neurodegenerative mechanisms are involved in multiple sclerosis (MS). Growing evidences highlight the role of HSP70 genes in the susceptibility of some neurological diseases.
In this explorative study we analyzed a polymorphism (i.e. HSP70-hom rs2227956) of the gene HSPA1L, which encodes for the protein hsp70-hom.
We sequenced the polymorphism by polymerase chain reaction (PCR), in 191 MS patients and 365 healthy controls. The hsp70-hom protein expression was quantified by western blotting.
We reported a strong association between rs2227956 polymorphism and MS risk, which is independent from the association with HSP70-2 rs1061581, and a significant link between hsp70-hom protein expression and MS severity.

1. Introduction

Multiple sclerosis (MS) is a disimmune-mediated neurodegenerative disease characterized by demyelinated lesions scattered throughout the white and the grey matter of the brain and the spinal cord (Compston and Coles, 2008). The precise aetiology of MS is actually unknown but evidences suggest that demyelinated lesions are induced by different immunological mechanisms, including components of both the adaptive and the innate immune system (Mallucci et al., 2015). Oxidative stress is also involved in MS pathogenesis and progression by direct and indirect mechanisms of action(Lassmann, 2014), which also encompass the heat shock proteins 70 (hsp70s) family(Mansilla et al., 2012).

Intracellular hsp70s act as chaperones (i.e. assistant proteins that prevent proteins mis-folding and/or aggregation) and anti-apoptotic proteins (Mayer, 2013). Extracellular hsp70s process and present antigens, promoting the activation of immune system (Li et al., 2002). Polymorphisms leading to either quantitative or qualitative change in hsp70 expression likely affect both the hsp70 cyto-protective and/or immune-modulatory effects.

Among the several proteins included in hsp70s family, the two major stress-inducible members (i.e. the Hsp70-1 and Hsp70-2) are encoded by HSPA1A and HSPA1B gene respectively, and the constitutively expressed non-inducible protein (i.e. Hsp70-hom) is encoded by HSPA1L gene (Brocchieri et al., 2008). These three genes are located on chromosome 6 (6p21.3) (Milner and Campbell, 1990), within the human leukocyte antigen (HLA) class III region. Polymorphisms of the HLA class III have not yet been included in genome wide association studies aimed at studying MS genetic susceptibility, which actually have found >110 known MS risk variants in and out HLA class I and II region (International Multiple Sclerosis Genetics Consortium, Nat Genet 2013; Moutsianas et al., 2015). The HSPA1L gene located within the HLA class III region may be a good candidate gene for the analysis of MS genetic susceptibility adding some information on MS genetic risk. Those results will be validated in bigger studies.

While polymorphisms within HSPA1A exons are silent (Milner and Campbell, 1992), we recently demonstrated that + 1267 A/G HSPA1B (rs1061581) polymorphism is associated with an increased MS risk and MS patients with GG or GA genotype display a significant reduction of hsp70-2 expression compared to patients with AA genotype (Boiocchi et al., 2014). Polymorphisms in the HSPA1L gene are mainly located in the region coding for the substrate-binding domain. Among

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these, HSP70-hom rs2075800 has been associated with rheumatoid arthritis (Jenkins et al., 2000) and uveitis in patients with sarcoidosis (Spagnolo et al., 2007) and HSP70-hom rs2227956 has been linked to Loegren's syndrome and sarcoidosis (Bogunia-Kubik et al., 2006).

The aims of this study were to analyze the effect of +2437 HSP70-hom (rs2227956) polymorphism on MS risk and MS severity and its contribution to oxidative stress, in order to better define the real contribution of the gene HSPA1L as a MS onset/severity genetic susceptibility locus.

2. Material and methods

2.1. Subjects and ethics statement

We consecutively included in this study 195 Caucasian patients with a diagnosis of MS according to the 2010 revised McDonald Criteria (Polman et al., 2011), patients were recruited from the MS Centre of the National Neurological Institute "C. Mondino" (Pavia, Italy). The neurological disability of MS patients was quantified by the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) while the clinical impact of MS was calculated applying the Multiple Sclerosis Severity Score (MSSS) (Roxburgh et al., 2005) which relates scores on EDSS to disease durations. From our extended clinical experience, we arbitrary chose a cut–off value of an MSSS of 3: MS patients with a MSSS < 3 were considered affected by a mild form of disease; MSSS ≥ 3 reflected instead patients with a moderate to severe form of MS. The control population includes 439 Caucasian subjects. Controls were randomly selected from healthy individuals, as judged by regular checks, attending the National Neurological Institute C. Mondino. MS patients and controls demographical and clinical characteristics were recorded when blood samples were collected and are listed in Table 1 for the subjects with no missing information included in the analyses.

The study has been approved by local ethics committees and has been conducted in accordance with principles expressed in the Declaration of Helsinki.

2.2. Gene polymorphism analysis

Human genomic DNA was obtained from 200 μl of whole blood [collected by venipuncture in Vacutainer tubes containing ethylenedinitrilotetraacetic (EDTA, BD)], using the QIAamp DNA Blood Mini Kit (QIAGEN) following the manufacturer's instructions. The concentration and purity of DNA was determined by spectrophotometric analysis. In order to establish alleles and genotypes for the investigated polymorphism, +2437 HSP70-hom (rs2227956 C) and +1267 HSP70-2 (rs1061581 G) we used a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Amplification was performed using I-Cycler (BioRad). For the +12437 T/C HSP70-hom and +1267 A/G HSP70-2 polymorphisms, and the following amplification protocol was applied: initial incubation at 95 °C for 5 min, followed by 35 cycles each of 95 °C for 30 s, 53 °C for 1 min, 72 °C for 1 min and 30 s; final extension is carried out at 72 °C for 7 min. The PCR products were visualized by electrophoresis in 2% agarose gel (Agarose Standard, Eurobio). Genotypes were determined by digestion with an appropriate restriction enzyme. The restriction patterns were obtained by gel electrophoresis in 3% agarose gel (Table 1 supplement). To confirm the reliability of our data obtained by PCR- RFLP we performed further genotyping by RealTime PCR. Genotyping was performed with TaqMan® SNP Genotyping Assays on LightCycler 480 system (LC480) (Roche S.p.A., Milano, Italy). Assays were purchased from Applied Biosystem (C__25630755_10). The overlap of the data was 100%.

2.3. Peripheral blood mononuclear cells (PBMCs) isolation from whole blood

Five milliliter of blood were diluted 1:1 with Ficoll (Histopaque-1077, Sigma-Aldrich) and centrifuged at 450 ×*g* for 30 min. PBMCs above the Ficoll ring were harvested and washed twice with phosphate buffered saline 1× (PBS). The cellular pellets were stored at −80 °C until further analysis.

2.4. Western blotting

PBMCs from MS patients and controls were collected and homogenized in a buffer containing 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 50 mM 2-mercaptoethanol, 0.32 mM sucrose, and a protease inhibitor cocktail at the dilution suggested by the manufacturer (Roche Molecular Biochemicals), by using a Teflon/glass homogenizer and sonicating twice for 10 s. The protein content was measured via Bradford's method using bovine serum albumin (Sigma Aldrich) as standard. Proteins were diluted in sodium dodecyl sulfate (SDS) protein gel loading solution 2×, boiled for 5 min, separated by 12% SDS-polyacrylamide gel electrophoresis, and then processed as previously described. The mouse anti-HSP70-hom antibody (Enzo Lifescience) was diluted at 1:1000, and the mouse anti-α-tubulin (Sigma-Aldrich) at 1:1000. All the antibodies were diluted in TBST buffer [10 mM Tris-HCl, 100 mM NaCl, 0.1% (*v*/*v*) Tween 20, pH 7.5] containing 6% (*v*/*v*) milk. The nitrocellulose membrane signals were detected by chemiluminescence. The experiments were performed in duplicate for each sample using α-tubulin to normalize the data. The analysis was performed on the densitometric values obtained using the V1.62 NIH Image software after image acquisition.

2.5. Statistical analysis

To assess the association between the +2437 T/C HSP70-hom polymorphism and MS we performed an unconditional logistic regression analysis adjusted by sex and age; adjusted Odds Ratios (OR) with 95% confidence intervals (95% CI) were derived and used as measure of effect. An additive allelic model and a genotypic model were fitted to estimate i) the C allele risk; ii) the heterozygous TC versus wild type TT risk and iii) the homozygous CC versus wild type TT genotype risk. Moreover, allele frequencies in controls were examined to detect any significant deviation from the Hardy–Weinberg Equilibrium using a

Table 1
Demographic and clinical characteristic of MS patients and controls. Data are expressed as number of subjects and percentage (%) or mean ± standard deviation. *t*-Test for age and χ^2 test for gender are calculated to compare cases and controls, $p < 0.05$ is considered statistically significant. § Comparison of age or sex among MS patients and MS patients sub-group is not significant ($p > 0.05$). † Comparison of age or sex among controls and controls sub-group is not significant ($p > 0.05$).

	MS patients ($n = 191$)	Controls ($n = 365$)	*p*-Value	MS patients sub-group ($n = 47$)	Controls sub-group ($n = 29$)	*p*-Value
Age (years)	44.1 ± 10.7	35.7 ± 8.6	<0.001	45.4 ± 11.33§	39.3 ± 13.1†	>0.05
Sex (F/M ratio)	1.81	1.31	<0.05	1.61§	0.70†	<0.01
MS duration (years)	12.6 ± 8.3			12.0 ± 7.5§		
EDSS	2.66 ± 2.14			2.97 ± 2.19§		
MSSS	3.0 ± 2.4			3.30 ± 2.35§		

Abbreviation: EDSS = expanded disability status scale; F/M = female to male ratio. MS = multiple sclerosis; MSSS = multiple sclerosis severity score.

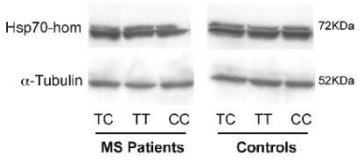

Fig. 2. Western blot analysis. Immunoblotting analysis of the HSP70-hom rs2227956 protein is performed on PBMC from MS patients and controls. α-tubulin serve as the loading control. Data shown are a representative Western blot of performed experiments and show that expression of hsp70-hom does not differ between MS patients and controls and between the three genotypes (TC, TT and CC). Abbreviation: HSP = heat shock protein; MS = multiple sclerosis; PBMC = peripheral blood mononuclear cell.

the patients with hsp70-hom below the 25th percentile had a high value of MSSS (i.e. high MS severity) (Fig. 3B).

4. Discussion

The presence of a predisposing effect on disease development and a detrimental effect on disease evolution is uncommonly found in MS genetic association studies. Comi et al. 2012 studied the Impact of Osteopontin Gene Variations on MS. Their work confirms that osteopontin and the *OPN* gene may be involved in MS development and, especially, progression. Similarly, in our study we are interested to study the impact of hsp70 on MS. In particular, we document an association between a variation in HSP70-hom gene (i.e. rs2227956 C) and MS risk. Additionally, we report that hsp70-hom expression is connected with MS impact.

The HSP70-hom (rs2227956) polymorphism increases about seven times the risk of MS in CC carriers compared to the wild-type TT carriers. Interestingly, the multilocus analysis including the HSP70–2 (rs1061581,(Boiocchi et al., 2014) and HSP70-hom (rs2227956) polymorphisms, using only the 365 controls successfully genotyped for rs2227956, shows that the combination of the two risk alleles (G and C, respectively) increases MS risk and shows that HSP70-hom rs2227956 affects MS risk independently from HSP70-2 rs1061581).

Due to lack of functional studies, we can only speculate on the biological consequences of the association between HSP70-hom rs2227956 and MS. The T-to-C HSP70-hom gene polymorphism leads to a novel amino acid at residue 493 of HSP70-hom (i.e. Met to Thr amino acid substitution). According to the model structure by Zhu (Zhu et al., 1996), HSP70-hom residue 493 lies within one of the β-sheets and form the floor of the peptide-binding grove. This non-synonymous polymorphism might therefore affect the functional efficiency of peptide-binding hsp70-hom specificity leading to an accumulation of misfolded proteins into neurons and glia. This will induce a primary cytodegeneration which will release antigens and, in predisposed individuals, promote autoimmune and inflammatory MS response (Stys et al., 2012).

However, the alteration in hsp70-hom chaperoning ability is only one of the possible mechanism through which HSP70-hom polymorphism might lead to an increased MS risk. Hsp70s not only prevent protein misfolding but also induces antiapoptotic mechanisms in both neurons and glia, suggesting that mechanisms that affect hsp70s hinder both reparative and regenerative processes. Hsp70s exhibit immunomodulatory functions and trigger both innate and adaptive response. Macrophages and dendritic cells are stimulated via the toll-like receptors to release pro-inflammatory cytokines [such as interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α)], chemokines (such as RANTES) and nitric oxide (Arango Duque and Descoteaux, 2014). T cell response is induced by the hsp70 adjuvant ability to bind immunogenic peptides. These peptides are internalized into cells and further presented via MHC-I and MHC-II molecules (Li et al., 2002). The aberrant hsp70 expression and/or a perturbation of hsp70 functions likely affects MS pathogenesis by exacerbating the immune response or eliciting the presentation of autoantigens.

Our results are in contrast with previous studies that do not detect any significant association between HSP70-hom rs2227956 and MS

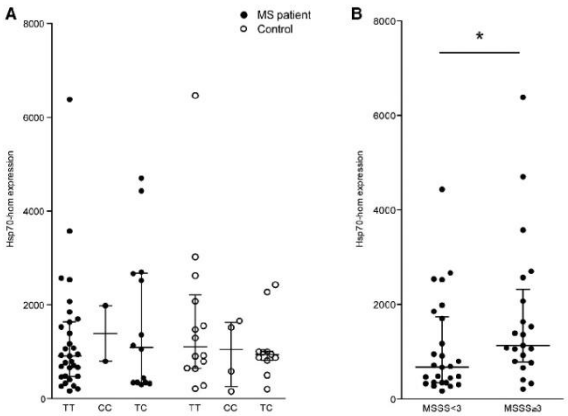

Fig. 3. Hsp70-hom protein expressions. A) MS patients (black dots) and controls (white dots) are grouped by HSP70-hom genotype; 25th percentile, median and 75th percentile are reported on the dot plot (black bars). No statistically significantly difference between MS patients and controls or within HSP70-hom genotypes are observed, $p > 0.05$. B) MS patients (black dots) are grouped by MS severity (i.e. MSSS), 25th percentile, median, 75th percentile are reported on the dot plot (black bars). Hsp70-hom protein expression is significantly reduced in MS patients with low MS severity (i.e. MSSS < 3) compared to the other MS patients (i.e. MSSS ≥ 3), $^*p < 0.05$. Abbreviation: HSP = heat shock protein; MS = multiple sclerosis, MSSS = multiple sclerosis severity scale.

risk. Ramachandran and Bell studied this polymorphism in 59 Canadian MS patients ethnically matched to healthy controls (Ramachandran and Bell, 1995); and Niino and colleagues studied this polymorphisms in 107 Japanese MS patients ethnically matched to healthy controls (Niino et al., 2001). The small size of Canadian sample and the ethnical diversity of the Japanese sample, might explain the discrepancy with our sample.

Further we have explored PBMC-derived hsp70-hom expression in a sample of the MS and control groups, and the relationship between HSP70-hom genotypes and hsp70-hom expression. Though, the hsp70-hom expression does not correlate with HSP70-hom rs2227956 variation in both MS patients and controls, we have found a direct significant relationship between hsp70-hom expression and the degree of MS severity, measured by the MSSS. While MS patients with a mild impact disease (i.e. MSSS < 3) display low levels of hsp70-hom, the other MS patients (i.e. MSSS ≥ 3) have higher hsp70-hom expression. Weather the severity of the disease is the cause or the effect of hsp70-hom expression has still yet to be elucidated. The data on hsp70-1 and hsp70-2 (hsp70-1, -2) expression show that PBMCs produce same baseline levels of stress-inducible hsp70-1,-2 in MS patients and controls; and that hsp70-1, -2 are overproduced in MS patients in comparison to controls when under stress conditions (Cwiklinska et al., 2010). Additionally, hsp70-hom is relatively more expressed, compared to hsp70-1, -2, in response to interferon-γ (Fourie et al., 2001) studies on mRNA show enhanced levels of the mRNA for hsp70-hom and hsp70-1, -2 in response to lipopolysaccharide (LPS) (Fourie et al., 2001). Despite further experiments are needed to understand the exact mechanisms and pathways involved in hsp70-hom regulation and expression, our results are encouraging and also supported by data from the experimental model of MS that show a mild form of the disease in hsp70-deficient C57BL/6 (Mycko et al., 2008).

These results indicates that hsp70-hom plays a more relevant role in promoting a pro-inflammatory immune system activation and an effective T cell response against the myelin antigens compared with its role in protecting CNS cells from inflammatory injury; however the underlying mechanisms involved in this unfavorable outcome are not clear yet and additional studies are necessary to disclose them.

In conclusion, this work outlines the increased risk of MS in HSP70-hom rs2227956 C carriers and the enhanced expression of hsp70-hom in MS patients with a not mild form of the disease. These results document the harmful effect of the HSP70-hom gene polymorphism and its related protein. Further studies are required to clarify the exact roles of hsp70-hom and its possible applications as biomarker and/or as target therapy in MS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2016.07.011.

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FINAL

RESEARCH ARTICLE

Complement C4A and C4B Gene Copy Number Study in Alzheimer's Disease Patients

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Abstract: ***Background/Objectives:*** Increasing evidence suggests the importance of neuroinflammation in the pathogenesis of Alzheimer's disease (AD), which is a complex neurodegenerative disorder. Complement activation occurs in the brain of patients with AD and seems to contribute to an important local inflammatory state. Increased expression of the fourth serum complement component 4 (C4) has been observed in AD patients in many studies. This protein has two isoforms, encoded by two genes: C4A and C4B localized to the HLA class III region. These genes exhibit copy number variations (CNVs) and this different gene copy number can influence C4 protein levels. We focalized our attention on these two genes, determining the distribution of CNVs in AD patients, compared with healthy controls, in order to analyse their possible involvement in AD pathogenesis.

Methods: We investigated 191 AD patients and 300 healthy controls. The C4A and C4B copy numbers were assessed by quantitative PCR (qPCR).

Results: The results obtained showed a statistically significant increase in the number of copies for both C4A and C4B in AD patients, compared with healthy controls ($p<0,001$).

Conclusion: The presence of high C4A and C4B copy numbers in AD patients could explain the increased C4 protein expression observed in AD patients, thus highlighting a possible role for C4A and C4B CNVs in the risk of developing AD.

Keywords: Alzheimer's Disease, Complement system, C4A, C4B, inflammation.

INTRODUCTION

Alzheimer's Disease (AD) is a complex multifactorial and probably multigenic neurodegenerative disorder, that is clinically associated with the progressive loss of consciousness and memory [1]. This disease represents the most common cause of dementia in elderly adults and can be classified as sporadic or familial. The majority (95%) of AD cases belong to the sporadic type, which is strictly correlated with age [2]. Various factors seem to be involved in AD development, such as oxidative stress, neuroinflammation and gender. AD pathogenesis is complex and characterized by three different types of injury: injury due to extracellular deposition of beta amyloid peptide and tau protein; injuries due to loss of neurons in specific areas of the brain and, injury due to reactive processes, such as inflammation.

The pathogenic "inflammation hypothesis" which has recently emerged, is based on evidence of altered immune processes in AD patients that cannot be ignored [3]. According to this hypothesis, during the early stages of the inflammatory process, beta amyloid peptides lead to the activation of microglia within the central nervous system. This triggers increased expression of inflammatory cytokines in the brain, and results in the release of potentially neurotoxic substances, including reactive oxygen species (ROS), reactive nitrogen species (RNS) and various proteolytic enzymes which can in turn bring about degenerative changes in neurons. Activated microglia, elements of the complement cascade and pro-inflammatory cytokines are markers for the presence of inflammatory processes in senile plaques, a characteristic post-mortem feature of the disease. Recently, Xu and colleagues, in 2012, confirmed that several inflammatory mechanisms are involved in the pathogenesis of cognitive dysfunction in AD [4]. The inflammation hypothesis is also supported by epidemiological observations, which have provided evidence that anti-inflammatory approaches may be protective against AD progression [5].

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Despite numerous studies supporting the role of inflammation in AD pathogenesis, it is currently unknown whether brain inflammation in patients is the cause or only a secondary consequence of the disease. Investigation of genetic polymorphisms, that are linked to the modulation of induction and regulation of inflammatory processes, would be useful to understand AD pathogenesis and its correlation with possible risk factors. Different studies have identified genetic polymorphisms that are in part responsible for susceptibility to AD [3, 6]. The presence of alleles that increase the expression of genes encoding for pro-inflammatory mediators or that reduce the production of anti-inflammatory mediators seems to be more frequent in AD patient genomes. In the general population, where these allelic variants are quite frequent, there is a high probability that an individual will inherit at least one or more of these "risk alleles" [7].

The complement system is composed of about 50 serum and membrane proteins: these proteins can be activated following three different pathways and all lead to the osmotic lysis of pathogens [8].In degenerative disorders, a deregulation of the classical complement pathway, and in particular C4 protein, has been studied [9]. The fourth serum Complement component 4 (C4) protein is a betaglobulin which in humans exists in two isoforms: C4A and C4B. C4 genes are located in the Human Leukocyte Antigen (HLA) class III region, between HLA class II and class I. One peculiarity of C4 genes is their high degree of polymorphism: allelic variants are associated with particular HLA class III haplotypes whose frequency is significantly increased or reduced in affected individuals with respect to healthy controls [10, 11]. At the genomic level, C4 exhibits copy number (CNV) and size variations, that seem to confer different levels of immunity or different susceptibility to autoimmune diseases. From this point of view, a variable number of C4 gene copies has been observed in some autoimmune diseases, such as systemic lupus erythematosus [12] and in other types of disease [13]; in systemic erythematosus lupus a deficit in C4 protein has also been observed [10]. Recent studies also indicate a high expression of C4 protein in AD patients [14, 15], thus validating the pathogenic "inflammation hypothesis" [3].

In this study, we investigated the CNV of both C4A and C4B genes of AD patients in order to evaluate their possible role in AD pathogenesis.

MATERIAL AND METHODS

Study Samples

In this study, 191 Caucasian AD patients (71 males [37%] and 120 females [63%]) were enrolled by the 1) Neurologic Science Department, IRCCS National Neurological Institute "C. Mondino", Pavia, Italy; 2) Department of Internal Medicine and Therapeutics, Section of Geriatrics and Gerontology, IDR S. Margherita, University of Pavia, Italy; 3) "Conte Franco Cella di Rivara Foundation", Broni, Italy.

To determine the clinical presence of AD, the NINCS-ADRDA diagnostic criteria were used.

Three hundred healty Caucasian controls (135 males [45%] and 165 females [65%]), matched for sex and ethnicity, were analysed. All healthy controls were provided by the Immunogenetics Laboratory, Immunohematology and Transfusion Centre, Fondazione IRCCS, Policlinico San Matteo, Pavia, Italy. At the time of blood sampling, all of the healthy controls were assessed, to confirm the absence of any kind of disorder, physical or mental. In addition, the healthy subjects analysed in this study were periodically followed-up. If a subject in the healthy control group presented with a serious disease, such as diabetes, cardiovascular disorders, cancer, or neurological problems, he/she was removed from the analysis.

For patients with mild or moderate cognitive impairment (Mini Mental State Examination (MMSE) > 18), written informed consent was obtained from the patient and their families. For patients with severe cognitive impairment (MMSE ≤ 18), written consent was obtained only from the patient's relatives, who were informed about the study. The Ethics Committee of each institution approved the study and all of the clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

C4A and C4B CNV Analysis

Eight samples with the most common C4A and C4B CNVs were used for assessment (Table **1**). These particular CNVs were previously measured and characterized in a study by Wu and colleagues [16]. The authors used TaqI RFLP-southern blot analysis and PvuII-PshAI RFLP-Southern blot analysis. one sample was characterized during the VII Complement Genetics Workshop of Mainz [17].

Table 1. DNA Samples characterized for C4A and C4B CNV, Obtained from the 2nd International Histocompatibility Working Group (www.ihvg.org) and VII Complement Genetics Workshop.

IHW Code	Copy number C4A	Copy number C4B
9020	2	0
WT51	4	0
9005	3	2
9017	1	2
9106	2	3
9023	0	2
9016	2	4
BS*	2	1

*Sample from the VII Complement Genetics Workshop.

Whole blood was collected by venepuncture, in vacutainer tubes containing EDTA. Human genomic DNA was extracted from 200 µl of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN) following the manufacturer's protocol. The concentration and purity of the DNA were determined by spectrophotometric analysis.

To evaluate the copy number variations (CNV) of both the C4A and C4B genes, quantitative PCR (qPCR) was performed using Taqman probes. The protocol has been previously described in the study by Wu and collaborators [16].

Briefly, the primers were selected, based on published sequences, in order to ensure specific amplification and the distinction between C4A and C4B, which differ in five-base pairs on exon 26. ***C4***-specific Taqman probes (Eurofins Genomics, Ebersberg, Germany) were labelled with the fluorescent dye 6-FAM, while ***RP1***, was used as an endogenous reference in multiplex reactions and labelled with the dye VIC. Each reaction was carried out in a total volume of 20 µl, containing the forward and reverse primers for both target and control amplicons (1µM), 100 nM of the target probe and the endogenous control probe, 15 ng of test genomic DNA and 2X Master mix Probe master (Biorad). Each sample was analysed in triplicate. Real-time PCR was performed using the LightCycler 480 (Roche) with PCR cycles of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The sequences of the PCR primers and probes are reported in Table **2**.

To validate the results, a second qPCR test was performed. Copy number status was determined by TaqMan-based genomic qPCR using 2 TaqMan assays, Hs07226349_cn and Hs07226350_cn (Life Technologies), which are specifically designed for analysis of ***C4A*** and ***C4B***. For this assay a 10 µl reaction mixture, containing 10 ng of genomic DNA, 2X Master mix Probe master (Biorad, Hercules (CA), USA), ***C4A*** or ***C4B*** TaqMan probe, and ***RNaseP*** TaqMan probe, was used. Thermal cycling conditions consisted of 1 cycle of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Statistical Analysis

Categorical variables were described as counts and percentages. A multivariate logistic regression model (with and without interaction) was fitted with Alzheimer presentation as a dependent variable and the number of C4A or C4B copies as independent variables. The Odd's ratio (OR) and 95% Confidence Interval (CI) were calculated using two copies as a reference value. A model selection was based on the likelihood-ratio test. Comparison of C4Aand/or C4B copy numbers were performed with the non parametric Mann-Whitney test, a p value of <0.05 was considered significant. All analyses were performed using Stata 14.1 (StataCorp College Station, USA).

RESULTS

Determination of C4A and C4B Number Variation

In this study attention was focused on C4A and C4B copy number variations in AD patients in comparison with healthy controls. The distributions of CNV frequencies for C4A and C4B in AD patients as compared with healthy controls are listed in Table **3** and illustrated in Fig. (**1**).

C4A and C4B are encoded by different genes, thus each may exhibit gene duplication; and null genes are also possible. The number of C4A or C4B gene copies for each individual was assessed.

The analysis of C4 CNV reveled, a significant increase in copy number for C4A and C4B in AD patients with respect to healthy controls as reported in Table **3** and in Fig. (**1**).

Since the likelihood-ratio test of the comparison of the two models with and without interaction terms was not significant, the model without interaction terms was considered and the results showed that C4A and C4B were associated with AD following an independent fashion. In particular, AD patients with three copies of C4A or C4B were more prevalent, compared to healthy controls (OR= 5.784664; p< 0.001 and OR= 6.659737; p< 0.001 respectively). There were 31 AD patients with 4 C4A copies (21.7%), whereas only 18 healthy controls presented 4 C4A copies (6.3%) (OR= 7.066464; p< 0.001). No more than three C4B copies in a single subject were observed.

The median number of both C4A and C4B copies was three in AD patients and two in healthy controls (p<0.001); median total number (C4A+C4B) was five in AD and four in heathy subjects (p<0.001; Mann-Whitney test).

DISCUSSION

AD is a progressive form of dementia, associated with neuroinflammation and neurodegeneration. Recently, greater emphasis has been placed on finding unique biochemical changes in peripheral tissue, that may represent biomarkers for the early identification of this disease. Our research group is interested in the possible role of HLA class III genes in AD pathogenesis [3, 11]. Among genes localized to

Table 2. Primers and Probes Used for the Amplification of C4A, C4B and the Reference Gene RP1.

Gene	Probe	Primers	
C4A	**C4AB**	**FORWARD C4F2**	**REVERSE C4A32**
	VIC-CCAGGA GCA GGT AGG AGG CTC GC	5'-CCTTTGTGTTGAAGGTCCTGAGTT-3'	5'-TCCTGTCTAACACTGGA CAGGGGT-3'
C4B	**C4AB3**	**FORWARD C4BF**	**REVERSE C4BR2**
	VIC-AGC AGG CTG ACG GC	5'-TGCAGGAGACATCTAACTGGCTTCT-3'	5'-CATGCTCCTATGTATC ACTGGAGAGA-3'
RP1	**RP1**	**FORWARD RP1E4F**	**REVERSE RP1E4R**
	FAM-AGG GAC TCA GAAATC ACG T	5'-GACCAAATGACACAGACCTTTGG-3'	5'-GACTTTGGTTGGTTCCACAAGTC-3'

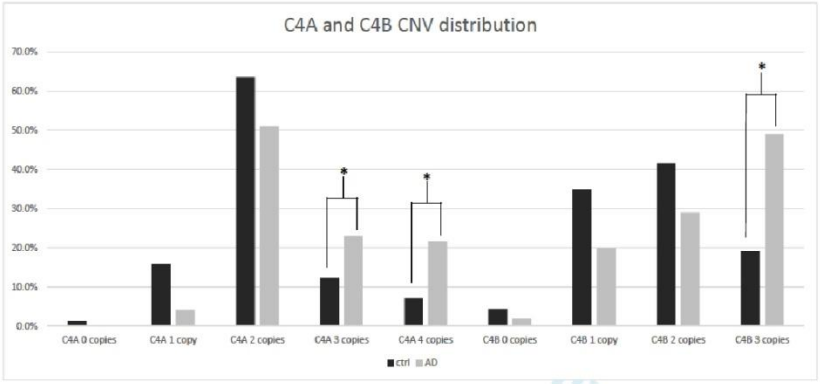

Fig. (1). Bar graph showing the frequency of C4A and C4B gene copies in AD patients (dark colum) compared to healthy controls (grey colum).* p< 0.001.

Table 3. C4A and C4B results of healthy controls and AD patients.

	HEALTHY CONTROLS (n°/%)	ALZHEIMER (n°/%)
C4A COPY NUMBER		
0	3 (1.2%)	0
1	40 (15.9%)	6 (0.7%)
2	160 (63.5%)	73 (51 %)
3	31(12.3%)	**33 (23.1%)***
4	18 (7.1%)	**31 (21.7)****
TOTAL	252	143
C4B COPY NUMBER		
0	12 (4.41%)	3 (1.9%)
1	95 (34.92%)	31 (20%)
2	113 (41.54%)	45 (29.03%)
3	52 (19.11%)	**76 (49.03%)*****
TOTAL	272	155

*OR= 5.784664; p< 0.001; C.I.= 2.610431 12.8187.
**OR= 6.659737 : p <0.001 C.I.=2.944987 15.0602.
***OR= 7.066464; p< 0.001 C.I. 3.396723 14.70091.

the class III region, C4A and C4B encode for proteins of the serum complement system. The complement system comprises more than 50 proteins that circulate in the bloodstream and become activated in response to infection [18].

There are several data in the literature suggesting a possible role for the complement system in the pathogenesis of AD, in particular regarding C4A and C4B [15, 19]. Other data have also linked neuroinflammation to psychiatric disorders: a role for C4 in schizophrenia has been established [13]. Recent proteomic studies have shown increased levels of C4 in the cerebrospinal fluid of AD patients: one of these studies reported that C4 expression was increased according to patient age and disease stage [14]. Others proteomic studies regarding C4 have been performed comparing different protein purification techniques: in a study by Bennett and collaborators, increased plasma C4 protein levels were found [15]. Furthermore, different authors have evaluated an association between C4 genes and plasma protein levels [20, 21].

In this study191 AD patients were tested. The analysis of C4 CNV reveled a significant increase in C4A and C4B copy

number in AD patients, in comparison with healthy controls. This is also consistent with increased protein in cerebrospinal fluid and elevated plasma levels of C4 protein observed in AD patients [14, 15].

Copy number variants have been predicted to have an important role in genetic susceptibility to common diseases: several associations between CNVs and the presence of inflammation associated disorders have been described [22]. The role of CNVs has been investigated in late-onset AD [23] and, more recently, a study, performed in a Caucasian population, found a novel APP (amyloid beta precursor protein) gene duplication, that requires further investigation [24]. These studies suggest the possible involvement of CNVs in the susceptibility to AD, in particular the role of C4 CNVs could be important.

Regarding C4 CNVs, an association with other autoimmune diseases has been established: low C4 copy numbers are a risk factor for systemic lupus erythematosus [25, 26] and C4B deficiency has been reported in rheumatoid arthritis [27]. Together with the report of Yasojima and colleagues [28], who showed that mRNA levels for C3 and C4 were doubled in post-mortem AD brains, our findings of a higher C4 copy number in AD patients supports the notion that there is increased production of complement in AD patients. In addition, we would like to highlight the Margery-Muir study wich showed that in healthy subjects the plasma level of C4A and C4B is genetically predetermined by the C4 gene copy number [21].

Interestingly, a study by our group showed that the presence of the C4B null allele is less frequent in healthy elderly subjects, as compared with younger individuals. A high percentage of healthy elderly show high C4B protein levels, suggesting a selection for individuals with a more efficient complement system [29]. This result is consistent with a study performed in 2003, that showed an age-associated decrease in the frequency of the C4B null allele. The null allele seems to be a negative selection factor for health and survival [30]. Furthermore, the possible involvement of linkage disequilibrium between C4 copy number variations and other HLA genes can not be excluded. However, in a previous study, only a slight correlation between C4 copy number and class I and II HLA genes was demonstrated [30]. Cleynen and collaborators in fact found a low level of positive linkage between C4 copy number and alleles of the classical HLA genes [31].

In conclusion an elevated number of C4 genes was observed in AD patients as compared with healthy controls. Considering the limitations of this study, independent confirmation of our results in a larger cohort is necessary. The differences we observed should encourage further studies on markers of complement activation in AD. Such studies could address the question of whether complement activation contributes to or is a defense response against further neurodegeneration.

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AUTHORS' CONTRIBUTIONS

Michele Zorzetto designed the study, together with Francesca Datturi. Ilaria Campo and Laura Divizia performed all of the experiments. Cristiana Pistono contributed to the draft and the editing of the manuscript. Annalisa De Silvestri analysed the data. Mariaclara Cuccia contributed to the design of the study (she is an expert on HLA class III genes). Giovanni Ricevuti is on expert on complement inflammation and Alzheimer's disease.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Review

What's new about oral treatments in Multiple Sclerosis? Immunogenetics still under question

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ABSTRACT

Multiple Sclerosis (MS) is a chronic pathology affecting the Central Nervous System characterized by inflammatory processes that lead to demyelination and neurodegeneration. In MS treatment, disease modifying therapies (DMTs) are essential to reduce disease progression by suppressing the inflammatory response responsible for promoting lesion formation. Recently, in addition to the classical injectable DMTs like Interferons and Glatiramer acetate, new orally administered drugs have been approved for MS therapy: dimethyl fumarate, teriflunomide and fingolimod. These drugs act with different mechanisms on the immune system, in order to suppress the harmful inflammatory process. An additional layer of complexity is introduced by the influence of polymorphic gene variants in the Human Leukocyte Antigen region on the risk of developing MS and its progression. To date, pharmacogenomic studies have mainly focused on the patient's response following admission of injectable drugs. Therefore, greater consideration must be made to pharmacogenomics with a view to developing more effective and personalized therapies.

This review aims to shed light on the mechanism of action of the new oral drugs dimethyl fumarate, teriflunomide and fingolimod, taking into account both the importance of immunogenetics in drug response and pharmacogenomic studies.

Contents

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1. Introduction

Multiple Sclerosis (MS) is a chronic disimmune disease of the Central Nervous System (CNS) characterized by demyelination and neurodegeneration, which affects approximatively 2.5 million people worldwide. This disease is the most common cause of permanent physical disability in young adults, with an onset between 20 and 40 years [1], displaying a greater prevalence in women (3:1) [2].

Like other chronic neurological disorders, MS represents a serious health problem. Many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), are characterized by progressive dysfunction of the nervous system, with neuroinflammation often making an important contribution to the neurodegenerative course [3]. Rather, in MS inflammation plays a central role.

MS is a complex disease and its pathogenesis involves different mechanisms including demyelination, neurodegeneration, and inflammatory phenomena, leading to the formation of plaques localized both in the white and in the grey matter of the CNS [4].

MS is not only a physically disabling disease, but it also affects the cognitive ability of the patients [5]. The symptoms are heterogeneous and depend on the location of the demyelinated area within the CNS [1,6]. The disease course is highly variable, although two main types are recognizable: relapsing-remitting MS (RRMS) and progressive MS (PMS) [7].

MS treatment consists of administrating immunomodulating agents to postpone and reduce the risk of relapses. Specifically, an acute treatment using corticosteroids is important to decrease the relapses duration, while disease modifying therapies (DMTs) are long-term treatments essential to suppress the inflammatory response that promotes lesions formation, thus reducing disease progression [8]. Typically, DMTs are used for patients with RRMS [9], where patients with a mild or moderate active disease start the therapy with a first-line DMT, whereas patients with an aggressive form of the disease start with a second-line DMT [10]. Notably, patients treated with a first-line DMT that do not show an effective response, are switched to a second-line DMT. Classical MS first-line treatments include the use of Interferons (IFNs) and Glatiramer acetate (GA), two injectable drugs that reduce the annual relapse rate by approximately 30% [11].

In recent years, the number of drugs that can be used in the treatment of MS has increased. The new orally administered drugs approved for MS treatment, dimethyl fumarate, teriflunomide (first-line treatments) and fingolimod (second-line treatment), offer new therapeutic options. In this review we will focus on the above-cited orally administered drugs and their mechanism of action, and will additionally examine their efficacy in the context of immunogenetics.

1.1. Insights on MS: from genetics to environmental factors

Several genetic and environmental factors influence the risk of developing MS. However, although the environment seems to play a role in terms of disease progression [12,13], the aetiology of this pathology is still poorly understood.

The majority of the genetic risk factors are related to the immune system, more specifically, the HLA (Human Leukocyte Antigen) region. This genomic region of about 4000 kb is located on the short arm of chromosome 6, and includes more than 200 highly polymorphic genes that are divided into three classes [14]. The HLA-DRB1*15:01 allele continues to represent the major genetic risk factor for MS, with an odds ratio (OR) of 3.08 [15]. In addition, other polymorphisms have been associated with MS, such as HLA-A*02:01, which is considered a protective allele for MS risk [16]. Moreover, our research group has recently described two polymorphisms, HSP70-2 +1267 A/G (rs1061581) and HSP70-HOM +2437 T/C (rs2227956), that are related to the risk of developing MS [17,18]. However, several environmental factors may contribute to disease susceptibility, including vitamin D deficiency, infections and smoking [12]. Interestingly, MS prevalence increases at higher latitude and its distribution seems to be influenced by sunlight exposure – solar light is the principal factor for the production of vitamin D_3. Vitamin D deficiency, due to a reduced exposure to solar light, is therefore a possible risk factor for the development of MS [19]. Vitamin D acts as an important immuno-modulating factor influencing the transcription of several immune-related genes, and thus the differentiation of immune system cells [20]. Smoking is another factor that may affect both the risk of developing MS and the disease progression [21]. In recent years, attention has been focused on the gut and its microbiota, which is important for the proper function of the immune system [22]. Different factors, such as diet, medications or stress, can alter the gut microbiota, leading to gut inflammation and alterations of intestinal immunity [23]. In particular, diet can alter the commensal gut microbiota and also affect cellular metabolism, with consequences for the immune system [24]. Within this context, an association between obesity and a higher risk of developing MS has been demonstrated [25], where the accumulation of white adipose tissue may lead to a systemic inflammation [26].

Epigenetic mechanisms also seem to play a role in MS, promoting a pro-inflammatory phenotype and influencing demyelination and remyelination processes. In this regard, different environmental agents, such as Epstein-Barr virus, smoking and diet, are able to induce epigenetic modifications, thus contributing to the development of the disease [27,28].

2. Focus on oral treatments for MS

2.1. Dimethyl fumarate

Dimethyl fumarate (DMF) is a first-line oral drug for the treatment of RRMS, approved by the FDA in 2013 [29]. Fumaric acid esters, including DMF, have been previously employed for the management of psoriasis.

After administration, DMF is metabolized at gut level to produce the active compound monomethyl fumarate (MMF), that is

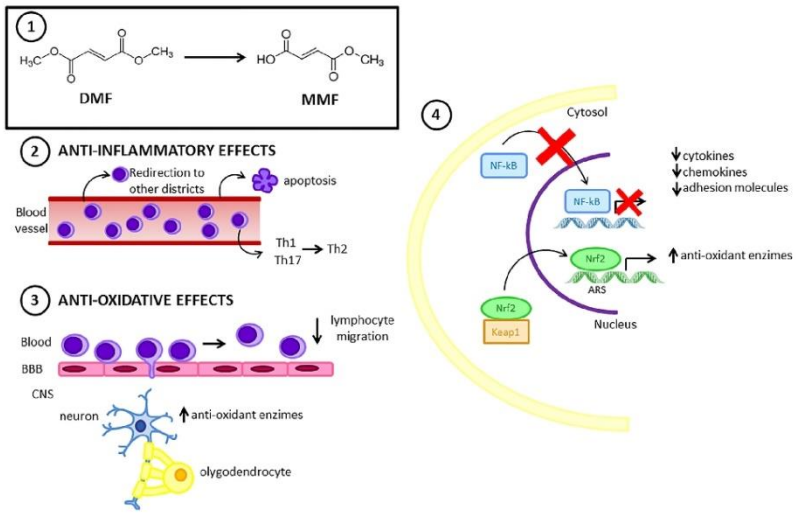

Fig. 1. ① Molecular structure of dimethyl fumarate (DMF); after administration, DMF is metabolized at gut level to produce the active compound monomethyl fumarate (MMF). ② DMF is able to reduce the number of circulating T lymphocytes both by redirecting the lymphocytes to other districts or by inducing the apoptosis of the activated T cells. Furthermore, DMF can induce a shift from Th1 and Th17 (pro-inflammatory) phenotypes to Th2 (anti-inflammatory) phenotype. ③ The reduction of the expression of adhesion molecules affects the migration of activated T lymphocytes through the blood brain barrier (BBB). ④ At cellular level, DMF is able to inhibit the translocation of NF-kB into the nucleus, thus suppressing the transcription of genes controlled by this transcription factor. The inhibition of NF-kB results in the reduction of pro-inflammatory cytokines, chemokines and adhesion molecules. DMF triggers the translocation of Nrf2 into the nucleus, thus promoting the production of anti-oxidant enzymes.

endowed with anti-inflammatory and anti-oxidative properties [30] (Fig. 1), capable of reducing MS activity and progression [29].

2.1.1. Clinical trials

The efficacy and safety of DMT was established by two-phase III studies: DEFINE and CONFIRM. DEFINE (Determination of the efficacy and safety of oral fumarate in relapsing-remitting MS), a 2-years phase III study, demonstrated positive outcomes after treatment with DMF, such as reduction of relapses, decreased disability progression and reduction in the number of lesions measured by magnetic resonance imaging (MRI) [31]. CONFIRM (Comparator and an oral fumarate in relapsing-remitting multiple sclerosis) showed positive results consistent with those obtained in the DEFINE study [32]. Furthermore, both studies demonstrated the acceptability, safety and tolerability of DMF [31,32]. However, some side effects can occur in treated patients such as flushing and gastrointestinal symptoms, in particular upper abdominal pain, diarrhoea and nausea. In some case, other adverse reactions, like lymphopenia and an increase in hepatic transaminases, can arise [33]. Additionally, progressive multifocal leucoencephalopathy (PML), a rare severe adverse reaction, has been related to DMF treatment [33,34,35].

2.1.2. Mechanisms of action

2.1.2.1. Anti-inflammatory effects. DMF acts on different cells of the immune system that have a fundamental role in MS pathogenesis. Specifically, DMF is able to decrease the number of circulating lymphocytes, in particular T cells, and to reduce their migration towards the CNS (Fig. 1). A study performed in patients with psoriasis showed a reduction in the number of circulating lymphocytes, in particular CD8 cells [36]. Two more recent studies on MS patients treated with DMF further documented the drop in the number of circulating lymphocytes [37,38]. Notably, depleting memory T cells may be vital for decreasing the immune response against myelin, thus reducing MS progression. An hypothesis to explain the reduction of circulating memory T cells is that these lymphocytes are redirected towards other locations inside the body [38]. Another mechanism may rely on T cell apoptosis, indeed, *in vitro* experiments showed that DMF is able to induce apoptosis in a subpopulation of activated T cells [39].

In addition, DMF can exert an anti-inflammatory function by altering the maturation process of T lymphocytes. Accordingly, several studies show a shift in T cell differentiation, from T helper 1 (Th1) and Th17 phenotypes to Th2 cell phenotype upon DMF exposure [40,41]. Th1 cells, together with Th17, exert a pro-inflammatory role in the pathogenesis of MS [42,43], whereas Th2 cells exhibit an anti-inflammatory function [44]. The increase in Th2 cell levels generally leads to an augmented production of IL-4, IL-5 and IL-10 and a decrease in IFN-γ [45,46].

The variation in cytokine production and in maturation of T lymphocytes might be due to the ability of DMF to inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [47,48] (Fig. 1). NF-kB transcription factor regulates the transcription of many genes essential for the immune system such as pro-inflammatory cytokines, chemokines and adhesion molecules.

Furthermore, it plays a central role in the activation, differentiation and proliferation of the immune system cells [49].

Recently, an *in vitro* study demonstrated that the release of pro-inflammatory cytokines from isolated human peripheral blood mononuclear cells (PBMCs) following stimulation with lipopolysaccharide is reduced in a dose-dependent manner in the presence of DMF. The study also showed the capability of DMF to inhibit NF-kB translocation [48]. As stated above, DMF, via NF-kB inhibition, is also able to decrease the expression of adhesion molecules [50,51], thus reducing the migration of autoreactive immune system cells through the blood brain barrier (BBB), an additional mechanism which contributes to the positive effects of this drug.

Justified text2.1.2.2 Anti-oxidative effects. DMF is also endowed with a neuroprotective effect. The main mechanism involved in neuroprotection is linked to the ability of DMF to induce the production of anti-oxidant enzymes through the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) [30,52] (Fig. 1).

Specifically, DMF acts by promoting the translocation of Nrf2 from the cytoplasm to the nucleus, thus inducing the expression of anti-oxidant enzymes [53]. Moreover, DMF modifies specific cysteine residues on the actin-associated protein kelch-like ECH-associated protein 1 (KEAP1), the protein responsible for Nrf2 ubiquitination-dependent degradation. This KEAP1 modification inhibits Nrf2 degradation, thus allowing the translocation of the transcription factor to the nucleus where it can modulate gene expression [54]. Nrf2 controls the transcription of several genes containing the antioxidant response element (ARE) that are involved in the control of the *redox status*, in anti-inflammatory pathways and detoxification pathways in different tissues and organs, including the CNS. Among them, some encode for anti-oxidant enzymes, such as superoxide dismutase, catalase, sulfaredoxin, thioredoxin, other are important for the glutathione synthesis and metabolism, like glutathione peroxidase, glutathione reductase, γ-glutamine cysteine ligase and synthase, and for iron homeostasis, such as heme-oxygenase 1 and ferritin [55]. DMF seems also to influence the *redox status* of the cells by modulating the amount of cellular glutathione (GSH) [56]. In this regard, data obtained *in vitro* on human astrocytes suggest that the treatment with DMF initially depletes GSH storage but that after 12 h of treatment there is a recovery and a subsequent increase in GSH levels [54]. This increase, after the initial depletion, may be a compensatory mechanism against GSH loss and may exert a protective role against oxidative stress [54]. Accordingly, increased GSH levels have been reported after DMF treatment in both MO3.13 human oligodendrocyte cells [57] and in neurons [30]. Taken together, these results suggest that DMF is able to both influence the Nrf2 pathway and modulate GSH levels. However, the upregulation of Nrf2 may contribute to upregulate GSH itself, since the enzymes involved in glutathione synthesis and metabolism are modulated by this transcription factor, as previously described.

Given the ability of DMF to induce the activation of Nrf2, it is important to understand the potential oncological risk associated with this drug: on one side Nrf2 protects against chemical-induced carcinogenic processes (probably because of its ability to reduce the amount of ROS and thus DNA damage), on the other, the prolonged activation of this transcription factor may favour the progression of cancer, enhancing cell proliferation and promoting chemo- and radio-resistance [58]. Nevertheless, to date there is no evidence that the use of Nrf2 activating drugs promote tumour growth in patients [58]. Conversely, it should be emphasized that multiple studies suggest that DMF may have anti-cancer properties [59], likely related to its ability to inhibit NF-kB [60]. Further research is needed to better elucidate the possible influence of DMF on tumour growth.

2.2. Teriflunomide

Teriflunomide is an oral drug approved by the FDA in 2012 for RRMS treatment. Teriflunomide is the active metabolite of leflunomide, approved by the FDA in 1998 for the treatment of rheumatoid arthritis (RA) [61].

2.2.1. Clinical trials

The efficacy and safety of terifluonomide has been verified in several clinical trials. The TEMSO (teriflunomide multiple sclerosis oral) study was the first phase III clinical trial demonstrating the effectiveness of teriflunomide. The reported effects were a reduction in relapse rate, in progression of disability and in disease activity [62]. The TOWER (teriflunomide oral in people with relapsing multiple sclerosis) trial showed a reduction in relapse rate and in the accumulation of disability [63]. The TENERE (teriflunomide versus Rebif) study, that compared teriflunomide treatment *versus* subcutaneous interferon β-1a (IFNβ-1a) treatment, showed beneficial effects of this oral drug and similar risks of treatment failure [64]. The most common adverse effects associated with teriflunomide are headache, increase in alanine aminotransferase, alopecia and gastrointestinal symptoms (diarrhea and nausea). Peripheral neuropathy, hypertension and rash are less common [33].

2.2.2. Mechanisms of action

2.2.2.1. Inhibitor of the dihydroorotate-dehydrogenase. The exact mechanism of action of teriflunomide is poorly understood, but it is known that it mainly acts as an inhibitor of the dihydroorotate-dehydrogenase (DHODH) enzyme [65,66] (Fig. 2). The mitochondrial DHODH is important for *de novo* synthesis of pyrimidines [65,67] and for cellular proliferation. The inhibition of DHODH displayed by teriflunomide has a cytostatic effect on activated T and B lymphocytes [67–69], resulting in a reduction of the inflammatory response [70]. Despite the inhibition of lymphocyte proliferation, the patients maintain a normal activation of the adaptive immune responses [71]. The block of *de novo* synthesis of pyrimidines only affects proliferating activated lymphocytes, which require a higher amount of pyrimidines for DNA synthesis, but not resting lymphocytes and homeostatically proliferating hematopoietic cells. These latter cells can obtain pyrimidines from the salvage pathway, which generates pyrimidines from nucleosides or nucleobases deriving from the endogenous recycling of nucleic acids and do not depend upon DHODH [65,72]. Furthermore, the propagation of other actively proliferating cells, such as gastrointestinal mucosal cells, is not inhibited by teriflunomide since they express lower levels of DHODH [73,74].

Notably, *de novo* synthesis of pyrimidines is not only crucial for DNA and RNA production, but it is also involved in other cellular functions, such as phospholipid synthesis, and protein/lipid glycosylation [66]. In particular, the reduction of the phospholipid pool may influence the generation of bioactive lipid messengers, such as diacylglycerol and arachidonic acid, thus affecting cellular activation, proliferation and inflammatory processes. Furthermore, it may alter protein glycosylation processes: in this way cell surface molecules are modified and cell–cell interactions are impaired, thus altering T and B cells functions [66,75].

Although teriflunomide inhibition of lymphocyte proliferation is well characterized, its effect on the microglia in the CNS is only poorly understood and has been investigated only *in vitro* [76].

2.2.2.2. Other effects. A more recent study, performed *in vitro* on human PBMCs, better elucidated the effect of teriflunomide on T and B cells proliferation and activation [77]. The work confirms that teriflunomide is able to reduce the expansion of autoreactive lymphocytes involved in the pathogenesis of MS, by inhibiting T and B cells proliferation in a dose-dependent manner; however, it

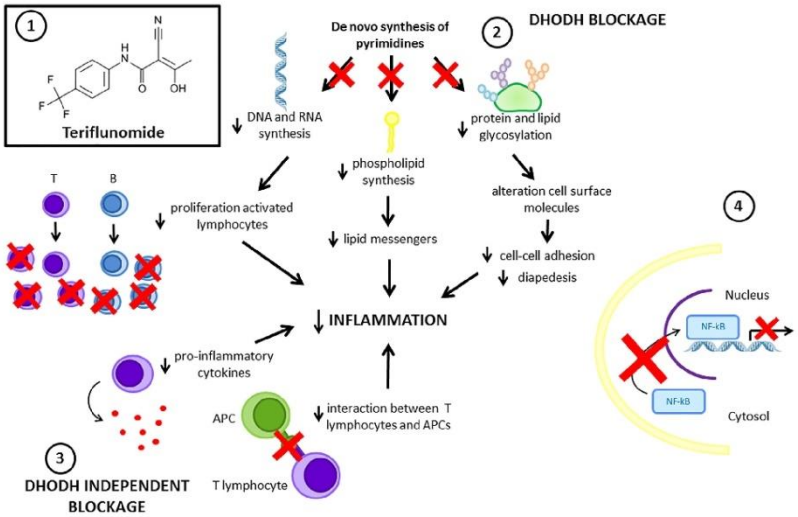

Fig. 2. ① Molecular structure of teriflunomide. ② This drug inhibits dihydroorotate-dehydrogenase (DHODH), a key enzyme for *de novo* synthesis of pyrimidines. The blockage of this pathway, involved in the synthesis of DNA and RNA, leads to the reduction in the proliferation of activated T and B lymphocytes. *De novo* synthesis of pyrimidines is also important for phospholipid synthesis and for protein/lipid glycosylation. As a consequence, the production of bioactive lipid messengers and surface molecules is altered, thus affecting T and B cell functions and subsequently inflammatory processes. ③ Teriflunomide is able to induce anti-inflammatory effects independently from its ability to inhibit DHODH. It decreases the production of pro-inflammatory cytokines and interferes with the interaction between T lymphocytes and antigen presenting cells (APCs). ④ At cellular level, teriflunomide is able to suppress the activation of NF-kB induced by TNF-α.

displays a limited impact on lymphocyte activation. Furthermore, the study highlights the ability of teriflunomide to decrease the expression, and the subsequent release, of some pro-inflammatory cytokines such as IL-6, IL-8 and monocyte chemotactic protein-1 (MCP-1) that are important in MS pathogenesis. This reduction seems to be independent from the inhibition of DHODH [77]. Interestingly, IL-6 has been shown to be essential for the development of experimental autoimmune encephalomyelitis (EAE), an animal model that reproduces the major clinical and histopathological features of MS [78,79], where it modulates the balance between pro-inflammatory Th17 cells and T regulatory (Treg) lymphocytes. The decrease of IL-6 after teriflunomide treatment has the potential to reduce Th17 cells and their negative impact on MS pathology [77]. Even the inhibition of IL-8 is crucial: this cytokine is a chemotactic factor for monocytes and neutrophils [80], and its reduction may decrease the entry of these immune cells inside the CNS. Finally, considering that MCP-1 is fundamental for monocytes and T cells migration into the CNS [81], its reduction causes a decreased infiltration of these cells.

Other studies show that teriflunomide may act not only on DHODH, but might also influence the activity of other enzymes, thus affecting different aspects of the immune response (Fig. 2). For example, *in vitro* studies suggest that this drug inhibits the activity of tyrosine-kinase, an enzyme important for triggering the cellular responses mediated by the activated T cell receptor-CD3 complex, and decreases the production of cytokines, such as IL-2 [82,83]. Other anti-inflammatory effects depend on the ability of teriflunomide to inhibit cyclo-oxygenase-2 [84] and to suppress TNF-α induced gene expression by blocking NF-kB activation [85]. An *in vitro* study suggests that teriflunomide also interferes with the interaction of T cells with antigen presenting cells (APCs) [86], a key mechanism for the activation of T cells themselves and the subsequent adaptive immune response.

2.3. Fingolimod

Fingolimod, also known as FTY720, is an oral drug employed for RRMS treatment. It was approved by the FDA in 2010 as the first oral drug employed for the treatment of MS [33].

2.3.1. Clinical trials

The efficacy and safety of fingolimod have been evaluated by two-phase III clinical trials: FREEDOMS (FTY720 research evaluating effects of daily oral therapy in multiple sclerosis) and TRANSFORMS (trial assessing injectable interferon *vs* FTY720 oral in RRMS). The 24-months FREEDOMS trial revealed a reduction in relapse rate, in disability progression, in disease activity and in brain volume loss [87]. The 12 month TRANSFORMS trial, which compared fingolimod treatment with intramuscular IFNβ-1a therapy, showed a stronger reduction in relapse rate in the patients treated with fingolimod. Furthermore, fingolimod was more effective with respect to IFNβ-1a in reducing lesion activity and brain volume loss [88]. Although the FREEDOMS extension trial concluded that the rates and the types of adverse events are similar across placebo and fingolimod groups [89], in some cases this drug can result in an increase in transaminases, infections, macular

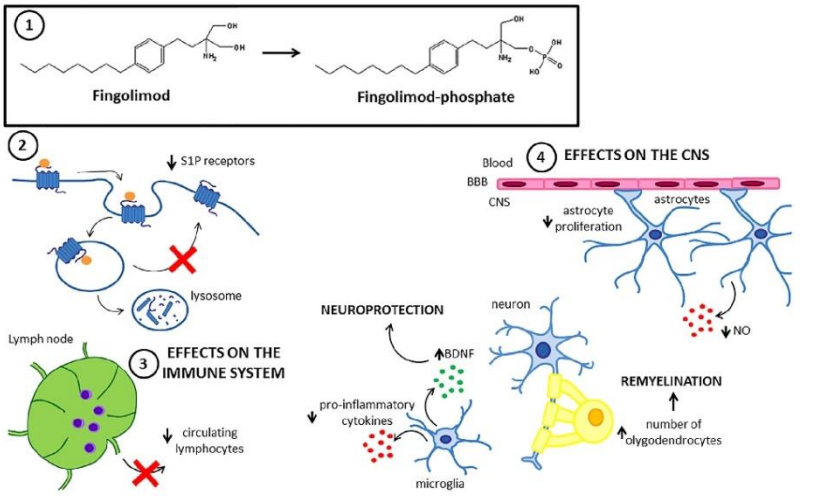

Fig. 3. ① Molecular structure of fingolimod, which is phosphorylated in fingolimod-phosphate (fingolimod-p) once it enters the cells. ② The phosphorylated form competes with sphingosine 1-phosphate (S1P) for the binding to S1P receptors, in particular $S1P_1$ receptors located on the surface of T lymphocytes. Initially, fingolimod-p induces the activation of the receptor, which is internalized and directed to lysosomes for degradation. ③ In this way, T lymphocytes express on their surface lower levels of $S1P_1$ receptors and they are unable to properly respond to S1P gradient, being sequestered inside the lymph nodes. ④ Fingolimod is able to cross the blood brain barrier (BBB), thus acting directly within the CNS. Fingolimod impairs the ability of astroglia to respond to pro-inflammatory cytokines, likely by blocking NF-kB activation and the production of NO, thus limiting neuroinflammation and neurodegeneration. At the level of microglia, fingolimod can reduce the release of pro-inflammatory cytokines and increase the production of BDNF, having a neuroprotective effect. Finally, this drug can contrast demyelination increasing the number of oligodendrocytes.

oedema, and a decrease in peripheral lymphocytes. Besides, bradycardia and atrioventricular conduction disturbances can occur following the first dose of fingolimod thus requiring pulse and blood pressure monitoring [33].

2.3.2. Mechanisms of action

Fingolimod is an immunomodulatory drug that plays a role as a structural analog of sphingosine 1-phosphate (S1P) [90], a signaling sphingolipid produced by the phosphorylation of sphingosine which is involved in anti-apoptotic, proliferative, and inflammatory cascades [91] (Fig. 3).

2.3.2.1. Sphingosine and the immune system. S1P binds to a family of five G proteins-coupled S1P receptors: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$ [92]. Notably, immune system cells express different S1P receptor types: T and B lymphocytes mainly express $S1P_1$ and $S1P_4$ receptors, while natural killer (NK) cells mainly express $S1P_5$ receptor [93]. The signaling of S1P receptors is critical for the regulation of adaptive immunity; indeed, the activation of S1P receptors expressed on the surface of T lymphocytes is essential for their maturation and passage from the lymph node to the lymphatic circulation [94,95]. Moreover, S1P is involved in regulating the migration of both B cells and cells of the innate immune system (e.g. monocytes, macrophages, NK cells and eosinophils) [94]. Beyond the immune system, S1P receptors are expressed in neurons, microglia, oligodendrocytes, astrocytes [96,97] and are present in other organs too [91].

S1P takes part in several immunoregulatory pathways: differentiation, maturation and entrance of naïve and Central Memory T cells (TCMs) from the lymph nodes into the blood and the lymphatic circulation, and their subsequent migration within the CNS. Consequently, aberrant S1P receptor signalling is a risk factor for autoimmune diseases, including MS [98].

2.3.2.2. Fingolimod as sphingosine analog: effects on the immune system. As a sphingosine analog fingolimod is a substrate for sphingosine kinase 2 (SPHK2). Phosphorylation of fingolimod by SPHK2 generates the bioactive form fingolimod-phosphate (fingolimod-p), which competes with S1P for the binding to all S1P receptors, except $S1P_2$ [99,100] (Fig. 3). In particular, fingolimod-p has been shown to selectively target naïve T cells and TCMs [101] acting as an antagonist of the $S1P_1$ receptor [91]. Following the initial receptor activation, fingolimod-p induces the physiological internalization of $S1P_1$ receptor; however, contrary to what occurs with the physiologic ligand, the receptor cannot be recycled on the cellular surface and it is degraded [102,103]. In this way, T lymphocytes express lower levels of $S1P_1$ receptor and are unable to adequately respond to S1P gradient [102]. Fingolimod is able to decrease the migration of autoreactive T lymphocytes, thus reducing their infiltration into the CNS and their negative effects on MS progression.

2.3.2.3. Effects on the CNS. It should be emphasized that the action of fingolimod is not restricted to the immune system, but acts directly on the CNS itself, where it contributes to a reduction of the neurodegenerative processes and promotes repair mechanisms

Table 1
Major effects of the three oral DMTs on immune system cells, cytokine production, blood brain barrier (BBB) and central nervous system (CNS). NA: not available, only *in vitro* studies are present [76].

	Effects			
Drug	Immune system cells	Cytokine production	Blood brain barrier	Central nervous system
Dimethyl fumarate	Reduction in the number of circulating lymphocytes, in particular T cells [37,38]. Shift from Th1 and Th17 to Th2 [40,41].	Reduction of pro-inflammatory cytokines [47,48].	Reduction of the migration of autoreactive immune system cells through the BBB [50,51].	Neuroprotective function thanks to the expression of anti-oxidant enzymes [53]
Teriflunomide	Reduction of T and B lymphocytes proliferation [65–69] and alteration of their functions [66,75,86].	Reduction of pro-inflammatory cytokines [77].	Reduction of cell–cell contact and diapedesis [75].	NA
Fingolimod	Reduction of circulating T lymphocytes [102].	Reduction of pro-inflammatory cytokines produced by microglia [114].	Direct effect at the level of the BBB, this increases the delivery of fingolimod itself into the CNS [91,108].	Neuroprotection: reduction of astrogliosis [113], decrease in NO production [113], increase in BDNF production [114] and in the number of oligodendrocytes [115].

(Fig. 3). Indeed, $S1P_1$ receptors are expressed on the surface of endothelial cells (EC), where they modulate EC cytoskeletal forces, thus increasing their barrier properties [104]. Within this context, fingolimod has been shown to be effective directly at the level of the BBB. Research carried out on rats demonstrated that this drug decreases the activity of P-glycoprotein (P-gp) by acting on $S1P_1$ signaling [105]. P-gp is an efflux transporter localized on the luminal and abluminal membranes of BBB endothelium [106], whose function is to pump exogenous substances out of cells. In particular, P-gp is crucial for the maintenance of the homeostatic environment within the CNS and it is able to protect it from potentially neurotoxic molecules [107]. The reduced activity of P-gp due to fingolimod, which is lipophilic, indirectly improves the delivery of therapeutics into the CNS [91,108].

$S1P_1$ receptor expression has been observed in astrocytes [109], and exposure to S1P increases their proliferation [110] and induces the release of trophic factors, such as fibroblast growth factor 2, glial cell-derived neurotrophic factor and nerve growth factor, promoting astrocyte growth and neuronal survival [111,112]. A recent study showed that fingolimod inhibits astrocytes proliferation, thus reducing the astrogliosis in EAE mice [113]. The authors identified NF-kB activation and nitric oxide (NO) production as specific targets of fingolimod. Accordingly, blocking the NF-kB pathway in astrocytes limits tissue damage, neuroinflammation, and neurodegeneration, while the decrease in NO production contributes to the reduction of neuroinflammatory processes [113].

S1P receptors are also expressed on microglia, oligodendrocytes and neurons [97], however, a direct neuroprotective effect mediated by fingolimod has not been reported. It is known that fingolimod can reduce the microglial production of pro-inflammatory cytokines and increase the production of brain-derived neurotrophic factor (BDNF) [114]. Several *in vitro* studies reported that fingolimod exposure seems to increase the number of progenitor and mature oligodendrocytes, protecting them from cytokine-induced cell death [115], thus likely inhibiting demyelination and promoting remyelination [115]. A recent publication showed that fingolimod might be able to reduce excitotoxic neuronal death *in vitro* [116]. In addition, other *in vitro* experiments support the hypothesis of a direct neuroprotective action of this drug: fingolimod may modulate the inflammatory phenotype of activated microglial cells, inhibiting pro-inflammatory signalling pathways, and protecting neuronal cortical cultures from NMDA-excitotoxicity [116].

2.4. Common features of the three oral drugs

All these three oral drugs act on the immune system in order to decrease the auto-inflammatory process that occurs at the level of CNS during MS pathogenesis. They decrease the number of circulating T cells, thus limiting the harmful inflammatory process mediated by these cells in the CNS.

Notably, all three drugs are able to interfere with the production of pro-inflammatory cytokines and chemokines [48,77,114]. This way, they influence different inflammatory mechanisms and contribute to the decrease of the neuroinflammatory process (see paragraphs 2.1.2, 2.2.2 and 2.3.2). A summary table describing the common features of the oral drugs has been produced (Table 1).

3. Drug responses, adverse reactions and immunogenetics

The approval of these new oral drugs will provide a substantial benefit for MS patients since the oral route of administration increases the therapeutic compliance and simultaneously broadens the panel of available drugs. When choosing a therapy, it is important to balance drug efficacy against the probability of the patient developing adverse drug reactions (ADRs). This consideration will be examined closely in the following section.

3.1. Immunogenetics and adverse drug reactions

The patients' response to therapy and the probability of developing ADRs are variable. It is well established that some of the variability in drug response, and the risk of developing a serious adverse reaction, is due to genetic variations [117] especially those at the HLA locus [118].

The presence of certain polymorphic variants of genes located within the HLA region leads to serious safety problems, including mortality [119]. A well-known example is abacavir, a nucleoside reverse-transcriptase inhibitor that is used in the treatment of HIV [118]. Different studies linked the presence of HLA-B*57:01 allele to a significant risk of developing abacavir hypersensitivity syndrome (AHS) [120,121] that, in the most severe form, can lead to hypotension and even to death [122]. Fortunately, a more sensitive test to detect the presence of HLA-B*57:01 has been developed, which reportedly enabled the reduction of AHS incidence from 5 to 8% to less than 1% [123,124].

Another example is given by carbamazepine (CBZ), an aromatic amine used as anticonvulsant for the treatment of epilepsy, trigeminal neuralgia and bipolar disorder [125–127]. Up to 10% of the

Table 2
HLA alleles related to a different response to the injectable drugs employed in MS treatment. Nabs = neutralizing anti-drug antibodies; OR = Odd Ratio.

Drug	Genes	Alleles	Response to treatment	OR	Studied populations	References
Natalizumab	HLA-DRB1	DRB1*13 DRB1*14	Higher risk of developing natalizumab-related anaphylactic/anaphylactoid reactions	8.96	French, Spanish, German	[141]
	HLA-DRB1	DRB1*15	Protective effect on the development of anaphylactic/anaphylactoid reactions	0.2		
Glatiramer acetate	HLA DRB1	DRB1*1501	Better response	–	Italian	[144]
	HLA-DRB1	DRB1*1501	Better response	–	American	[145]
	HLA-DRB1	DR15	Positive response	–	American	[146]
	HLA-DQB1	DQ6	Positive response	–		
	HLA-DRB1	DR17	No response	–		
	HLA-DQB1	DQ2	No response	–		
Interferon β	HLA-DRB1	DRB1*0401 DRB1*0408	Increased risk to produce NAbs	5.159.6	German	[151]
	HLA-DRB1	DRB1*04:01 DRB1*04:08 DRB1*16:01	Increased risk to produce NAbs	–	German	[152]
	HLA-DRB1	DRB1*15	Risk to produce biologically relevant NAb titers following intramuscular IFNβ-1a administration	4.36	Swedish	[153]
			High risk to produce NAbs titres following subcutaneous IFNβ-1a administration	4.15		
			Risk to produce biologically relevant NAb titres following subcutaneous IFNβ-1a administration	8.16		
	HLA-DQA1	DQA1*05	Decreased risk to produce biologically relevant NAb titres following subcutaneous IFNβ-1a administration	0.29		
	HLA-DRB1	DRB1*04	High risk to produce NAbs following subcutaneous IFNβ-1b administration	3.53		

patients treated with this drug can experience cutaneous ADRs [125]. In different Asian populations HLA-B*15:02 allele has been strongly associated with the development of both Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), where the presence of this allele leads to about 100-fold increased risk of developing SJS/TEN following CBZ administration [128]. Due to this strong association between HLA-B*15:02 and SJS/TEN, patients from certain areas of Asia need to be genotyped before starting the therapy with CBZ [129]. More recently, in European and Japanese populations, HLA-A*31:01 allele has been related to the onset of ADRs like maculopapular exanthema and hypersensitivity syndrome [130,131].

Another well-established example of association between ADRs and the presence of a peculiar HLA allele is given by allopurinol, a xanthine oxidase inhibitor that has been primarily used to treat gout and hyperuricemia [132,133]. Allopurinol hypersensitivity leads to a variety of cutaneous manifestations, from benign maculopapular rashes to more severe forms, including SJS/TEN [134]. A strong association between HLA-B*58:01 and SJS/TEN has been detected in different Asian populations [118] and also in Caucasian [135] patients, albeit to a lesser degree.

It should be stressed that the presence of specific HLA alleles is also linked to variable patient responses to treatment regimens [136,137].

3.2. HLA alleles and responses to injectable drugs in MS therapy

Pharmacogenomic studies regarding MS therapy focused mainly on patient's response to treatment, with particular attention paid to injectable drugs like GA, IFNβ, and natalizumab [138–141]. Several genetic polymorphisms related to the probability of developing ADRs and to the efficacy of drugs, are located in immune-related genes [142,143] in the HLA region (examples are listed in Table 2).

It has been noted that Natalizumab-related anaphylactic reactions are exacerbated in patients with DRB1*13 and DRB1*14 alleles, whereas the DRB1*15 allele has been shown to exert a protective role [141].

Regarding GA, an early study conducted by Fusco and collaborators in 2001 showed a significant correlation between DRB1*1501 and the response to GA therapy. This correlation is significant considering the rate of responders [144]; furthermore, DRB1*1501 positive patients show a greater reduction in relapse rate compared to DRB1*1501 negative subjects [144]. A more recent study confirmed the association between DRB1*1501 and a better response to GA treatment [145]. Specifically, this study suggested that only patients homozygous for HLA DRB1*1501 have a longer event-free interval when compared to other subjects treated with GA [145]. Another study investigated whether the presence of HLA-DR and HLA-DQ alleles and their haplotypes could predict the response to GA therapy [146]. The presence of DR15 and DQ6 was significantly associated with the responder *status*, whereas DR17 and DQ2 were associated with a non-responder *status*. Furthermore, the combination of DR15 and DQ6, but the absence of DR17 and DQ2, were predictive for a favourable clinical response to GA treatment. Conversely, the absence of DR15 and DQ6, but the presence of DR17 and DQ2 were predictive for a poor clinical response to the drug [146].

Studies regarding IFNβ treatment were mainly focused on the production of neutralizing anti-drug antibodies (NAbs) that are found in a subgroup of MS patients receiving this treatment [147]. At high titres, NAbs block the biological response of IFNβ [148], likely affecting its therapeutic efficacy [149]. The activation of B lymphocytes and the production of these specific antibodies depend on the help of CD4 T cells, and the development of antigen-specific CD4 T cell responses is influenced by the individual repertoire of HLA II class molecules [150]. Several studies have tried to identify a possible association between HLA polymorphic variants and the development of NAbs in patients receiving IFNβ

Table 3
Efficacy and possible side effects of injectable and oral drugs employed in MS treatment. The efficacy of the drug is assessed by clinical signs and by MRI. ARR = Annualized Relapse Rate; MRI = Magnetic Resonance Imaging; ISR = Injection Site Reaction; IPSRs = Immediate Post-Injection Systemic Reaction; PML = Progressive Multifocal Leucoencephalopathy.

Drug	Efficacy		Adverse effects
	Clinical	MRI	
Beta Interferon	32–37% reduction in ARR over 2 years [155,156,158]	50–60% decrease in new/enlarging T2 lesions and gadolinium-enhancing lesions [155,156,158,159]	Flu-like symptoms (fever, chills, headache, myalgia, arthralgia, fatigue), ISRs (bruising, pain, rarely skin necrosis and atrophy) [162,163]
Glatiramer acetate	29% reduction in ARR over 2 years [157]	11% decrease in new/enlarging T2 lesions and gadolinium-enhancing lesions [160]	IPISRs (flushing, chest tightness, dyspnea, palpitations, anxiety), ISRs (bruising, erythema, pain, pruritus, induration, rarely skin necrosis) [157,163–165]
Natalizumab	68% reduction in ARR over 1 year [161]	83% reduction in new or enlarging hyper-intense lesion on T2-weighted over 2 years. 92% reduction in lesions detected by gadolinium-enhanced MRI over 2 years [161]	Infusion-associated reactions, PML, infections [161,166,167]
Dimethyl fumarate	44–53% reduction in ARR over 2 years [31,32]	74–85% reduction in the number of new or enlarging hyper-intense lesions on T2-weighted images at 2 years. 73–90% reduction in the odds of an increase in the number of gadolinium-enhancing lesions at 2 years [31]	Flushing, gastrointestinal symptoms (upper abdominal pain, diarrhea and nausea), lymphopenia, increase in transaminases, PML [33,34,168]
Teriflunomide	31–36% reduction in ARR over 2 years [62,63]	39–67% reduction in the total lesion volume. Less gadolinium-enhancing lesions *per* T1-weighted scan [62]	Headache, increase in alanine aminotransferase, alopecia, gastrointestinal symptoms (diarrhea and nausea), peripheral neuropathy, hypertension, rash [33]
Fingolimod	48–54% reduction in ARR over 2 years [87,154]	75% reduction in MRI lesion activity [154]	Bradycardia and atrioventricular after the first dose. Increase in transaminases, macular edema, decreased peripheral lymphocyte count, infections, PML [33,169]

therapy. A study performed in 2008 demonstrated an association between HLA class II alleles and the production of NAbs [151]. In particular, patients carrying the HLA-DRB1*0401 allele showed a 5-fold increased risk to develop antibodies against IFNβ while patients with the HLA-DRB1*0408 allele had a 14-fold increased risk. Furthermore, sera from HLA-DRB1*0401- and *0408-positive patients showed, *in vitro*, a significant increase in the ability to neutralize the activity of IFNβ compared to sera from HLA-DRB1*0401- and *0408-negative patients [151]. Buck and collaborators confirmed that HLA-DRB1*04:01 and HLA-DRB1*04:08 were linked to the development of antibodies against IFNβ therapy, and that HLA-DRB1*16:01 allele is associated with the production of antibodies [152]. Recently it has been reported a panel of associations for different IFNβ molecules, specifically IFNβ-1a and IFNβ-1b [153]. In patients treated with intramuscular and subcutaneous IFNβ-1a, HLA-DRB1*15 is linked to the development of Nabs (which reach biologically relevant titers), whereas for patients receiving subcutaneous IFNβ-1b the presence of HLA-DRB1*04 was associated with development of biologically relevant titers [153]. Conversely, DQA1*05 was correlated with a decreased risk of developing biologically relevant titres in patients receiving subcutaneous IFNβ-1a.

3.3. In the "jungle" of MS treatments

Despite expanding the therapeutic choices available with these newly approved oral drugs, finding better treatments is still a challenging prospect.

Due to their ability to interfere with immunological responses, all of these drugs reduce the rate of relapses and forestall the decline in neurological functions (Table 3).

Compared to injectable drugs, the oral drugs show a greater capacity to reduce the Annualized Relapse Rate (ARR), although their MRI outcomes are similar. Among oral drugs, teriflunomide shows a modest effect on ARR [62,63] relative to DMF and fingolimod [31,32,87,154]. Teriflunomide's ARR effects are comparable to those observed with injectable drugs like IFNβ and GA [155–158]. DMF on the other hand, induces a strong reduction of the ARR [31,32], comparable to fingolimod [87,154]. Regarding MRI outcomes, the effect of teriflunomide [62] is analogous to that observed for IFNβ [155,156,158,159], but more pronounced with respect to GA [160]. DMF and fingolimod show better results in relation to MRI outcomes [31,154]; in particular, DMF treatment provides comparable results to Natalizumab treatment with respect to the number of new or enlarged hyper-intense lesions (on a T2-weighted basis), and the number of lesions detected via gadolinium-enhanced MRI [31,161]. These results suggest that, of the three oral treatments, DMF and fingolimod seem to have the most positive impact on the disease.

However, the choice of the most appropriate treatment for a patient must also take into account the ADRs. So far, all the compounds used to treat MS induce multiple side effects (Table 3). For the injectable drugs, there are ADRs created by the mode of administration itself, for example, IFNβ and GA can induce injection site reactions (ISRs) [157,162–165]. Infusion associated reactions (such as headache, flushing, dizziness and allergic dermatitis) may occur after natalizumab [161,166,167]. Certainly, while exempt from the effects described above, the oral treatments may cause other ADRs [33,34,168,169], such as PML [33,34].

Indisputably, a better understanding of all the factors associated with the risk of developing ADRs may be useful in selecting the most personalized therapeutic choice. Therefore, future studies should focus their efforts on understanding the role of immunogenetics on the probability of developing ADRs following the administration of oral drugs.

3.4. Associations studies on the response to oral treatments in MS therapy

The available data regarding the link between HLA variants and the onset of ADRs following MS therapy are limited in comparison to other drugs.

To our knowledge, no association has been established so far between genetic polymorphisms and efficacy or adverse reactions to DMF, teriflunomide and fingolimod in MS patients.

The literature suggests DMF is the most encouraging and promising drug, showing good results in terms of ARR reduction and MRI outcomes. Currently, only a few studies analysing the impact of genetic variations on the efficacy of MS oral drugs are present in the literature, mainly focusing on teriflunomide and fingolimod. Some of these studies focus on the efficacy and tolerability of leflunomide in RA [170]. This pro-drug is metabolized to the active metabolite teriflunomide. The rs3213422 single nucleotide polymorphism (SNP) in the DHODH gene may be associated with the therapeutic effects; in particular, a study reported that the frequency of remission was increased in RA patients with the C allele, compared to A allele carriers [171]. Furthermore, some SNPs haplotypes in DHODH gene may predispose to reduced leflunomide efficacy [172].

Regarding fingolimod, a recent study investigated the polymorphisms of $S1P_1$ receptor, finding three missense mutations located in the transmembrane helixes that seem to affect the receptor's function [173]. These polymorphisms alter the action of fingolimod on the receptor and can be risk factors for the development of MS. Two SNPs lead to an impaired S1P-induced endocytosis; notably, rs148977042 causes an amino acid substitution from Ile to Thr at position 45 and rs146890331 causes a replacement of Gly with Cys at position 305. SNPs that affect $S1P_1$ internalization may influence the risk of develop the disease or its severity, as shown in point mutation mutant mice. The phosphorylation of S351 is important for receptor internalization as mice with phosphorylation-defective $S1P_1$ receptor showed a severe form of EAE and enhanced Th17-mediated neuroinflammation [174]. In contrast, mutants lacking $S1P_1$ receptor on astrocytes showed attenuated EAE [175], suggesting that the type of alteration in $S1P_1$ receptor is important in influencing the progression of the disease. Furthermore, the impaired endocytosis can cause a receptor resistance to fingolimod-induced receptor degradation. The third polymorphism, rs149198314 (R120P), leads to defects in the ligand-induced receptor activation and endocytosis. This loss-of-function mutation can be a risk factor for various diseases, including those affecting the immune system [173].

Certainly, the research described above present interesting starting points for further studies to gain insight into the complexity of MS therapy and drug responses with respect to the impact of genetic variants within the population

4. Conclusions and future perspectives: implications of pharmacogenetics for prediction and personalization of MS treatments

In recent years, new oral treatments have expanded the panel of drugs available for MS therapy. Indeed, DMF, teriflunomide and fingolimod are now options as DMTs for use alongside more traditional injectable drugs, like IFN-β and GA. Obviously, the oral route of administration tends to be favoured by patients. Moreover, it should be highlighted that the availability of a wider array of drugs for MS treatment allows a more personalized therapy. When treating a patient, it is fundamental to consider the severity of the disease in order to choose the best therapy, while keeping in mind that not all patients respond in the same way to the same treatment. In this way, the patient will immediately start the most appropriate therapy for him/her-self, thus avoiding changes to the treatment regime at a later date that cost time and money. In general, this approach would represent not only an advantage for the patients, but also would positively affect the economy; indeed, choosing the right therapy would lead to a tremendous reduction of the costs with benefit for the national health systems. In this regard, a recently published paper reported the rising costs in the United States of 20 years' therapy for MS [176]. Specifically, these data show that the costs for all MS DMTs, including the more recently FDA approved oral treatments, have dramatically increased since 2002, reaching billions of dollars. For instance, between 2008 and 2012, the cost of MS DMTs doubled from 4 billion dollars to nearly 9 billion dollars annually.

Within this scenario, a key step towards a more selective therapy will be the recognition of how polymorphic variants relate to drug efficacy: knowing ahead if a patient will be able to respond favourably to a specific treatment will allow more cost-effective therapy. Furthermore, identifying the presence of variants associated with an increased risk of developing ADRs may be useful in selecting the appropriate treatment to minimize side effects and make the therapy safer, leading to further benefits for the patients.

Conflict of interest

Dr. Roberto Bergamaschi has received honoraria for scientific boards, lectures, travel and registration coverage for attending several national or international congresses or symposia, from Almirall, Bayer Schering, Biogen Idec, Genzyme, Merck Serono, Novartis, Sanofi-Aventis, Teva.

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INVOLVEMENT OF HSP70-2 PROTEIN IN OXIDATIVE STRESS: A STUDY ON MULTIPLE SCLEROSIS

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ABSTRACT

The complex scenario of Multiple Sclerosis (MS) pathology involves several mechanisms, among which oxidative stress response and Heat Shock Proteins (Hsp) are implicated. Our research has been focused on MHC class III, on gene HSPA1B encoding for the inducible Hsp70-2.
We investigated the influence of oxidative stress (hydrogen peroxide) on PBMCs from MS patients and healthy controls. PBMCs mitochondrial activity, Hsp70-2 protein expression and the production of intracellular ROS was assessed and the expression of Hsp70-2 protein was related to the HSP70-2 rs1061581 polymorphism.
Overall, our results indicate that Hsp70-2 does not seem central in the protection towards oxidative stress.