University of Sassari

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Subject areas: Microbiology and Immunology

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Exploring the role of *Mycobacterium avium* subsp. *paratuberculosis* in multiple sclerosis

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Cycle XXVIII (Years 2012-2015)

Foreword

This thesis is based on several manuscripts that were published during my PhD.

The work of present PhD thesis has been completed during my enrolment as PhD student at the Department of Biomedical Sciences, Section of Microbiology and Virology, University of Sassari, Italy, in the period from November 2012 to October 2015 under the supervision of Professor Leonardo Sechi. The studies of the thesis were also conducted for a period of one year in Japan, from October 2014 to October 2015, under the supervision of Professor Eiichi Momotani. All the subjects enrolled in the study were kindly provided by the collaboration with the Multiple Sclerosis Centre, Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, Italy.

Abbreviations

Abs: Antibodies

APC: Antigen presenting cell

BAFF: B-cell activating factor

BBB: Blood brain barrier

CD: Crohn's disease

CNS: Central nervous system

CSF: Cerebrospinal fluid

EAE: Experimental autoimmune encephalomyelitis

EBNA-1: *Epstein-barr* nuclear antigen 1

EBV: *Epstein-barr* virus

EDSS: Expanded Disability Status Scale

ELISA: Enzyme-linked immunosorbent assay

GI: Gastro Intestinal

HCs: Healthy controls

HLA: Human leukocyte antigen

HT: Hashimoto thyroiditis

IFNG: Interferon-gamma

IgG: Immunoglobulin G

IL: Interleukin

IM: Infectious mononucleosis

IRF5: Human Interferon regulatory factor 5

JD: Johne's disease

MAC: *Mycobacterium avium* complex

MALT: Mucosa associated lymphoid tissue

MAP: *Mycobacterium avium* subsp. *paratuberculosis*

MBP: Myelin basic protein

MHC I-II: Major histocompability complex I-II

MOG: Myelin oligodendrocyte glycoprotein

MS: Multiple sclerosis

NRAMP1: Natural Resistance-Associated Macrophage Protein

OR: odds ratio

PBS-T: PBS-Tween 20

ROC: Receiver operator characteristic

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PBMCs: Peripheral blood mononuclear cells

PPMS: Primary progressive multiple sclerosis

RRMS: Relapsing remitting multiple sclerosis

SPMS: Secondary progressive multiple sclerosis

T1D: Type one diabetes

TCR: T-cell receptor

Th: T-helper

TLR: Tool-like receptor

TNF: Tumor necrosis factor

Treg: Regulatory T cells

Summary

Multiple sclerosis (MS) is a complex inflammatory disease of the central nervous system (CNS). Even though rigorous research was performed in MS field, its etiology as well the exact pathogenic mechanisms remain not well understood so far. Nevertheless it is believed to be a multifactorial disease, caused by environmental factors acting on a genetic predisposition. Several studies suggest that different microorganisms could play a role in triggering autoimmunity, through immunological cross-reactivity or molecular mimicry.

Recently it was reported an association between *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and MS in Sardinia, proven both with the detection of bacterial DNA in the peripheral blood of MS patients, and with the finding of a strong humoral response against some MAP proteins which share homology with human proteins MS-related. Sardinians have a genetically isolated background characterized by a unique high-rate incidence of several autoimmune disorders such as MS. Taken together, this particular genetic makeup of Sardinians and the wide presence of a potentially infective microorganism such as MAP, could act synergistically in predisposing the population to develop MS.

We will try to understand if MAP is necessary for the development and progression of the disease, and if in case of co-infection, a synergism between MAP and others pathogens takes place.

Riassunto

Nonostante la Sclerosi Multipla (SM) sia tutt'oggi una patologia autoimmune ad eziologia sconosciuta, numerosi dati suggeriscono che sia una malattia multifattoriale causata dall'interazione di fattori ambientali e genetici. Recenti studi evidenziano che numerosi geni conferiscono suscettibilità alla SM, tra di essi i geni HLA di classe II sono responsabili di circa il 50% del rischio. I Sardi hanno un background genetico isolato caratterizzato da un'elevata incidenza di malattie autoimmuni, in particolare il diabete di tipo 1 e la SM. Questa particolare struttura genetica dei Sardi insieme all'ampia presenza nell'isola di microorganismi potenzialmente infettivi come il *Mycobacterium avium* subsp. *paratuberculosis* (MAP), potrebbe agire sinergicamente nel predisporre la popolazione a sviluppare la SM.

I nostri dati confermano tale associazione e questo potrebbe implicare che, in individui geneticamente predisposti, più di un fattore di natura ambientale possa essere necessario per scatenare la risposta autoimmune. Cercheremo di scoprire se e in che modo un patogeno come il MAP giochi un ruolo nell'eziologia della SM.

Index

Chapter 3: Identification of new antigenic MAP proteins and homologous epitopes belonging to MAP, EBV virus and human proteins MS-related

Chapter 4: MAP and Genetics

Chapter 1: General Introduction

Part of this chapter is based on the following articles:

1. Cossu D, Masala S, Sechi LA. A Sardinian map for multiple sclerosis. Future Microbiol, Vol. 8(2), pp. 223-232, February, 2013.
2. Otsubo S, Cossu D, Eda S, Otsubo Y, Sechi LA, Suzuki T, Yumiko I, Shizuo Y, Takashi K, Momotani E. Seroprevalence of IgG1 and IgG4 class antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in Japanese population. Foodborne Pathog Dis, August, 2015 *(in press).*

Multiple Sclerosis (MS)

MS is a disabling disease of the CNS (brain, spinal cord and optic nerves) that disrupts the ability of parts of the nervous system to communicate, resulting in a wide variety of symptoms. Within the CNS, the immune system attacks myelin sheet as well as the nerve fibers themselves, forming glial scars or sclerosis, which gives the diseases its name.

Epidemiology

MS is the most prevalent neurodegenerative disorder of the CNS in Europe and North America, affecting about 0.1% of the population living in high-prevalence areas [1]. MS is most frequently diagnosed in young adults, typically between 20 and 40 years of age, although individuals of any age may be diagnosed with this neurological condition. Women are affected twice as often as men. The total number of individuals suffering from MS in the world is approximately 2.5-3 million, 600,000 of whom are in Europe and 72,000 in Italy [1]. Geographically, people who live farther from the equator have a higher risk of developing MS than people living in hot areas near the equator, or in very cold areas near the north or south poles (**Figure 1**).

MS distribution in Europe follows a north-south gradient, with the notable exception of the Mediterranean island of Sardinia [2]. Sardinians have a genetically isolated background characterized by a unique high rate of incidence of several autoimmune disorders, among which MS presents one of the highest incidence rates worldwide [2].

The most common types of MS are: relapsing-remitting MS (RRMS), with about 80% of patients affected by it, characterize by defined attacked of neurologic deficits followed by partial or complete recover periods; secondary-progressive MS (SPMS), 50% of people initially affected by RRMS; primary-progressive MS (PPMS), about 10% of people with MS are diagnosed with this clinical disease course; and progressive-relapsing MS (PRMS), characterized by steadily progressing disease from the beginning without remissions.

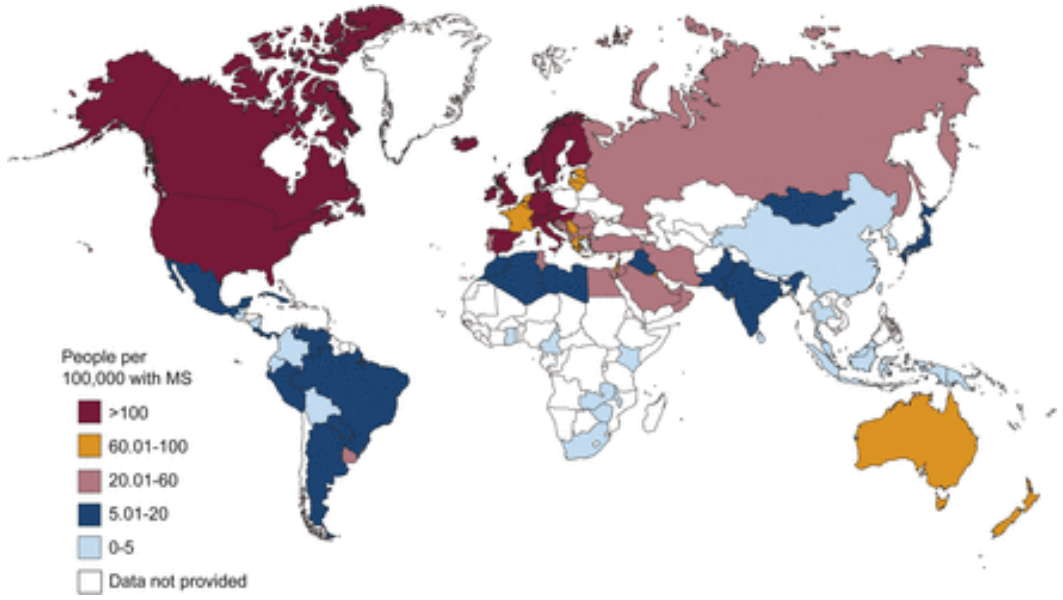


Figure 1: Global prevalence of MS © www.atlasofms.org

Pathogenesis

The hallmark of this disease is the loss of the protective myelin sheath surrounding the axons of neurons, resulting in neurological damage [3]. Inflammatory demyelination is mainly due to the infiltration of myelin-specific T-cells, which are activated from the periphery and are attracted to the blood brain barrier (BBB) by a chemokine gradient.

In the perivascular compartment, they are reactivated by local antigen presenting cells (APC) presenting their target antigens. This leads to the initiation of an immune response directed against constituents of the axonal myelin sheath and other elements of the CNS, which is accompanied by a breakdown of the BBB and recruitment of additional immune cells such as B-cells and peripheral macrophages [4].

Etiology

MS is a complex disease, the etiology of which remains elusive, but according to current data, MS is considered, in many aspects, to be the result of an interaction between genetic and environmental factors.

Genetic susceptibility is strongly correlated with the human leukocyte antigen (HLA) class II locus, in particular the alleles *DR* and *DQ* account for 10-60% of MS genetic risk [5].

More recently, genome-wide association studies have identified approximately 50 potential genes associated with MS; nonetheless, taken individually, each gene has only a weak effect [6]. Thus, it is clear that genetics exert a role in conferring MS susceptibility, and in any case, there is a growing body of epidemiological evidences supporting an equally important role for the environment as an inducing stimulus [7].

There are multiple theories regarding how infections may induce autoimmunity, and different pathogens have been reported to be linked to MS over the past four decades [8, 9]. However, confirmation of the vast majority of these findings is lacking. Among the viruses, *Epstein-Barr* virus (EBV) is the best candidate, and it has been thoroughly described elsewhere [10].

Concerning bacteria, several data support the involvement of both *Helicobacter pylori* and *Chlamydia pneumoniae* in MS [11, 12]. Recent findings propose that *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection **(Figure 2)** could act as risk factor favoring MS progression in the Sardinian population [13-15].

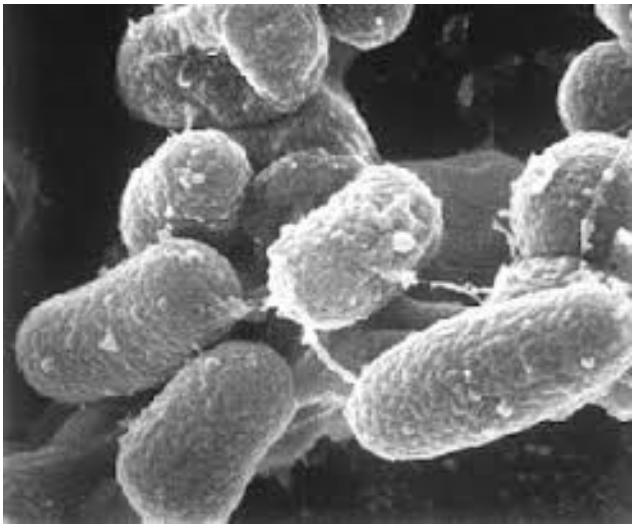


Figure 2: *Mycobacterium avium* subsp. *paratuberculosis*

***Mycobacterium avium* subsp. *paratuberculosis* (MAP)**

MAP is a member of the *M. avium* complex (MAC), it belongs to the family of *Mycobacteriaceae*, which are defined by their acid-fast properties, having a cell wall containing mycolic acids, and a high genomic C + G content [16]. *In vitro* doubling time vary between subspecies from 12 to over 20 h, with MAP being one of the slowest growers (over 48 h). MAP is an obligate pathogenic parasite of animals and protozoa; the only place it can multiply in nature is within a susceptible host, inside their macrophages [16]. When MAP leaves an animal, it can survive for extended periods in soil and water, but it is unable to multiply outside the host. MAP can survive pasteurization, as demonstrated in different studies [17], and milk, dairy products, meat and vegetables can be contaminated [18]. Moreover, MAP has been found in powdered milk for infants [19]. There is evidence that MAP can exist in vegetative, cell wall-deficient, dormant forms, and it has been shown to form spores [20]. MAP is the established causative agent of debilitating chronic granulamatous enteritis in cattle and other domestic and wild ruminants, called Johne's disease (JD) or paratuberculosis [21].

MAP is present worldwide, but epidemiological studies are lacking regarding the true MAP distribution [22]. In northern Italy, it has been reported that approximately 70% of bovine herds are infected with MAP [23] and approximately 60% of Sardinian sheep flocks are infected by this pathogen [24].

MAP and human diseases

MAP has been linked to Crohn' disease (CD) in human, an inflammatory bowel disease of still unresolved etiology [21]. The link between MAP and CD pathogenesis is based on 2 keys hypotheses which result from: MAP persistent infection or the constant exposure of MAP antigen [25]. Further, a number of studies have also linked MAP presence to other human autoimmune diseases such as type 1 diabetes (T1D), Hashimoto Thyroiditis (HT) and MS [16, 26-27]. The association between MAP and autoimmunity was explained in light of the molecular mimicry theory, and it was supported by both the presence of a strong humoral response against MAP specific antigens and the detection of MAP DNA in the peripheral blood mononuclear cells (PBMCs) of the patients [15, 26]. Therefore, it was hypothesized the existence of a cross reaction between non self-antigen and self-components, and how this mechanism potentially biased the immune system towards autoimmunity [15].

Regarding MAP and T1D, it has been shown that MAP could play a role in triggering autoimmunity towards pancreatic β-cells [24]. Indeed, anti-MAP and anti- zinc transporter 8 (ZnT8) Abs were present in T1D adults, newly diagnosed T1D children and in an Italian cohort of pediatric T1D patients [24, 26]. In addition, a significant correlation was found between anti-MAP heparin-binding hemagglutinin Ab-positivity and the presence of HLA DQA1 *0201/DQB1*0202 [26].

A link between MAP and HT has been initially postulated as the presence of viable MAP was ascertained by reverse transcription-PCR in a few Italian family members suffering from the aforementioned pathology [16]. Successfully, recent studies have also reported a significantly high positivity to ZnT8A and Abs against homologous peptides derived from MAP in HT patients [27].

Aims of the study

MS is a complex human disease of unknown etiology. Evidence for genetic contribution is not strong enough to account for MS incidence. This legitimates a deeper study of the environmental factors at play. We will attempt to shade light into the environmental pathogens possibly involved in the pathogenesis of this disease. Inasmuch as Sardinia is characterize by an isolated and homogeneous genetic background and its population show one of the highest rate of MS incidence, it is fertile material for studying this pathology.

MAP is a potential infective candidate, an obligate intracellular pathogen able to persist inside the host cells and to alter the immune system function particularly in genetically predisposed individuals. MAP is the causative agent of paratuberculosis in ruminants and it is also has been associated with several human autoimmune diseases, and more or less 60% of Sardinian flocks are infected by this pathogen.

In this thesis, we propose that MAP infection could act as risk factor in favoring MS progression in Sardinian population. The particular genetic makeup of Sardinians and the wide presence of potentially infective microorganisms such as MAP could act in predisposing the population to develop MS.

The main aims of the thesis are:

- Genetic study to evaluate the genetics factors associated to pathogen recognition predisposing to MS.
- Environmental study to further characterize infective agents involved in MS pathogenesis
- Study of the T-cell driven immune response against MAP

Hypotheses behind the study

A recent study looking at the prevalence of 12 autoimmune diseases in Sardinia showed that 5% of the general population is affected by one or more autoimmune diseases [28]. The most common disease is HT, whereas T1D and MS are respectively the forth and the fifth more prevalent diseases. The results showed that individuals affected by one autoimmune disease are most likely to develop a second autoimmune disorder, suggesting that there might be a common pathogenic mechanism behind them [28]. Sardinians are probably prone to develop MS due to their particular genetic structure. Several factors, such as malaria, have led to the fixing of a unique distribution of alleles [29]. It is also plausible that environmental factors, such as bacteria, play a major role in shaping such a unique distribution. It has been hypothesized that autoreactive T-cells activated against MAP antigens might cross-react with human myelin epitopes sharing similar amino acid sequences [16].

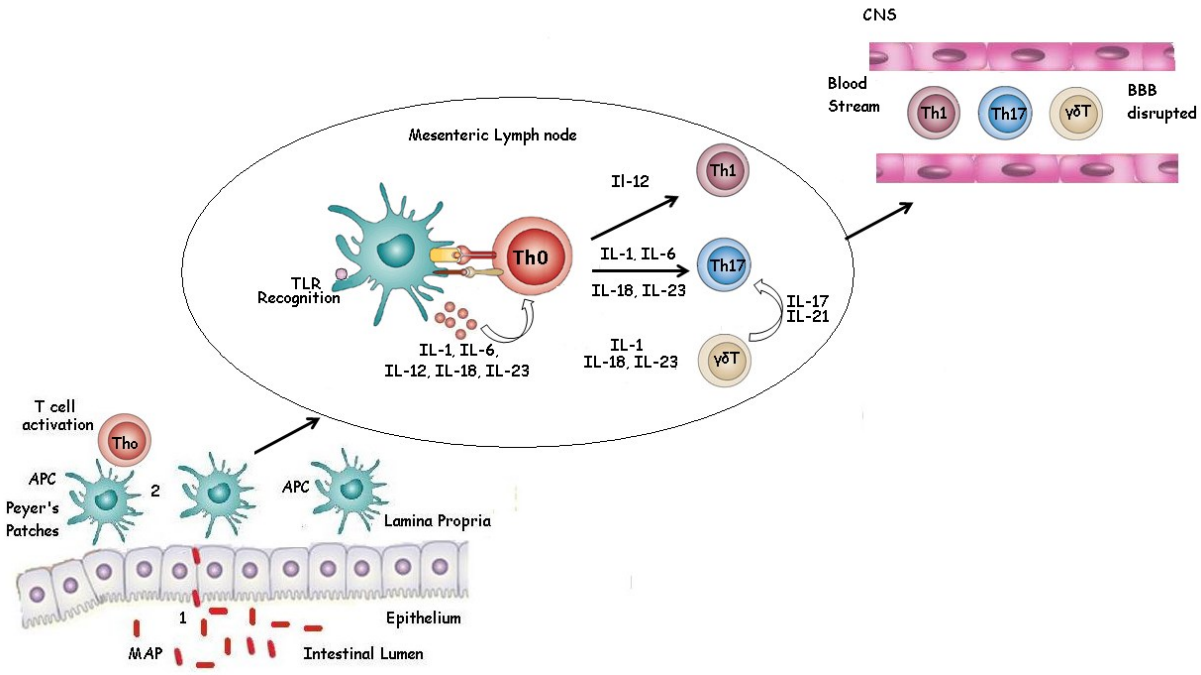
Regarding the transmission of MAP from cattle to humans, there is no doubt that milk is a vehicle for the diffusion of the bacterium through consumption of dairy products. Several study have demonstrated MAP survival after pasteurization because of its thermal-resistant characteristics [30-31]. Then, living MAP or most likely antigenic fraction of the bacilli could be acquired through milk products ingestion. So, natural infections with MAP primarily occur via the fecal-oral route. Assuming that MAP has a role in the pathogenesis of MS, then the infection has to be food-or water-borne. MAP is often acquired during childhood and its colonization's site is the GI tract. MAP resides and colonizes the Mucosa-Associated Lymphoid Tissue (MALT) of the small intestine [16, 25].

It passes the intestinal epithelium trough the M cells of Peyer's patches, after which it may be phagocytized by intra epithelial macrophages and/or dendritic cells.

The GI tract and CNS are strictly bidirectional connected through immune mechanisms [32]. Antigen-presenting cells process and expose MAP epitopes on MHC class I and II activating T-cells in the gut that may successively migrate to the periphery and the CNS. MAP persistence in the gut might cause the spreading of inflammatory mediators that, in turn are responsible for initiating inflammation in the brain. These soluble mediators orchestrate the immune response, attracting $CD4^+$ effector T-cells, accounting for inflammation, and driving the polarization of the immune response towards a Th1/Th17 phenotype. These cells may reach the CNS by the bloodstream penetrating the BBB in order to infect the CNS and create a milieu of Th1/Th17-type cytokines involved in the pathogenesis and apoptosis of neuronal cells [33]. At the same time, B-cells and macrophages also enter the CNS through the BBB, which further enhances the local immune response against myelin.

Notably, the existence of silent MS, an autoimmune process that operates in the pre-disease stage, has been proven [34]. It has been shown that during this latent phase, an abnormal immunological mechanism occurs, which in turn favors the phenomenon of epitopes spreading, which is often related to environmental and genetic factors in a time-dependent mechanism. MAP could be one environmental factor that is responsible for the activation of this apoptotic process during the early youth of the genetically predisposed subjects. Therefore, MAP persistence in the gut, together with its ability to induce the production of several inflammatory mediators, appears to be a common denominator influencing the pathophysiology of autoimmune diseases such as MS, T1D, HT and CD.

Figure 3: MAP proposed course of action. MAP colonizes the intestinal lumen and passes the epithelium trough M cells of Peyer Patches. Dendritic cells patrolling Peyer Patches sample MAP bacilli and, acting like APCs process and expose MAP epitopes (highly homologous with self-antigens) to naive T-cells. Naive T-cells (Th0) migrate to mesenteric lymph nodes, where Th0 cells differentiate into Th1-type and Th17-type cells, driven by both dendritic cells and γδ T-cell-secreted cytokine. Differentiated autoreactive T-cells exit the mesenteric lymph nodes and migrate through the blood. Once they have penetrated the BBB, they reach the CNS and attack myelin and host homologous antigens.



Cossu et al. Future Microbiology 2013

Chapter 2: MAP and MS

Part of this chapter is based on the following articles:

1. Cossu D, Cocco E, Paccagnini D, Masala S, Ahmed N, Frau J, Marrosu MG, Sechi LA. Association of *Mycobacterium avium* subsp. *paratuberculosis* with multiple sclerosis in Sardinian patients. Plos One, Vol. 6(4):e18482, April, 2011.
2. Cossu D, Masala S, Cocco E, Paccagnini D, Frau J, Marrosu MG, Sechi LA. Are *Mycobacterium avium* subsp. *paratuberculosis* and *Epstein-Barr* virus triggers of multiple sclerosis in Sardinia? Mult Scler, Vol. 18(8), pp. 1181-1184, August, 2012.
3. Frau J, Cossu D, Coghe G, Lorefice L, Fenu G, Melis M, Paccagnini D, Sardu C, Murru M, Tranquilli S, Marrosu M, Sechi L, Cocco E. *Mycobacterium avium* subsp. *paratuberculosis* and multiple sclerosis in Sardinian patients: epidemiology and clinical features. Mult Scler, 19(11), 1437-1442, February 2013.

IS900 and detection of MAP DNA

MAP DNA was detected in the PBMCs by PCR amplification of the *IS900* sequences. The *IS900* element is highly specific for the MAP organism and PCR targeting of this element is accepted to be the reference standard to discriminate MAP from other mycobacteria [35].

In a preliminary study, MAP DNA was detected in a total of 21 out of 50 (42%) MS patients, but only in 7 out of 56 (6.3%) of healthy controls (HCs) [13].

This first cohort of MS patients was chosen among patients that had been entirely drug free for at least 6 months before recruitment. This finding shaped the basis for the subsequent search for evidence linking MAP to MS.

In a second work, it was enlarged the sample size, obtaining that 27 out of 98 (27.5%) MS patients and 7 out of 110 (6.3%) HCs resulted positive for MAP DNA [14].

A recent study conducted on a larger sample size (436 patients and 264 HCs), confirmed the data previously reported, indeed MAP DNA was detected in 68 (15.5%) MS and 6 (2.3%) HCs had MAP DNA ($p = 1.14 \times 10^{-11}$) [36].

MAP possible involvement in the etiology of MS was further supported by the discovery of several MAP proteins sharing sequence homology with human proteins MS-related [13, 14]. It was then hypothesized that MAP may trigger autoimmunity trough a mechanism of molecular mimicry, leading to the activation of the relevant autoreactive T-cell clones [13, 14, 16, 36].

Chapter 3: Identification of new antigenic MAP proteins and homologous epitopes belonging to MAP, EBV virus and human proteins MS-related

Part of this chapter is based on the following articles:

1. Cossu D, Cocco E, Paccagnini D, Masala S, Ahmed N, Frau J, Marrosu MG, Sechi LA. Association of *Mycobacterium avium* subsp. *paratuberculosis* with multiple sclerosis in Sardinian patients. Plos One, Vol. 6(4):e18482, April, 2011.
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3. Frau J, Cossu D, Coghe G, Lorefice L, Fenu G, Melis M, Paccagnini D, Sardu C, Murru M, Tranquilli S, Marrosu M, Sechi L, Cocco E. *Mycobacterium avium* subsp. *paratuberculosis* and multiple sclerosis in Sardinian patients: epidemiology and clinical features. Mult Scler, 19(11), 1437-1442, February 2013.
4. Cossu D, Masala S, Frau J, Cocco E, Marrosu MG, Sechi LA. Anti-*Mycobacterium avium* subsp. *paratuberculosis* heat shock protein 70 antibodies in the sera of Sardinian patients with multiple sclerosis. J Neurol Sci, Vol. 335(1-2), pp. 131-133, September, 2013.
5. Cossu D, Masala S, Frau J, Mameli G, Marrosu MG, Cocco E, Sechi LA. Antigenic epitopes of MAP2694 homologous to T-cell receptor gamma-chain are highly recognized in multiple sclerosis Sardinian patients. Mol Immunol, Vol. 57(2), pp. 138-140, February, 2014.
6. Mameli G, Cossu D, Cocco E, Masala S, Frau J, Marrosu MG, Sechi LA. *Epstein-Barr* virus and *Mycobacterium avium* subsp. *paratuberculosis* peptides are cross recognized by anti-myelin basic protein antibodies in multiple sclerosis patients. J Neuroimmunol, Vol. 270(1-2), pp. 51-55, March, 2014.
7. Cossu D, Mameli G, Masala S, Cocco E, Frau J, Marrosu MG, Sechi LA. Evaluation of the humoral response against mycobacterial peptides, homologous to MOG_{35-55}, in multiple sclerosis patients. J Neurol Sci, Vol. 347(1-2), pp. 78-81, September, 2014.

8. Cossu D, Mameli G, Galleri G, Cocco E, Masala S, Frau J, Marrosu MG, Manetti R, Sechi LA. Human interferon regulatory factor 5 homologous epitopes of *Epstein-Barr* virus and *Mycobacterium avium* subsp. *paratuberculosi*s induce a specific humoral and cellular immune response in multiple sclerosis patients. Mult Scler, Vol. 21(8), pp. 984-995, November, 2014.

Molecular Mimicry

The association of MAP with MS is based on the concept of molecular mimicry, a mechanism by which MAP can induce autoimmunity. Structural similarity between MAP and self-component might be implicated in the mechanism of antibody (Ab) production [37].

Molecular mimicry occurs when an infectious agent, such as a bacterium, displays antigenic elements similar to host component. Due to this resemblance, the pathogen structure elicits an immune response capable of breaking the immune tolerance to the host component.

For example, T-cell receptors (TCR) specific for epitopes derived from MAP might be activated during the infection and cross-react with self-antigens, inducing autoimmune disease. Either linear amino acid sequences of the molecules or their conformational epitopes maybe shared, even though their origins are separate [15].

MAP_2694 protein and peptide library

MAP_2694 is a transmembrane protein of 351-aminoacid that shares sequence homologies with the T-cell receptor gamma-chain and the complement C1q of the host [13].

Gammadelta T-cells are a family of cells which takes part in both innate and adaptive immunity [38] playing a pathogenic role in CNS inflammation and autoimmunity.

These T-cells are known for their response to mycobacteria and their locations at mucosal sites [39]. Moreover, gamma delta T-cells have been located in the cerebrospinal fluid and in lesions of MS patients, where they seem to have a role in the regulation of autoimmune inflammation in the CNS [40]. This type of T-lymphocyte is the main producer of interleukin Th17, a pro-inflammatory autoimmune-associated cytokine [41].

Results and discussion

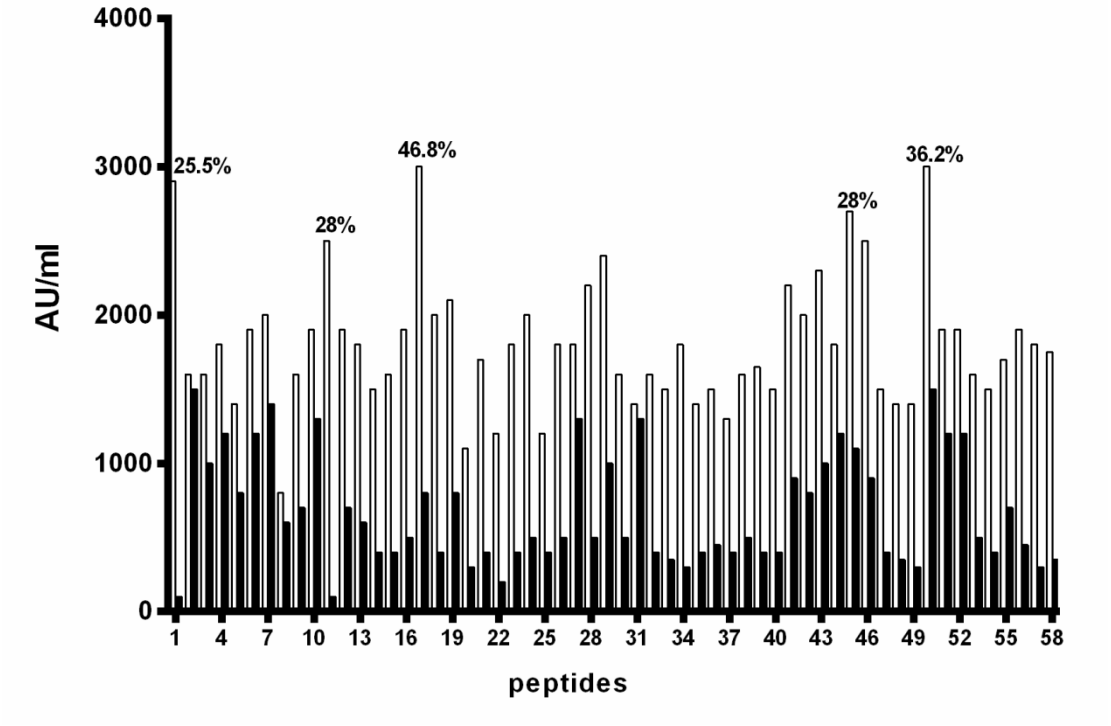
In a pilot study conducted in a Sardinian cohort of 50 MS patients and 56 HCs, this antigen gave strong ELISA values in 32% of the MS patients and only in 2% of the HCs [13]. Besides, in a different study carried out on a larger sample size, MAP_2694 positivity was detected in 33.7% of 436 MS patients and 3.8% of 264 HCs respectively [36].

In both studies it was tested the whole protein, which was fused with the maltose binding protein tag. Hence, we decided to identify the most immunogenic regions of MAP_2694 and to better understand the antigenic structure of the protein.

Firstly, it was performed a screening by ELISA to identify the most antigenic peptides and to determine the location of the relevant epitopes.

ELISA results revealed some reactive peptides (**Figure 4**). It was then decided to take into account only the peptides displaying a percentage of Ab+ higher than the one displayed by MAP_2694 protein. MAP_2694 $_{97\text{-}105}$ was recognized by 46.8% of the MS patients but only by 4.8% of HCs (AUC = 0.73, $p < 0.0001$).

Figure 4*:* ELISA-based screening of MAP_2694 peptide library.



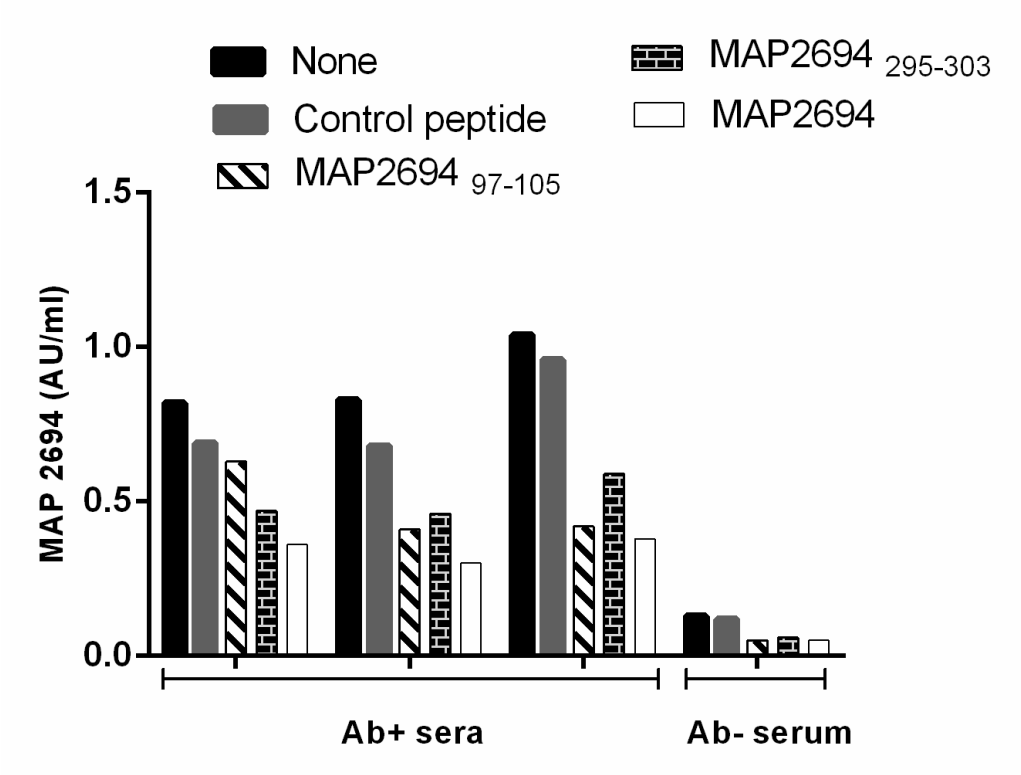
Cossu et al. Molecular Immunology 2013

The 58 overlapping 9-mer-peptides were screened against 47 MS patients and 42 HCs. The upright bars indicate the ELISA readout expressed in AU/ml. The white bars represent the mean value of MS patients, whereas the black bars stand for HCs mean value. The numbers on top of the bars represent the percent fraction of MS Ab+ sera (displayed only for the most recognized peptides). Peptides MAP_2694 $_{97\text{-}105}$ and MAP_2694 $_{295\text{-}303}$ correspond to peptides 17 e 50 respectively.

A slightly lower serum Ab reactivity was observed for MAP_2694 $_{295\text{-}303}$ peptide, as 36.2% of MS patients were Ab positive compared to 4.7% of HCs (AUC = 0.66, p = 0.0002). Interestingly, MAP_2694 $_{295\text{-}303}$ peptide covers the region of homology with the human T-cell receptor gamma chain C [13, 42].

Once identified, the inhibition activity of the positive peptides was assessed by a competitive inhibition assay. Regarding the inhibition experiments, three anti-MAP_2694 positive and one negative sera were pre-adsorbed overnight with the aforementioned reactive peptides, and then subjected to ELISA on MAP_2694-MBP coated plates. The control peptide caused only a little decline in signal whereas MAP_2694 $_{97\text{-}105}$ and MAP_2694 $_{295\text{-}303}$ inhibited the MAP_2694-MBP sero-reactivity to a similar extent (25-60% and 43-45% respectively) (**Figure 5**).

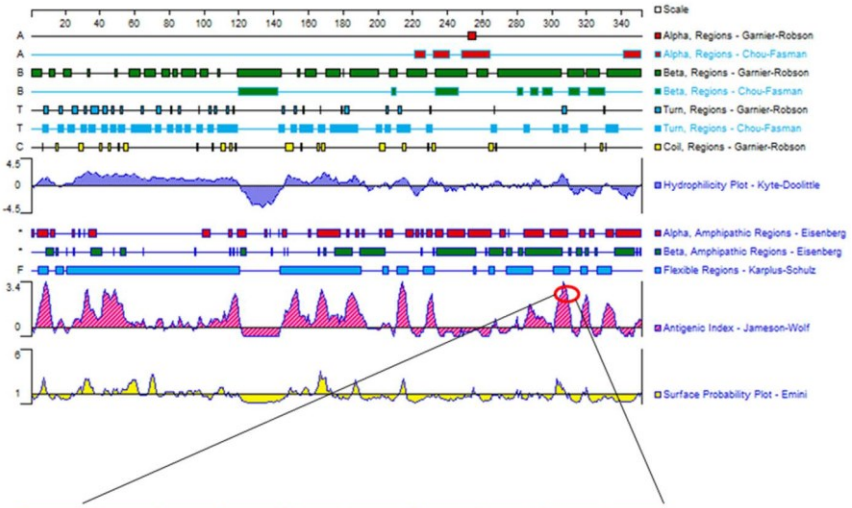
Figure 5: Competitive inhibition assay.



We demonstrated that these two peptides are potentially immunodominant epitopes of MAP_2694 protein. In fact, MAP_2694 $_{97\text{-}105}$ and MAP_2694 $_{295\text{-}303}$ bind specifically to Abs present in MS patients but not in healthy subjects, and the specificity of this binding was proven by competitive inhibiting assay.

In addition, the analyses of MAP_2694 predicted antigenic features previously performed by DNAstar package allow us to discover that MAP_2694 $_{295\text{-}303}$ fall within one of the protein region boasting one of the highest antigenic index and is likely to be exposed in the surface. Conversely, MAP_2694 $_{97\text{-}105}$ display both a very low antigenic index and a low probability to be located in the protein surface [13, 42] **(Figure 6)**.

Figure 6: Computational prediction of the MAP_2694 structure and properties such as different helices and turns, hydrophobicity, antigenicity and surface probability, etc. using Protean software from the DNAstar package (DNAstar Inc., Madison, USA).



Aa residue	Asp	Pro	Thr	Arg	Asn	Val	Lys	Gly	Asp
Aa location	301	302	303	304	305	306	307	308	309
Antigenic Index Value	1	1.34	1.98	2.32	2.46	3.4	3.06	2.27	1.68

Cossu et al. Plos One 2011

Ferredoxin MAP_FprB reductase

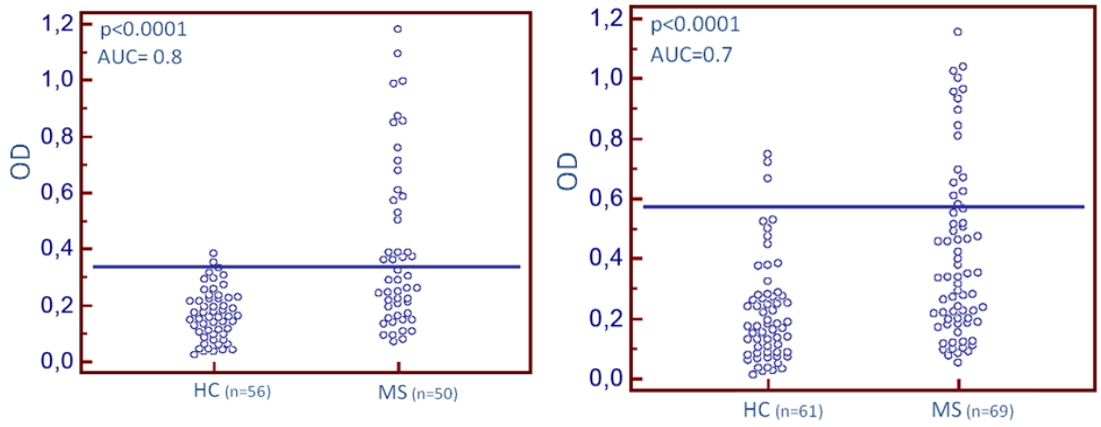
MAP_FprB is a Ferrodoxin NADP reductase characterized by the presence of a 4Fe-4S ferredoxin iron-sulfur binding domain. Comparisons between MAP proteome and human genome depicted that MAP_FprB is homologue to human myelin P zero (P0) [14]. Since myelin P0 has homophilic adhesive properties, it could have implications in host–pathogen interactions. Although the observed sequence similarities are not as high in percentage of identity, they could be relevant in the phenomena of molecular mimicry and receptor-binding that are potentially involved in neurodegeneration. P0 is the most abundant expressed peripheral nerve glycoprotein, and it has been implicated in promoting axonal regeneration in addition to its proposed structural functions in compact myelin [14].

Results and discussion

MAP_FprB Abs were searched in a total number of 119 MS patients and 117 HCs. Half of these were previously positive for MAP_2694 [13], therefore we report the results as two groups: group one (50 MS patients, 56 HCs) and group two (69 MS patients, 61 HCs). Concerning the first group, strong ELISA values were obtained in 42% of MS patients and only in 4% of HCs (chi squared = 20.754; p = 0.0001) whereas in the second group strong ELISA titers were observed in 25% of MS patients and 5% of HCs (chi squared = 8.216; p = 0.0042) (**Figure 7**).

Considering the two groups altogether the percentages reported were 31% for MS patients and 6% for the HC were positive (chi squared = 22.895; p = 0.0001;OR=7.1). In any case the association was extremely statistically significant.

Figure 7: Prevalence of anti-MAP_FprB Abs in Sardinian MS patients.



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This study was undertaken to evaluate the potential antigenic properties of a novel identified MAP_FprB protein which shares homology with host protein myelin P0. It was reported an association of MS in subjects affected with hereditary neuropathies due to mutations in the P0 gene [14]. Interestingly, P0 is homolog to myelin oligodendrocyte glycoprotein (MOG), a protein of the CNS**,** which is a minor component of myelin sheath. MOG is a CNS-specific autoantigen responsible for primary demyelination in multiple sclerosis as well as myelin associated glycoprotein (MAG), proteolipid protein (PLP) and myelin basic protein (MBP).

Heat shock proteins MAP_HSP70

Heat shock proteins (HSPs), are a group of highly conserved proteins which expression is promptly induced after a stress insult [43]. On one hand, HSPs have chaperone activity and possess anti-apoptotic properties, they promote cell survival after stressful conditions, and all these functions have beneficial effects in ameliorating neurodegenerative diseases [44]. On the other hand, HSPs are also involved in the phenomena of antigen processing and presentation by both MHC class I and MHC class II molecules [45]. Several data support the existence of a link between HSP70 over-expression and susceptibility to MS. In fact, it was reported that an up-regulation of inducible HSP70 in APCs improves the presentation of myelin autoantigens [43]. Moreover, it has been shown that HSP70 can form specific complexes with myelin proteins within the CNS in both MS and in EAE. A beneficial effects has also been postulated due to the fact that HSPs are capable of down-regulate the immune response [43]. This can be explained by the fact that HSP70 may have also a neuroprotective role in MS.

BLASTp based analysis highlighted that human HSP70 is highly homologue to the majority of mycobacterial HSP70 proteins, and specifically there is a 28% amino acid identity between human HSP70 and (MAP) dna K gene.

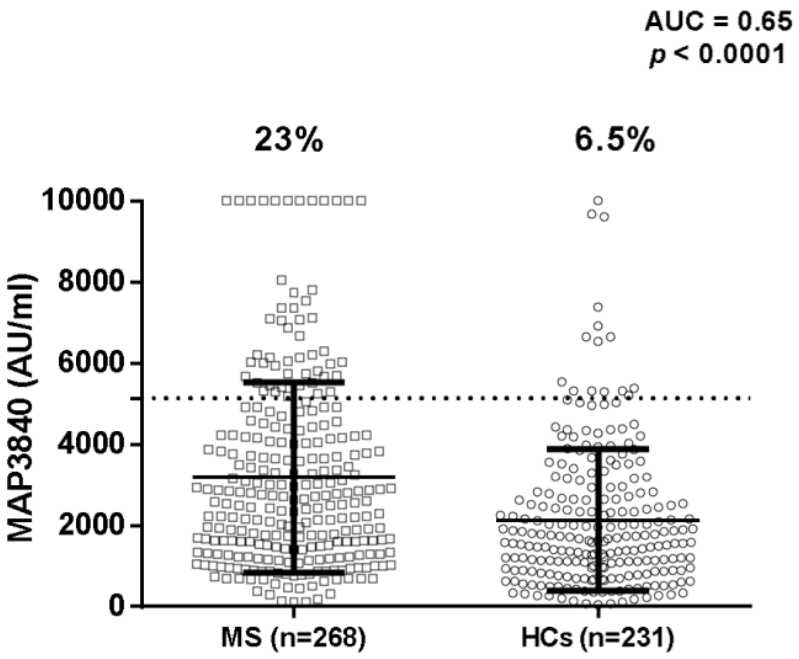
During an active immune response to infection, both the host and MAP synthesize HSPs, which being highly homologue might be cross-recognized by the immune system, leading to autoimmunity [43]. In addition it was also reported the presence of T lymphocyte-specific for sequences of both HSP65 and HSP70 shared by humans and mycobacteria [43] but it was not ascertained if the cross-recognition was due to an isolated or frequent event.

Aiming to decipher whether MAP HSP70 recombinant protein is recognize by MS patients, we searched for anti-MAP HSP70 antibodies in 268 MS patients and 231 age and sex-matched HCs.

Results and discussion

We have observed, for the first time, an increased humoral response against MAP_HSP70 in 62 out of 268 MS (23%) compared to 15 out of 231 HCs (6.5%) (AUC-ROC = 0.65, $p < 0.0001$).

Figure 8: Prevalence of anti-MAP HSP70 Abs in Sardinian MS patients and HCs.



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Not only do mycobacterial HSPs play a central role in stimulating the immune system, but they have also been found over-expressed in many autoimmune diseases [46]. HSP70 being able to bind a multitude of peptides or protein can acts as a carrier molecule of exogenous antigens. Moreover, the internalized HSP-peptide complex may lead to the processing of it making HSP epitopes available for assembly to MHC molecules I and II [47]. It was proven that when mycobacterial HSP70 carries an antigen, it could act as an adjuvant-inducing antigen specific T-cells as well as B-cells responses [48].

During an active immune response to MAP infection, the expression of both self and bacterial stress proteins sharply increase. Antibodies and T-cells generated against MAP HSP70 protein, due to the sequence homology between the bacteria and human HSP70, may target self-HSP70.

These cells may pass the BBB and in the presence of an inflammatory milieu they can cross-react with human HSP70 expressed in nervous cells causing the release of endogenous HSP70 which can perpetuate the inflammation. Moreover, classic molecules targets in MS such as PLP, MOG and MBP generated during myelin destruction may be bound by HSP70 which may act as an adjuvant and can be as well recognized by APC which in turn trigger the immune response against these self-peptides [49].

The aforementioned responses, which take place between MAP and mammalian HSP70 might be implicated in the pathogenesis of various autoimmune diseases, including T1D and MS [50-52].

EBNA1$_{400-413}$, MAP_0106c$_{121-132}$ and MBP$_{85–98}$

Amongst the environmental factors linked to MS, EBV is thought to be the best candidate. Epidemiological and immunological data showed altered immune reactivity to EBV in MS subjects [53]. In particular, elevated serum titers of anti EVB nuclear antigen-1 (EBNA) Abs have been associated with an increased risk of MS [54]. Moreover, a study conducted on a Sardinian cohort, highlighted a higher serum prevalence of EBNA1 IgG in MS patients compared to HCs [55].

MBP has been reported to be one MS related autoantigen and molecular dynamics simulation study suggested that MBP$_{85–98}$ epitope might share conformational homology with EBNA1$_{400-413}$ [56].

Blast analysis identified MAP$_{121-132}$ [PGRRPFTRKELQ] peptide belonging from MAP_0106c protein, which presents 6 conserved amino acids residues in sequence [PGRRPF], identical to EBNA1$_{400-413}$ [PGRRPFFHPVGEAD].

We searched for Abs against the homologous peptides EBNA1$_{400-413}$, MAP$_{121-132}$, and human MBP$_{85–98}$ on 47 MS patients free-therapy and 42 age and sex matched HCs. Concerning the clinical characteristic of the MS subjects, the disease course was relapsing-remitting in 90% and secondary progressive in 10%.

Results

EBV positivity was evaluated in all subjects enrolled in the study using the anti-EBV EBNA-1 IgG ELISA kit. Of the 53 MS patients and 53 HCs tested, 48 (91%) and 34 (64%) were EBV+ respectively, whereas only 5 MS patients (9%) and 19 HCs (36%) were EBV-.

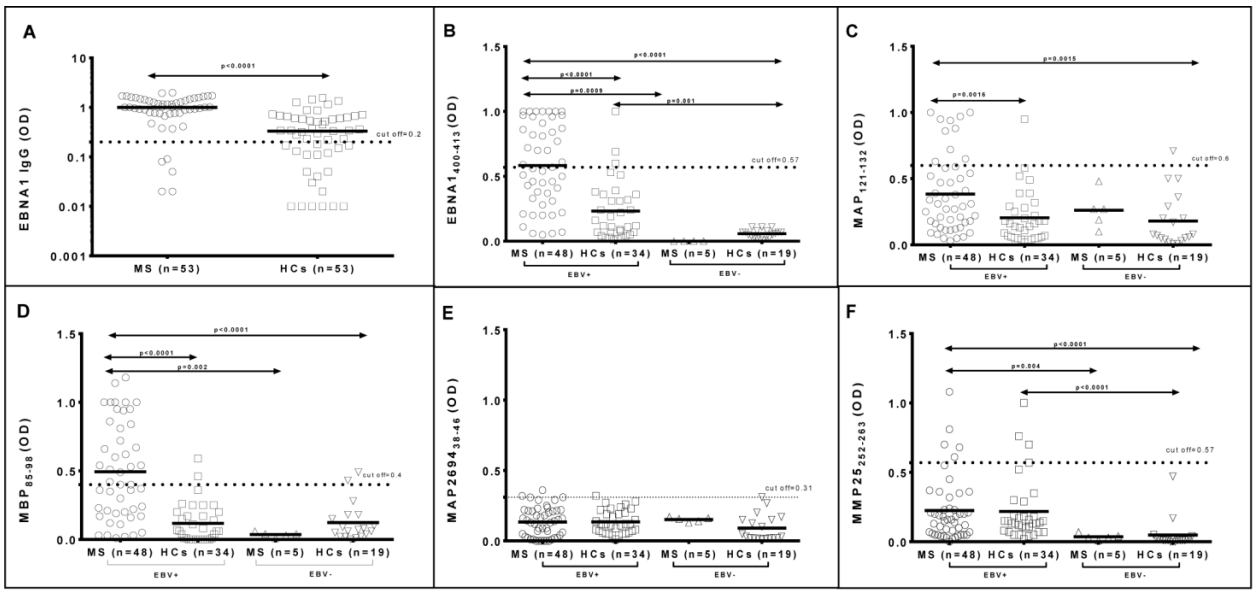
Then, we tested the humoral response against the selected peptides on all subjects, taking into account their positivity or negativity to EBV.

Among 48 patients EBV+, 22 (46%) were positive for $EBNA1_{400-413}$ whereas only 3 (9%) of the 34 HCs EBV+ were also positive for the same peptide (AUC=0.81, $p < 0.0001$). None of the remaining subjects EBV- (5 MS and 19 HCs) reacted against $EBNA1_{400-413.}$

Abs against $MAP_{121-132}$ were found in 12 out of 48 (25%) EBV+ MS patients and only in 1 out of 34 (3%) EBV+ HCs (AUC=0.70, $p = 0.0016$). Among the EBV- subjects, only 1 HC resulted Ab+ for $MAP_{121-132}$.

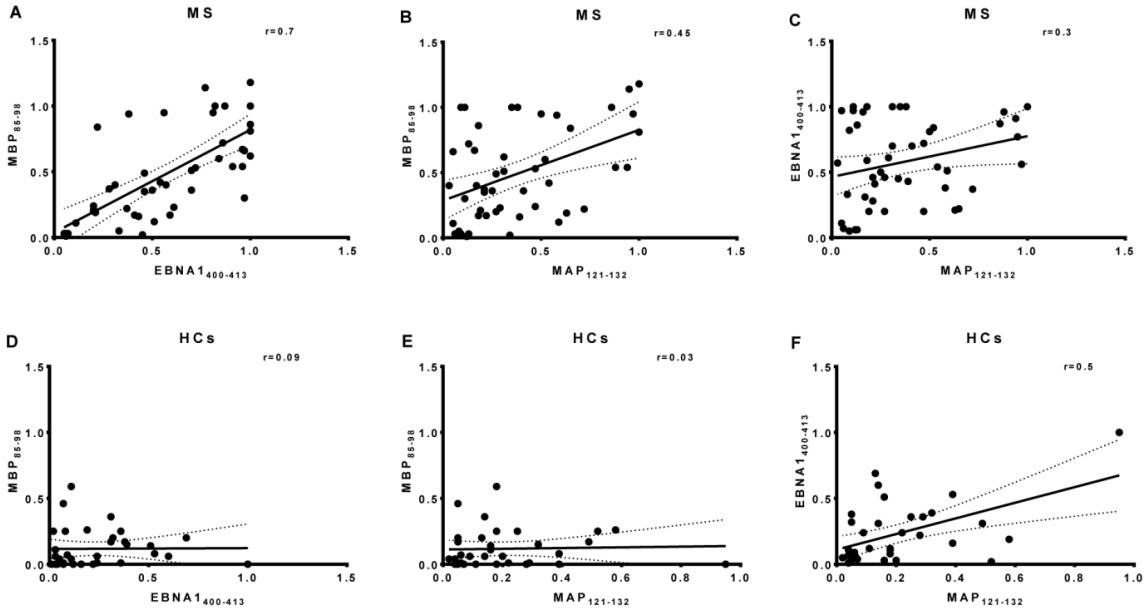
Concerning MBP_{85-98}, 23 out of 48 (48%) EBV+ MS patients were positive for this peptide, whereas only 2 out of 34 (6%) HCs EBV+ were recognized (AUC = 0.84, $p < 0.0001$). About the EBV- subjects, only 2 out of 19 (10%) HCs were positive for $MBP_{85-98.}$ Humoral response against the control peptides MAP_{38-46} and $MMP25_{252-263}$ was not statistically significant when comparing EBV+ subjects (MS versus HCs). All results are shown in **Figure 9**.

Figure 9: ELISA-based analysis.



Of interest, a correlation analysis of all EBV+ cases, showed a strong correlation between MBP_{85-98} and $EBNA1_{400-413}$ in MS (r = 0.7) but not in HCs (r = 0.09); a good correlation between MBP_{85-98} and $MAP_{121-132}$ in MS (r = 0.45) but not in HCs (r = 0.03); a weak correlation between $EBNA1_{400-413}$ and $MAP_{121-132}$ in MS (r = 0.3) but high in HCs (r = 0.5) **(Figure 10)**. These data point out that the $EBNA1_{400-413}$ and $MAP_{121-132}$ are related to MBP_{85-98}, but they act independently.

Figure 10: Correlation analysis.



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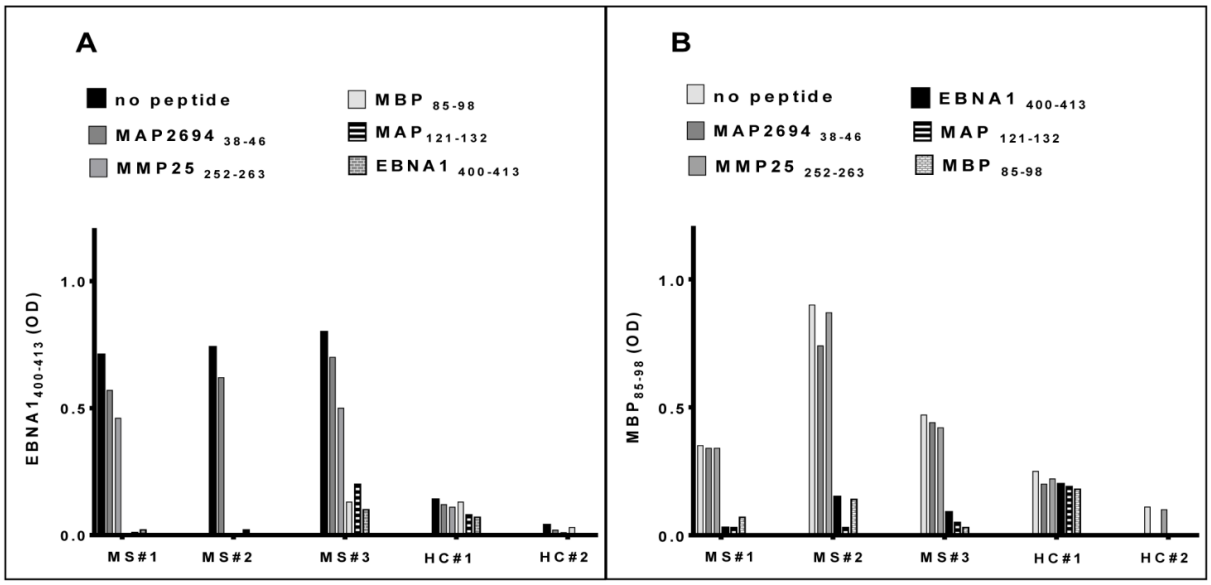
Correlation between titer Abs recognizing (A) MBP_{85-98} and $EBNA1_{400-413}$, (B) MBP_{85-98} and $MAP121-132$, (C) $EBNA1_{400-413}$ and $MAP_{121-132}$. The correlation was performed on 48 EBV+ MS subjects and 34 healthy controls EBV+ (D, E, F).

To confirm the data obtained by ELISA we developed two competition assays: One coating on the plate EBNA1$_{400-413}$ and the other coating MBP$_{85-98}$. The same patients were tested in both assays: (MS#1) a EBV+ MS patient displaying a strong reaction to EBNA1$_{400-413}$, MAP$_{121-132}$, MBP$_{85-98}$ and MMP25$_{252-263}$; (MS#2 - MS#3) 2 EBV+ MS patients showing a good reaction to MBP$_{85-98}$ and EBNA1$_{400-413}$ and a low reaction to MAP$_{121-132}$, MMP25$_{252-263}$ and MAP$_{38-46}$; (HCs #1) and (HC #2), one EBV+ and one EBV- HCs having a weak reaction to all the peptides. Concerning EBNA1$_{400-413}$-coated competition assay, in all the patients (MS#1, MS#2 and MS#3) both MBP$_{85-98}$ and MAP$_{121-132}$ efficiently inhibited Ab binding to EBNA1$_{400-413}$-coated peptide. Only in the case of MS#2, MMP25$_{252-263}$ was able to block the binding of EBNA1$_{400-413}$, probably due to the weak sequence homology.

In MBP$_{85-98}$-coated competition assay, both EBNA1$_{400-413}$ and MAP$_{121-132}$ efficiently inhibited MBP$_{85-98}$-coated binding in all the patients (MS#1, MS#2 and MS#3), while the control peptides MAP$_{38-46}$ and MMP25$_{252-263}$ did not cause any decrease in signal.

Taken together, these results demonstrate that Abs anti- EBNA1$_{400-413}$ and anti-MAP$_{121-132}$ targeting the same conformational MBP$_{85-98}$ epitope are cross-reactive, whilst both MAP$_{38-46}$ and MMP25$_{252-263}$ are not **(Figure 11).**

Figure 11: Competition assay with EBNA1$_{400-413}$ (A) and MBP$_{85-98}$ (B) coated ELISA plates.



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(A) Sera from 3 MS patients and 2 HCs were pre-incubated overnight with saturating concentrations [10µM] of MAP2694$_{38-46}$ (negative control), MBP$_{85-98,}$ MAP$_{121-132,}$ MMP25$_{252-263}$, and EBNA1$_{400-413}$ (positive control). The first bar represents a regular performed ELISA (1:100 sera in PBS-T) peptide. (B) The same sera were pre-incubated with MAP2694$_{38-46}$ (negative control), EBNA1$_{400-413}$, MAP$_{121-132,}$ MMP25$_{252-263}$, and MBP$_{85-98}$ (positive control). The first bar represents a regular performed ELISA (1:100 sera in PBS-T) peptide. Bars show means of triplicate wells and results are representative of two separately performed experiments.

Discussion

We investigated whether Sardinian population was consistent with the available epidemiological data related to EBV [53], which sees this virus spread in a uniform manner throughout the population. Not only did we confirmed the EBV distribution expected [55], but we also stratified the population according to virus positivity to have a more detailed analysis. Successively, we searched Abs against $EBNA1_{400-413}$, which is known to be an antigenic peptide relevant in MS pathology [54]. We established that $EBNA1_{400-413}$ peptide elicits a strong humoral response in MS EBV+ Sardinian patients. In addition, Abs against the homologous mycobacterial peptide $MAP_{121-132}$ were highly prevalent in MS patients but not in HCs. Thus, it is possible that MAP is able of igniting the production of autoantibodies targeting different MS correlated epitopes.

Kumar *et al.* studying MHC peptide binding groove dynamics, unveil that $EBNA1_{400-413}$ and MBP_{85-98} are both stably bound by HLA allele (DRB1*15:01) predisposing to MS, conversely the subjects carrying the protective allele (DRB1*16:01) are capable of interact stably only with MBP_{85-98} [56]. At this point, we asked whether a similar plasticity in peptide binding could be found at the more specific antibody recognition.

We demonstrated that MBP_{85-98} is strongly recognized by EBV+ MS Sardinian patients, this humoral response was somehow comparable to the one shown by $EBNA1_{400-413,}$ as demonstrated by the performed correlation analysis.

Furthermore, the statistical analysis has shown that $MAP_{121-132}$ is as well related to MBP_{85-98} ($r = 0.45$), even if at a lesser extent when compared to $EBNA1_{400-413}$ ($r = 0.7$).

After analyzing MS clinical characteristic in relation to the Ab-positivity, it was highlighted that 16 MBP_{85-98} Ab-positive MS subjects were in chronic phase of disease while only 7 in the early one, the latter were also positive for $EBNA1_{400-413}$ peptide but negative for $MAP_{121-132}$. These results seem to be in line with what reported in the EAE model, in which Abs are thought to be more important in the chronic phase of disease [57], but even if this could be also true for MS direct evidence is currently lacking.

Therefore, we set up competition assays between $EBNA1_{400-413}$ and $MBP_{85-98\ to}$ verify if they could be targeted by the same Abs in Sardinian MS patients. Our results highlighted that autoantibodies recognizing MBP_{85-98} are able to cross-react with $EBNA1_{400-413,}$ possibly by a molecular mimicry mechanism [57]. Because of the aforementioned cross-reactivity and given that MBP_{85-98} and $EBNA1_{400-413}$ do not share linear sequence homology, it is legitimate to speculate that these two peptides may share conformational homology.

Probably due to the high sequence homology, $MAP_{121-132}$ is capable of blocking the antibody binding to $EBNA1_{400-413.}$ Not surprisingly, $MAP_{121-132}$ was as well able to block the binding to $MBP_{85-98,}$ even if slightly less than $EBNA1_{400-413}$.

$MOG_{35–55}$, $MAP\_2619c_{352-361}$ and BCG_3329c_{64-74}

It was demonstrated that systemic infection with Bacille Calmette-Guerin (BCG) could suppress autoimmune responses in EAE, the animal model of MS [58]. It was showed that BCG induces apoptosis, which deletes $CD4^+$ T cells in the periphery and reduces autoreactive T cells in the CNS [59].

EAE can be induced by MOG-specific encephalitogenic T-cells. In this model, mice are immunized with $MOG_{35–55}$ peptide in complete Freund's adjuvant containing killed *Mycobacterium tuberculosis,* to develop inflammation and axon damage [60]. MOG, is one of the major antigen recognized by T-cells and a target of demyelinating autoantibodies [61], especially $MOG_{35–55}$ is an encephalitogenic peptide capable of inducing strong T and B-cell responses in several variants of EAE [60].

After performing an *in silico* analyses, two mycobacterial epitopes sharing linear homology with $MOG_{35–55}$ have been identified: $MAP\_2619c_{352-361}$ identical to $BCG\_1224_{355-364}$ and BCG_3329c_{64-74}, deriving from MAP and BCG, respectively.

We decided to explore the relationship between MS disease and the presence of anti-$MOG_{35–55}$ Abs in MS sera; moreover, we aimed to establish whether these mycobacterial homologues epitopes are capable of eliciting a strong humoral response in MS patients and, eventually, if they are able to be cross recognized by anti $MOG_{35–55}$ Abs.

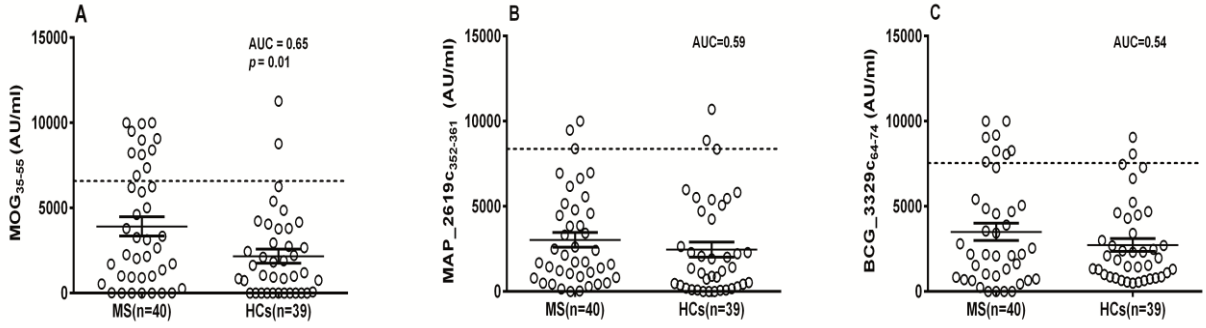
In order to achieve this goal, a peptide-based indirect ELISA was developed aiming at detecting Abs against human $MOG_{35–55}$, $MAP\_2619c_{352-361}$/$BCG\_1224_{355-364}$ and BCG_3329c_{64-74} epitopes in the peripheral blood of 40 MS patients and 39 healthy volunteers.

Results and discussion

Among the 40 MS patients, $MOG_{35–55}$, MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$., Abs were detected in the sera of 11 (27.5%), 3 (7.5%) and 8 (20%) patients, respectively (**Figure 12**). None of the HCs had high-titer Abs for these peptides (ranging from 5% to 8%). Compared to HCs Ab-positive subjects (5%), only the percent fraction of anti-$MOG_{35–55}$ Ab-positive MS subjects was statistically significant higher (p = 0.01). In contrast, the prevalence of MAP_2619c$_{352-361}$/BCG_1224$_{355-364}$ and BCG_3329c$_{64-74}$ Ab-seropositivity was found to be not statistically significant when comparing MS patients with HCs (p = 1; p = 0.2).

Concerning the clinical features, 10 out of 11 $MOG_{35–55}$ Ab-positive sera belonged to relapsing remitting MS patients, while only one positive serum belonged to a secondary progressive MS subject. In addition, the prevalence of $MOG_{35–55}$ Ab-seropositivity was found to be higher during acute phase than during remission periods.

Figure 12: Peptide-based indirect ELISA.



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We concluded that the mycobacterial epitopes homolog to $MOG_{35–55}$ are not recognized within MS patients, and therefore Abs against these epitopes are not MS correlated because there is no significant association between anti-MAP_2619c$_{352-361}$ and anti-BCG_3329c$_{64-74}$ Ab titers in MS subjects compared to HCs.

Taken together the results obtained let us conclude that sharing of highly conserved linear amino acidic sequences is relevant but not enough to elicit antibody-mediated cross-reactivity.

In order to clarify the pathogenic roles of autoantibodies in MS, future studies need to be performed with an assay system that reflects the *in vivo* environment.

If these mycobacteria are involved in MS disease, and are acting through a molecular mimicry mechanism as previously reported [14, 15, 42, 43], most likely the bacterial antigens involved are different, and they may generate Abs capable of cross-reacting with different self-antigens [57] or even with other MOG derived epitopes.

IRF5$_{424-434}$, BOLF1$_{305-320}$ and MAP_4027$_{18-32}$

We explored the humoral response against a putatively relevant EBV epitope deriving from EBV tegument protein (BOLF1$_{305-320}$) and two peptides homologous to BOLF1$_{305-320}$: MAP_4027$_{18-32}$ and Human Interferon regulatory factor 5 (IRF5)$_{424-434}$.

Noteworthy, EBV BOLF1 is one of the lytic antigens produced during EBV primary infection, asymptomatic or infectious mononucleosis (IM) [62]. It was demonstrated that Abs against BOLF1$_{305-320}$ epitope cross-react with the homologous self-epitope belonging to human transaldolase, one of the candidate auto-antigen in MS [63]. It is important to investigate EBV lytic antigens, as it was observed that having a previous history of IM increases dramatically the risk of developing MS [64, 65]. The immune reactivity towards EBV in MS subjects suggests that IM could contribute to MS through multiple mechanisms, including molecular mimicry [66]. Regarding MAP_4027$_{18-32}$, no information is available except that obtained *in silico*. It is a membrane protein and indeed one potential trans-membrane segment, spanning from residue 21 to 43. Therefore, it is a protein exposed and may continuously stimulate the host immune system in MAP infected individuals due to the persistent nature of the infection [16]. IRF5 is expressed in microglia after peripheral nerve injury, and it was recently demonstrated that IRF8–IRF5–P2X4R axis in microglia results in inducing IRF5-mediated P2X4R expression [67]. IRF5 is also involved in the polarization of M1 macrophages, which exhibit progressive expressions of inflammatory molecules, such as pro-inflammatory cytokines [68].

Molecular mimicry between immunodominant epitopes deriving from bacterial and viral persistent antigens may be a decisive factor in directing autoimmunity to self-antigens in MS patients. Therefore, it was important to explore if BOLF1 and the other homologous MAP antigens are able to induce a significant humoral and cell-mediated response in MS individuals compared to HCs, to assess if the same T-cell clones are capable of recognizing these peptides.

Results

Peptides' analyses

BLAST analysis identified three putatively mimicry peptides ($BOLF1_{305\text{-}320}$ $MAP\_4027_{18\text{-}32,}$ $IRF5_{424\text{-}434}$) originating from EBV, MAP and human proteins. $BOLF1_{305\text{-}320}$ is a 17-mer with 11 amino acids overlapping with $MAP\_4027_{18\text{-}32,}$ while $BOLF1_{305\text{-}320}$ and $IRF5_{424\text{-}434}$ boast 11 conserved amino acids even if gaps are present. The alignment of the three peptides revealed that they share 9 identical amino acids.

Concerning the nature of antigenic determinants recognized by Abs, both linear and conformational epitopes are capable of acting as antigen-binding molecule, for this reason the three peptides under scrutiny are capable of eliciting the secretion of cross-reactive Abs probably via molecular mimicry.

MHC class I molecules bind short peptides of 9 residues, whose N- and C-terminal ends are anchored into the pockets located at the ends of the peptide binding groove.

Longer peptides such as MAP_4027$_{18-32}$, BOLF1$_{305-320}$ and IRF5$_{424-434}$ can be accommodated by the bulging of their central portion, resulting in binding peptides of length 8 to 15 residues [69]. Conversely, the optimal length for peptides binding to class II proteins span from 12 to 16 residues.

T-cell receptors (TCR) are capable of binding linear determinants, and only 2 or 3 amino acids mediate peptide MHC binding. Moreover, many class I molecules have a hydrophobic pocket that recognizes one hydrophobic aminoacid, in MAP_4027$_{18-32}$, BOLF1$_{305-320}$ and IRF5$_{424-434}$ peptides the interaction might be probably due to the two leucine (L) residues at the C-terminal end of the peptides.

ELISA

EBV positivity in all subjects was evaluated by commercially available anti-EBV EBNA-1 IgG ELISA kit. Of the 47 MS patients, 40 (85%) were EBV+ and 7 (15%) EBV-, while concerning the 53 HCs, 40 (75%) were EBV+ whereas 13 (25%) were EBV-.

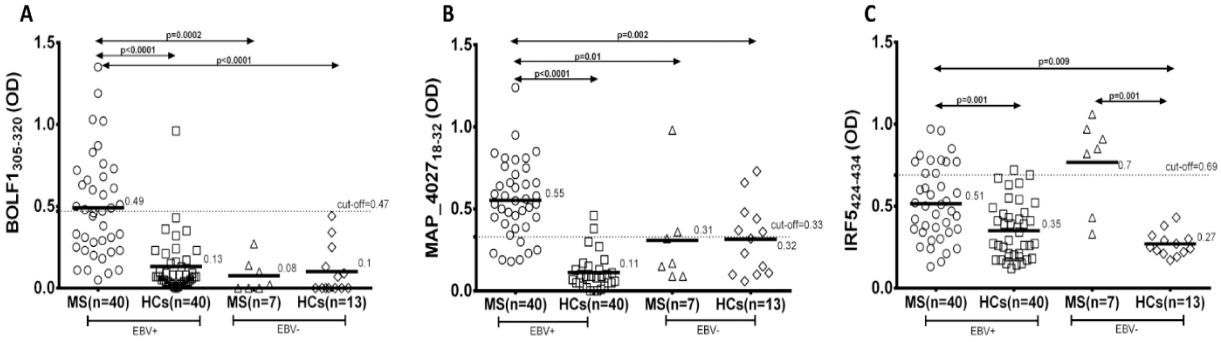
Abs against BOLF1$_{305-320}$ were found in 20 out of 40 (50%) MS EBV+ patients and only in 1 out 40 (2.5%) EBV+ HC (AUC=0.9, p >0.0001); none of the 7 MS and 13 HCs EBV- reacted against BOLF1$_{305-320}$ **(Figure 13A)**.

Regarding MAP_4027$_{18-32}$, 32 out of 40 (82.5%) EBV+ MS patients and 2 out of 40 (5%) EBV+ HCs were positive for this peptide (AUC=0.9, p >0.0001) whereas 2 out of 7 (28.5%) EBV- MS patients and 6 out 13 (46%) EBV- HCs reacted against MAP_4027$_{18-32}$ **(Figure 13B).**

Abs against IRF5$_{424-434}$ were found in 11 out of 40 (27.5%) EBV+ MS patients and in 2 out of 40 (5%) EBV+ HCs (AUC=0.7, p = 0.001).

Amongst the EBV- subjects, we observed the following situation: 5 out of 7 (71%) MS patients and none of the 13 HCs were Abs+ for IRF5$_{424-434}$ (p = 0.001) **(Figure 13C)**.

Figure 13: Ab titers against BOLF1$_{305-320}$, MAP_4027$_{18-32}$ and IRF5$_{424-434}$ measured by indirect ELISA.



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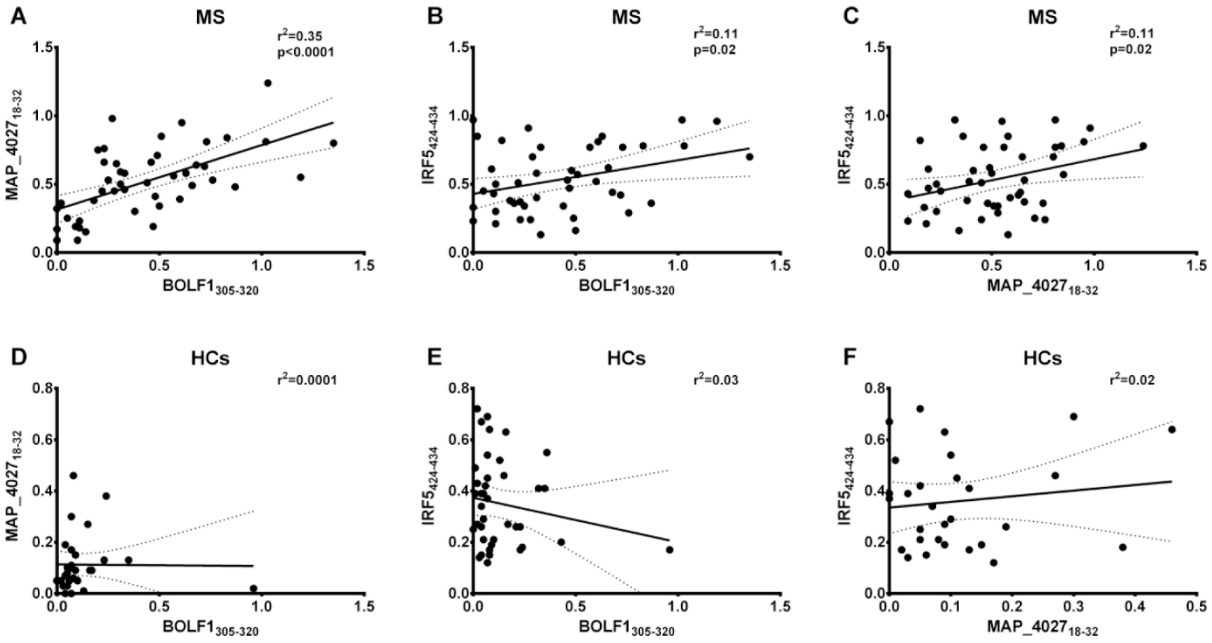
The horizontal black bars represent the mean value, while p values are indicated by two headed arrows drown on the top of each distribution. Cut-off values for positivity, calculated by ROC analysis, are indicated by dashed lines.

Finally, a correlation analysis showed a moderate degree of correlation between BOLF1$_{305-320}$ and MAP_4027$_{18-32}$ in EBV+ MS patients (r^2 = 0.35, p <0.0001) **(Figure 14A)**; a weak correlation between BOLF1$_{305-320}$/ MAP_4027$_{18-32}$ and IRF5$_{424-434}$ in EBV+ MS patients (r^2 = 0.11, p=0.02) **(Figure 14B-C)**. As regards EBV+ HCs, no correlation was found **(Figure 14D-E-F)**.

Figure 14: Correlations between BOLF1$_{305-320}$, MAP_4027$_{18-32}$ and IRF5$_{424-434}$ Ab titers.



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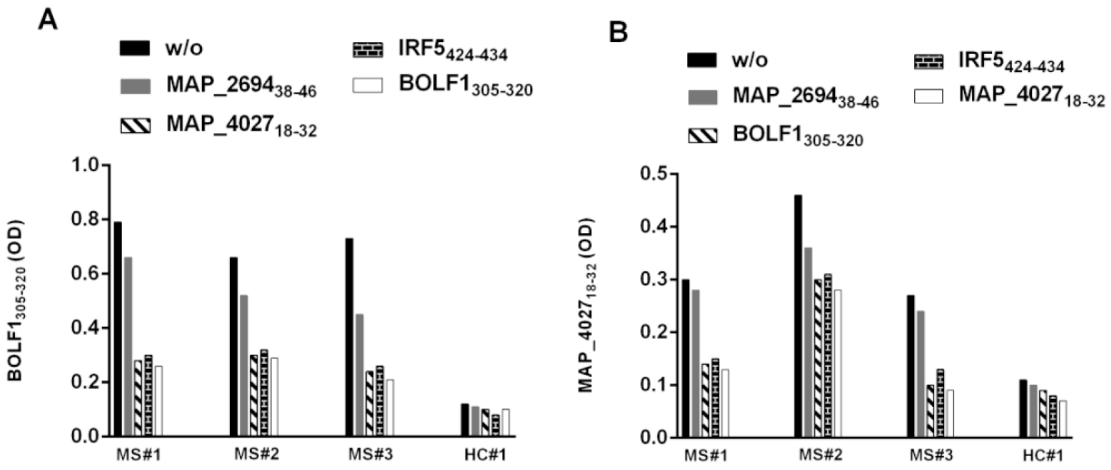
Competitive inhibition assays

Three MS patients (MS#1, MS#2 and MS#3) and 1 HC were screened by two different competitive inhibition assays. Regarding BOLF1$_{305-320}$-coated competition assay **(Figure 15A)**, in all the patients both MAP_4027$_{18-32}$ and IRF5$_{424-434}$ efficiently inhibited antibody binding to the BOLF1$_{305-320}$ -coated peptide. The same result was obtained in MAP_4027$_{18-32}$ -coated competition assay, where both BOLF1$_{305-320}$ and IRF5$_{424-434}$ efficiently inhibited the binding of antibodies to MAP_4027$_{18-32}$ -coated on the plate in all the MS patients **(Figure 15B).** In all experiments was added a control peptide (MAP_2694$_{38–46}$) but it did not cause any decrease in signal.

Figure 15: Competitive inhibition assays.



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Sera from 3 MS (MS#1-3) patients and 1 HC#1 were subjects to ELISA on plates coated with $BOLF1_{305-320}$ (A) and MAP_{18-32}, (B), respectively. The same sera were pre-incubated overnight with saturating equimolar concentrations of peptides ($BOLF1_{305-320,}$ MAP_4027_{18-32} and $IRF5_{424-434}$) and a sero-negative peptide ($MAP\_2694_{38–46}$). The first bar (w/o) represents a regularly performed ELISA (at 1/100 serum dilution).

Extracellular cytokines expression levels

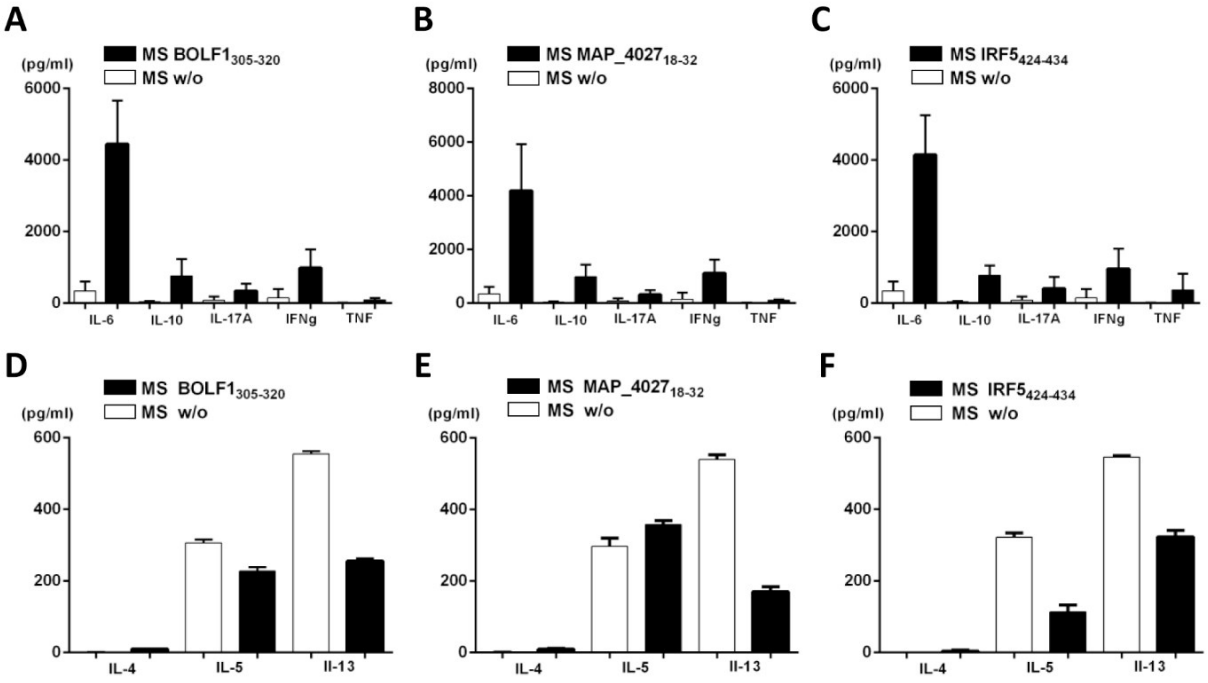
After stimulation of MS patients PBMCs (MS#1-4) with the identified peptides ($BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$) we observed production of IL-6, IL-10, IL-17A, IFN-γ, and TNF-α secretion **(Figure 16A-B-C)**, whereas the secretion of IL-4, IL-5, IL-13 in response to stimulation with all peptides was generally weak for all subjects **(Figure 16D-E-F)**.

Background values of cytokine levels (no peptide) expressed as mean ± standard deviation (SD) were the following: IL-6 (343±263.5 pg/ml), IL-10 (38.6±19.6 pg/ml), IL-17A (79.3±102.4 pg/ml), IFN-γ (149.3±249.1 pg/ml) and TNF-α (5.6±8.9 pg/ml).

The levels of cytokine expression observed after stimulation with each peptide ($BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$), followed a very similar trend. For instance, the values related to $BOLF1_{305-320}$ are the following: IL-6 (4446.66±1220.33 pg/ml), IL-10 (763.3±475.3 pg/ml), IL-17A (340.6±206.7 pg/ml), IFN-γ (989.3±516.5 pg/ml) and TNF-α (94±55.6 pg/ml) **(Figure 16A)**. Cytokine secretion in response to stimulation with peptides was defined as positive, when the level of each cytokine was more than ten times greater than those obtained as a background level, and/or the *p* value calculated by t test was <0.05. Compared to background level, a 13-fold increase ($p = 0.0006$) was observed for IL-6, a 20-fold increase ($p = 0.0226$) for IL-10, an 18-fold increase ($p = 0.0196$) for TNF-α, and a 6 fold increase ($p = 0.0262$) for IFN-γ. While concerning IL-17A, only a 4 fold increase was observed in cytokine production, therefore the result was not statistically significant ($p = 0.0636$). Thus, $BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$ peptides are capable of inducing the secretion of multiple MS-related cytokines.

Concerning the stimulation of HCs PBMCs (HCs#1-2) with $BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$, the values of IL-6, IL-10, IL-17A, IFN-γ, TNF-α, IL-4, IL-5 and IL-13 secretion was generally weak for all subjects. The tendency to induce expression of Th1 citokynes was the same for both HCs.

Figure 16: Bar graphs depicting secreted cytokine levels measured by a Multi-Analyte ELISArray kit.



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PBMCs from 4 MS patients were stimulated with 25µM of single peptides ($BOLF1_{305-320}$, MAP_4027_{18-32} and $IRF5_{424-434}$) for 48h, the experiment was carried out in duplicate**.** IL-6, IL-10, IL-17A, IFN-γ, TNF-α **(**A, B, C) and IL-4, IL-5 and IL-13 (D, E, F) levels was measured out after peptide-stimulation in MS#1-4. White bars represent the controls without peptide stimulation.

Flow cytometric quantitation of peptide-specific circulating T lymphocytes

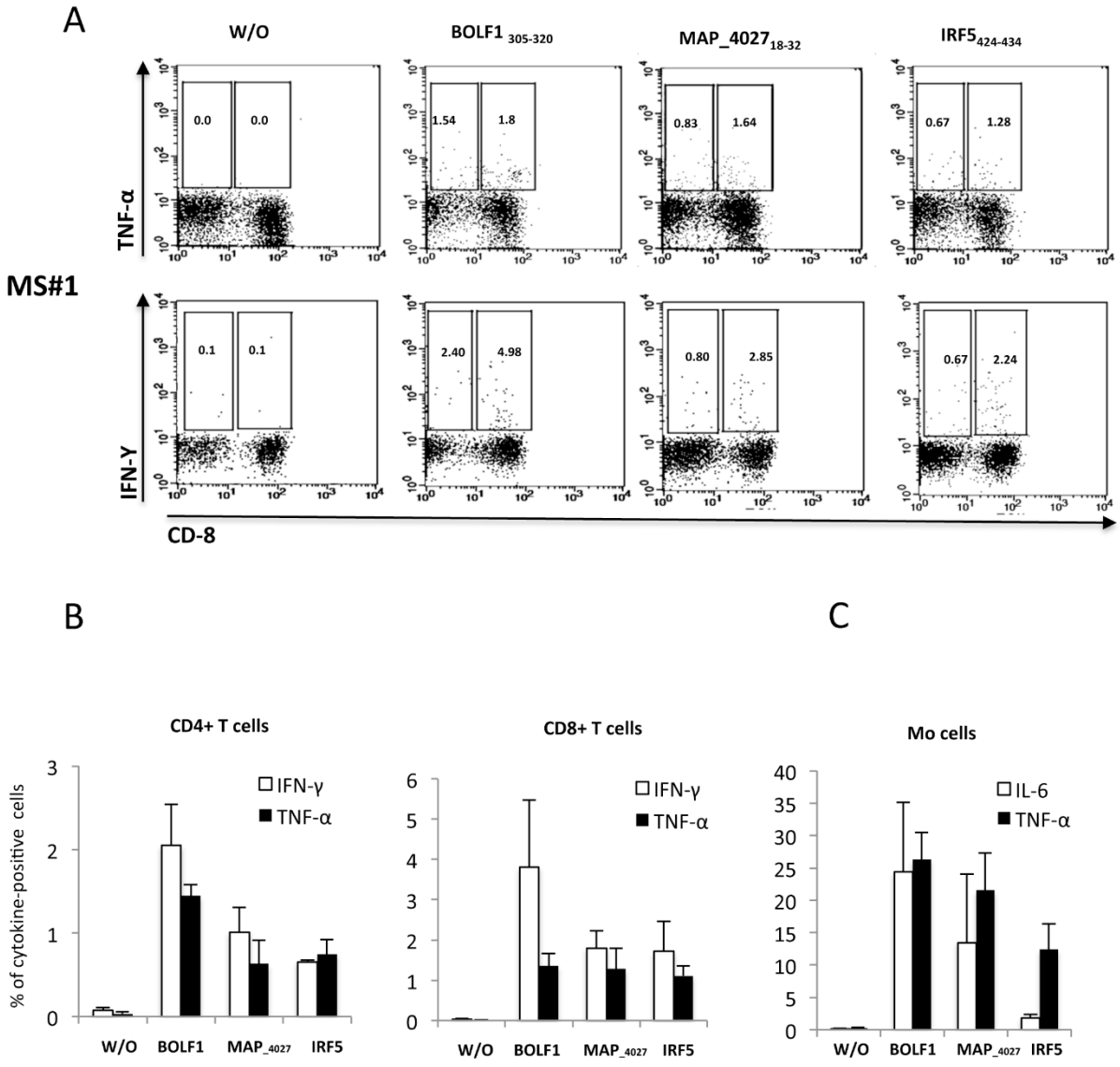
Flow cytometric evaluation of both peptide-specific CD8+ and CD8- (i.e. CD4) T lymphocytes upon 8-hour stimulation of peripheral blood from four MS patients and two HCs was performed. We analysed the expression of TNF-α and IFN-γ following stimulation with the peptides $BOLF1_{305-320,}$ MAP_4027_{18-32} and $IRF5_{424-434.}$ PBMCs from MS patients showed CD8- (i.e. CD4) and CD8+ T- cells expressing any of the both cytokines at a frequency higher of 0.5% and 1.0%, respectively **(Figure 17A)**. A statistical analysis showed that the frequency of both TNF-α and IFN-γ-expressing CD4 and CD8 T-cells was higher in MS PBMCs stimulated with the homologous peptides than in not stimulated ones **(Figure 17B)**.

Regarding the HCs, none of the peptides was capable of inducing cytokines production by $CD4^+$ and $CD8^+$ T-cells **(Figure 18)**.

Intracytoplasmic cytokine expression by peptide-stimulated monocytes

The cytokine expression pattern of $CD14^+$ monocytes in 4 MS patients and in 2 HCs was further analysed by FACS in the cytokine-driven assay. We analysed the expression of TNF-α and IL-6 by CD14+ monocytes following a stimulation of PBMC with the peptides $BOLF1_{305-320,}$ MAP_4027_{18-32} and $IRF5_{424-434}$. The peptides stimulate $CD14^+$ monocytes from MS patients increased both IL-6 and TNF-α expression **(Figure 17C)**, whereas IL-6 and TNF-α expression was absent in HCs.

Figure 17: Detection of BOLF1$_{305-320}$, MAP_4027$_{18-32}$ and IRF5$_{424-434}$ -specific T-cells and monocyte by intracytoplasmic cytokine expression assay.



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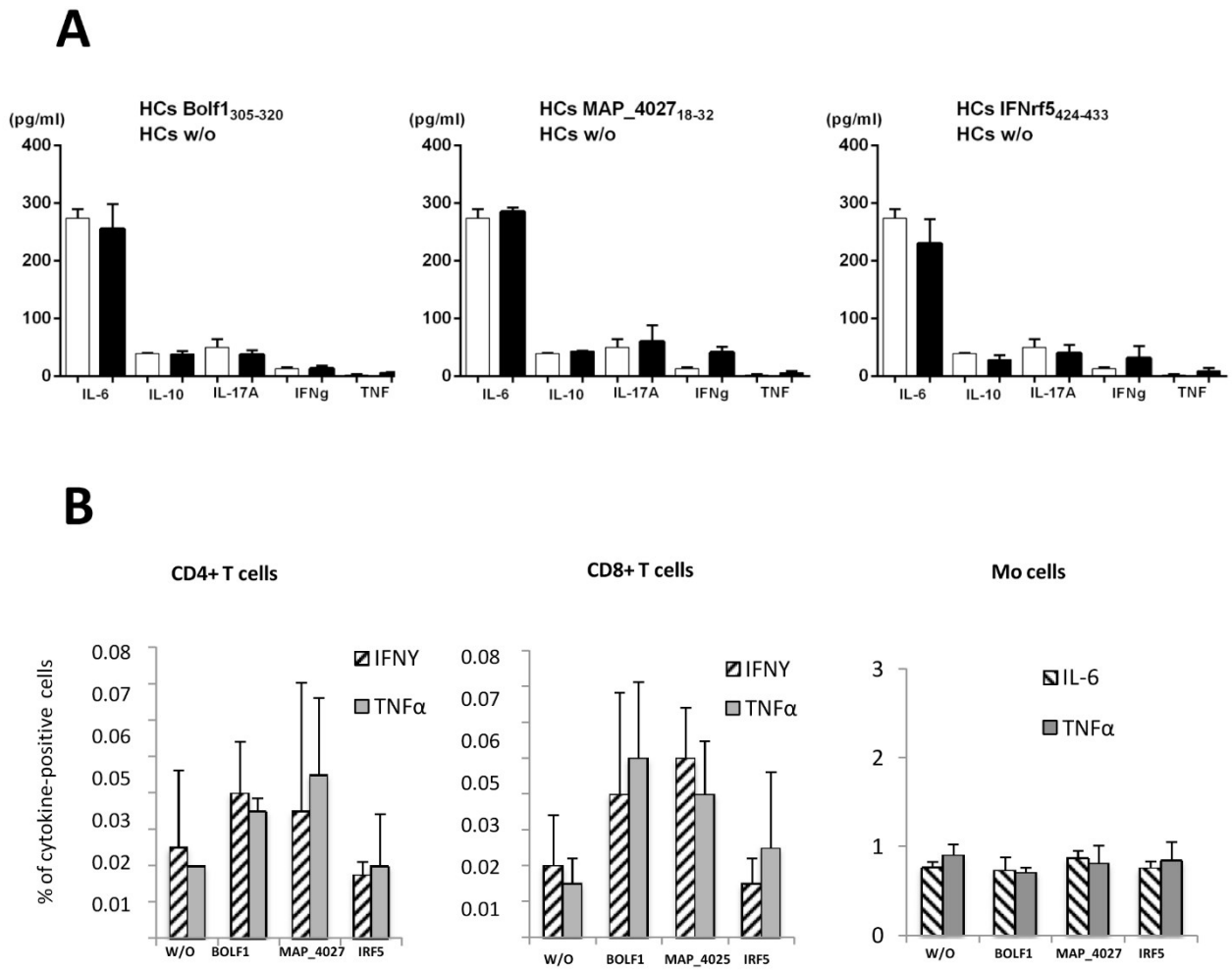
Gated to CD3$^+$CD8$^+$ cells, the expression of IFN-γ and TNFα in CD4$^+$ T cells (left) and CD8$^+$ T cells (right) was measured in MS#1 **(A)**. MS patients (MS#1-4) showed substantial expansions of CD8$^+$ and CD4$^+$ T-cells specific for all peptides **(B)**. (MS#1-4 showed an increase percentage of IL-6 and TNFα CD14$^+$ positive cells **(C)**.

Figure 18: Detection of extracellular cytokine levels, and quantitation of intracytoplasmic cytokine expression produced by peptide-stimulated T lymphocytes/monocytes in HCs.



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Discussion

MS has traditionally been considered a disease mediated by T-cells, in particular by CD4 helper T-cells, mainly due to the reported genetic association between MS and the MHC class II region [70]. Nevertheless, the role of CD8 T-cells has been re-evaluated [71], and recent studies reassigned an important role to the humoral responses driven by B-cells [72], hence it is important to clarify how B and T-cells interact in the complex pathogenesis of MS.

In this study we first reported the evaluation of the ability of $BOLF1_{305-320}$ and the homologues MAP_4027_{18-32} and $IRF5_{424-434}$ peptides to induce a strong humoral response in MS patients, and second the existence of a cross-recognition between Abs recognizing these homologues peptides. This data confirmed that EBV and MAP are capable of inducing the production of autoantibodies targeting different MS correlated epitopes.

IRF5 gene variation has a role in the pharmacological and clinical outcome of IFNβ therapy [73]. Furthermore, IRF5 is an important regulator of IFN-activity, it modulates immune signalling cytokine expression, apoptosis, cell cycle and it has been linked to MS development [67, 68]. Moreover, IRF5 regulates the toll-like receptor (TLR) dependent activation of inflammatory cytokines and functions downstream of the TLR-MyD88 pathway where it is activated by MyD88 and TNF receptor associated factor 6 (TRAF6) [74]. IRF5 was also proven to be a downstream mediator of TLR7 signalling in EBV infected B-cell lines with type III latency [75] and repressing the virus oncoprotein LMP1 [76].

Previous studies dealing with EBV and MAP derived peptides resulted to be informative, however the clinical parameters related to MS were restricted only to the humoral response (Ab positive/negative) [14, 15, 42, 43, 57].

For this reason, we investigated the existence of a cellular mediated immune response in MS patients against EBV and MAP epitopes. In order to verify that, we set up antigen specific T-cell stimulation experiments to detect extracellular and intracellular cytokine levels.

The extracellular cytokine levels was measured out by ELISA, and it showed that the cellular immune response induced by these peptides was mainly due to the Th1 subset, while there was a downregulation of the Th2 related cytokines.

The levels of IL-6, IL-10 and IFN-γ were upregulated upon stimulation with all the selected peptides. While IL-6 and IFN-γ are the clear signature of a Th1 response, IL-10 is a regulatory pleiotropic cytokine. Although IL-10 plays an important role in the onset and development of autoimmune diseases such as MS [77], the exact mechanisms by which this cytokine acts remain uncertain.

ELISA unveiled that IL-6 is the most secreted cytokine, and cytometry analysis showed as well a significant percentage of monocytes cells positive for this cytokine. IL-6 and TNF-α are pleitropic cytokines able to regulate both inflammatory and immunological responses, and are also capable of stimulating the terminal differentiation of activated B cells into immunoglobulin-secreting plasma cells [78]. IL-6 secretion could interfere with the suppressive activity of T-reg cells thus facilitating the priming of auto-reactive T-cells [79].

Cytometric analyses showed a significant increase of TNF-α $CD4^+$ and $CD8^+$ double-positive cells after stimulation with all peptides, while 25% of $CD14^+$ monocyte were also positive for TNF-α after $BOLF1_{305-320}$ and MAP_4027_{18-32} specific stimulation.

Altogether, the results produced by flow citometry highlighted that the selected peptides are able to induce a TNF-α mediated T-cell polarization.

TNF-α is the principal proinflammatory cytokine in MS and an important mediator of immunological and inflammatory responses; it can lead to the damage of myelin and oligodendrocytes in the CNS [80].

Both cytometry and ELISA revealed a significant IFN-γ production upon PBMCs stimulation with the three selected epitopes. IFN-γ response was mainly ascribable to $CD8^+$ T-cell, and in particular the main cytokine inducer was $BOLF1_{305\text{-}320.}$ Different studies support a prominent role of IFN-γ in driving MS disease, indeed it was reported that an increase of IFN-γ production precedes relapse phases in MS patients [81, 82]. Moreover, IFN-γ along with other type 1 cytokines was proven to be augmented in $CD4^+$, $CD8^+$, and $CD14^+$ cells in MS patients undergoing disease reactivation, whilst they were normalized in stable patients or patients under interferon-treatment [83]. In addition, it was also demonstrated, that IFN-γ levels are upregulated during RR exacerbations phases, whilst IL-17 levels are increased only in clinical isolated syndrome (CIS) patients [83]. These data are in lines with our results as our peptides are strong INF-γ inducer, thus suggesting that IFN-γ plays a more important role than IL-17 does in this subset of RR MS patients.

A possible explanation for the data obtained could be the following: in MS, T-cell attack might be directed to self-components, even if the primary target is yet to discover. Indeed, it is acknowledge that the autoimmune attack is directed against multiple targets [84].

When Abs against self-epitopes are produced, the phenomenon of the epitope spreading may contribute to tissue destruction and to the production of Abs against previously "sequestered" antigen, leading to a secondary autoimmune response against the newly released antigen.

Therefore, the specific response mounted against the non-self epitopes ($BOLF1_{305-320}$ MAP_4027_{18-32}) might be enhanced by the release of $IRF5_{424-434}$ from the intracellular compartment. We think that Abs production against $IRF5_{424-434}$, $BOLF1_{305-320}$ and MAP_4027_{18-32} does contribute to the MS pathogenesis but in a small part (e.g.Abs may act as ligands activating macrophages contributing to the tissue inflammation). Conversely, recent findings support the hypothesis that Abs have the ability to enter neurons in an epitope specific manner [85, 86]. The constant presence of cross-reactive Abs against $IRF5_{424-434}$, $BOLF1_{305-320}$ and MAP_4027_{18-32} may favor the entrance of this Abs into the cytosol, possibly via endocytosis. Therefore, Abs targeting these three homologues epitopes may alter the function of the IRF5 protein, modulating or inhibiting its activity.

In conclusion, the results of our experiments support the suggestion that cross reactivity of Abs targeting the non-self epitopes ($BOLF1_{305-320,}$ MAP_4027_{18-32}) with self $IRF5_{424-434}$, may mediate the immune dysregulation responsible in turn of favoring MS development.

Moreover, the same peptides are able to induce a Th1 immune response in MS patients highlighting the role of IL6, TNF-α and IFN-γ in the immune response to the described epitopes after PBMCs stimulation.

Chapter 4: MAP and Genetics

Part of this chapter is based on the following articles:

1. Cossu D, Masala S, Cocco E, Paccagnini D, Tranquilli S, Frau J, Marrosu MG, Sechi LA. Association of *Mycobacterium avium* ss. *paratuberculosis* and *SLC11A1* polymorphisms in Sardinian multiple sclerosis patients. J Infect Dev Ctries, Vol. 7(3), pp. 203-207, March, 2013.
2. Frau J, Cossu D, Sardu C, Mameli G, Coghe G, Lorefice L, Fenu G, Tranquilli S, Sechi LA, Marrosu MG, Cocco E. Combining *HLA-DRB1-DQB1* and *Mycobacterium avium* subspecies *paratubercolosis* (MAP) antibodies in Sardinian multiple sclerosis patients. (under review).

SNPs in the SLC11A1 gene and MAP

SLC11A1 is a gene located in chromosome 2q35 and encodes the natural resistance associated macrophage protein 1 (NRAMP1). This protein is a divalent transition metal transporter that regulates macrophage activation in infectious and autoimmune diseases; it also exerts a role in the resistance to intracellular pathogens including mycobacteria [87]. It also has been associated with human MAC infection [88].

We conducted a study aiming to understand in which way genes and environment interact with each other. Eight single nucleotide polymorphisms (SNPs) in the *SLC11A* gene (rs2276631, rs3731865, rs3731864, rs17221959, rs2695342, rs2279015, rs17235409, rs17235416) were searched by PCR RFLP-genotyping in 100 MS patients and 100 HCs [89].

Results and discussion

Only rs2276631 SNP was associated with MS. The genotype frequency for the allele AA was 40% in MS patients and 7% in the HCs ($\chi 2 = 28.5$, $p < 0.0001$).

The frequency of A allele was 60% in MS patients and 38.5% in HCs ($\chi 2 = 17.6$, OR = 1.95, $p < 0.0001$) (**Table 1**). The polymorphism rs2276631 is a nucleotide substitution that occurs in exon 3 (codon 66 for Phe). It consists of a C/T substitution at residue 274, which could impair iron transport through the channel. This in turn could avoid fagosome acidification and allow the mycobacterial survival inside the macrophage [89].

In the same population study, a strong humoral response against MAP_2694 was detected in 36% of MS patients and only in 3% of HCs. Binary logistic regression allowed us to observe a correlation between rs2276631 and MAP_2694 sero-positivity ($\chi 2 = 39$; MAP_2694 OR = 6, $p < 0.0001$; rs2276631 OR = 3, $p = 0.008$).

Table 1: Allelic and genotypic analyses of the associations between SLC11A1 SNPs and MS

SNP Identity		Allele frequency				Genotype frequency			
	Location	**A (%)**	**a (%)**	**OR (CI 95%)**	**p**	**AA (%)**	**Aa (%)**	**aa (%)**	**p**
rs2276631	Exon 3								
MS (n=100)		120 (60%)	80 (40%)	**1.95** (1.31-2.91)	**<0.0001**	40 (40%)	40 (40%)	20 (20%)	**<0.0001**
HCs (n=100)		77 (38.5%)	123 (61.5%)			7 (%)	63 (63%)	30 (30%)	
rs3731865	Intron 4								
MS (n=100)		135 (67.5%)	65 (32.5%)	NS	NS	46 (46%)	43 (43%)	11 (11%)	NS
HCs (n=100)		133 (66.5%)	67 (33.5%)			42 (42%)	49 (49%)	9 (9%)	
rs3731864	Intron 5								
MS (n=100)		5 (2.5%)	195 (97.5%)	NS	NS	0 (0%)	5 (5%)	95 (95%)	NS
HCs (n=100)		6 (3%)	194 (97%)			0 (0%)	6 (6%)	94 (94%)	
rs17221959	Exon 8								
MS (n=100)		97 (48.5%)	103 (51.5%)	NS	NS	19 (19%)	59 (59%)	22 (22%)	NS
HCs (n=100)		86 (43%)	114 (57%)			10 (10%)	66 (66%)	24 (24%)	
rs2695342	Exon 9								
MS (n=100)		122 (61%)	78 (39%)	NS	NS	22 (22%)	78 (78%)	0 (0%)	NS
HCs (n=100)		125 (62.5%)	75 (37.5%)			25 (25%)	75 (75%)	0 (0%)	
rs2279015	Intron 13								
MS (n=100)		68 (34%)	132 (66%)	NS	NS	1 (1%)	66 (66%)	33 (33%)	NS
HCs (n=100)		71 (35.5%)	129 (64.5%)			0 (0%)	71 (71%)	29 (29%)	
rs17235409	Exon 15								
MS (n=100)		13 (6.5%)	187 (93.5%)	NS	NS	4 (4%)	5 (5%)	91 (91%)	NS
HCs (n=100)		10 (5%)	190 (95%)			3 (3%)	4 (4%)	93 (93%)	
rs17235416	3'UTR								
MS (n=100)		5 (5%)	195 (97.5%)	NS	NS	0 (0%)	5 (5%)	95 (95%)	NS
HCs (n=100)		3 (3%)	197 (98.5%)			0 (0%)	3 (3%)	97 (97%)	

Our results suggest that rs2276631 SNP might contribute to increased susceptibility to MS. Furthermore, an association between rs2276631 SNP and T1D was recently reported [90], suggesting that the same polymorphism may be common to the major autoimmune diseases widespread in Sardinia.

HLA-DRB1-DQB1 and MAP

In Sardinia, the genetics of MS have some peculiarities. In particular, there is a negative association with the DRB1-DQB1 HLA haplotypes *15:02-*06:01, *16:01-*05:02 and *14:01-4-*05:03 and a positive association with *13:03-*03:01, *04:05-*03:01, *03:01-*02:01 and *15:01-*06:02 [91]. The last of these is the main genetic risk factor for MS in Northern European populations [92], it gives Sardinians a strong predisposition to MS, but is very rare in this population [93].

An interesting field of research is the possible interaction between the HLA haplotypes and the presence of anti-MAP Abs in conferring MS risk. Recently, a molecular modeling approach was used to find the differences between the Sardinian predisposing (*03:01 and *15:01) and protective (*16:01 and *15:02) HLA in recognizing peptides belonging to MAP_2694 protein. The former were found able to bind the MAP peptide better than the protective ones, suggesting a higher presentation efficiency, a higher probability of T-cell activation and autoimmune reaction [94].

For this reason, a group of 531 patients were searched for anti-MAP_2694 antibodies, and DRB1-DQB1 genotyping was performed.

The haplotypes were classified as predisposing (*13:03-*03:01, *04:05-*03:01, *03:01-*02:01, *15:01-*06:02) or protective (*15:02-*06:01, *16:01-*05:02, *14:01-4-*05:03), while other haplotypes were considered neutral.

Results and discussion

The analysis of the haplotypes (protective versus neutral versus predisposing) showed the presence of anti-MAP_2694 Abs in 23% of protective alleles, in 31% of neutral and in 31% of predisposing (p = 0.21%). Pearson's test showed a difference when considering protective HLA (33 haplotypes, 23%) versus combined neutral and predisposing ones (273 haplotypes, 31%) (p = 0.07). The analysis of the genotypes showed that the protective alleles were carried by 134 patients. The anti-MAP_2694 Abs were found in 31 subjects (23%) carrying at least one protective HLA and in 129 (32%) patients without a protective HLA (p = 0.04).

Summing-up, the analysis of the haplotypes showed no significant difference in the presence of anti- MAP_2694 Abs amongst the three haplotype groups (predisposing, protective and neutral), suggesting that MAP infection and predisposing haplotypes could be independent risk factors. Moreover, considering the demonstration of lower frequency of anti-MAP Abs in patients with protective HLA, it is possible that these haplotypes play a role in protecting not only against MS but against MAP infection as well.

Chapter 5: MAP and MS therapy

Part of this chapter is based on the following articles:

1. Frau J, Cossu D, Coghe G, Lorefice L, Fenu G, Melis M, Paccagnini D, Sardu C, Murru M, Tranquilli S, Marrosu M, Sechi L, Cocco E. *Mycobacterium avium* subsp. *paratuberculosis* and multiple sclerosis in Sardinian patients: epidemiology and clinical features. Mult Scler, 19(11), 1437-1442, February 2013.
2. Frau J, Cossu D, Coghe G, Lorefice L, Fenu G, Porcu G, Sardu C, Murru MR, Tranquilli S, Marrosu MG, Sechi LA, Cocco E. Role of interferon-beta in *Mycobacterium avium* subspecies *paratuberculosis* antibody response in Sardinian MS patients. J Neurol Sci, Vol. 349(1-2), pp. 249-250, February, 2015.

Steroids and disease-modifying drugs

We evaluated MAP positivity 436 MS patients in relation to therapeutic variables. Most MS subjects (294) had been treated with disease-modifying therapies for MS for at least three months at the time of the study: 277 patients took immunomodulant drugs (beta interferon, glatiramer acetate or natalizumab); 17 took immunosuppressive treatments (mitoxantrone or azathioprine). The numbers of patients who had used some immunomodulant or immunosuppressive drugs in their life before the study were 356 and 124, respectively. Only 60 subjects were free from any MS treatment.

Results and discussion

MAP DNA presence was higher in patients who used steroids in the 30 days before the sample collection compared to the steroid-free patients (31% vs. 14.5%, p = 0.03).

Logistic regression further confirmed this observation (OR = 2.65; CI 95% = 1.15–6.09; p = 0.02). **Table 2.**

This finding might be explained by the immunomodulatory/immunosuppressive effects of steroids, and it could be similar to what happens in viral infections. In fact, virus expression rises during or after steroid therapy [36].

Concerning MAP Ab-positivity, we found Abs anti MAP_2694 more frequently in patients who had received disease-modifying therapy for MS at the sample collection time.

In this case, logistic regression showed a significant association between the presence of anti-MAP2694 antibodies and MS therapy at study time (OR = 2.26; CI 95% = 1.21–4.25; p = 0.01). **Table 2.**

Table 2: presence of MAP DNA and anti MAP_2694 Abs in MS patients and HCS.

	Variables		Chi square
(A)	**OB neg**	**OB pos**	
MAP pos (2694 or PCR)	53.3%	37.2%	$p = 0.029$
MAP neg (2694 or PCR)	46.7%	62.8%	
(B)	**DMD neg**	**DMD pos**	
MAP2694 pos	33.1%	42.2%	$p = 0.08$
MAP2694 neg	66.9%	57.8%	
(C)	**Steroids neg**	**Steroids pos**	
MAPDNA pos	14.5%	31%	$p = 0.03$
MAPDNA neg	85.5%	69.0%	

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Because 277 subjects took immunomodulant drugs and 17 took immunosuppressive drugs, we hypothesised that the first class of drugs could influence MAP positivity, especially the host immunological response.

Influence of Interferon beta on MAP

In 120 patients analyzed in the previous study, the treatment was interferon beta (IFN-β), which is a cytokine involved in viral and intracellular infections. For this reason, we decided to perform a follow-up study, with the purpose to analyze the presence of anti-MAP2694 Abs in the blood of 89 MS patients before commencing IFN-β therapy and after at least six months of treatment.

Results and discussion

At the beginning of the study anti- MAP_2694 Abs were found in 21 subjects (18.69%), while the second sample was positive in 39 subjects (34.71%). In particular, 23 patients (20.47%) became positive after interferon-beta therapy and only 5 (4.45%) that were positive at the first sample collection time became negative (p = 0.001). The majority (45 patients, 40.05%) was always negative, while a group of positive patients did not change status (16 subjects, 14.24%).

It is noteworthy that IFN-β is a cytokine involved in infections, especially attributable to virus and intracellular agents. Anyway, its mechanisms of action are not fully understood.

IFN-β treatment could regulate many genes, some of which have anti-viral action, and could modify the level and the function of some cell populations [95]. In particular, were observed an increase of Th2, T-reg, NK-reg, dendritic cells and monocytes, and a decrease in CD4 and CD8 T- cells, B-cells and NK-cells [96]. Moreover, this therapy could enhance both blood leukocytes and serum levels of B-cell-activating factor (BAFF), which promote the inflammation supporting B-cell survival [97].

Indeed, it has been demonstrated that the induction of BAFF could lead to the production of autoantibodies and IFN-β neutralizing Abs [98].

Moreover, the upregulation of BAFF levels inducted by IFN-β and the consequently of autoreactive B-cells could lead to worsening in Devic disease [98]. Similar mechanisms could be the responsible for anti-MAP Abs production and for their higher frequency in subjects under IFN-β treatment.

The negative/positive status could change over an individual's lifetime. After commencing IFN-β treatment, a change from negative to positive is much more frequent than from positive to negative. It is to note that more than half of patients become positive had a higher OD in ELISA.

This supports the hypothesis that this drug could interact with the host immune system enhancing to enhance the immunological response to an intracellular pathogen such as MAP. The anti-MAP Abs negativization observed in a small number of persons could be explained as a physiological fluctuation in those with low Abs titer. This phenomenon has previously been observed in clinical practice for other common viruses. Our results are of particular importance for the interpretation of anti-MAP Abs test results. The timing of the sample collection and the possible presence of concomitant therapy should be taken into account in the interpretation of these results.

Chapter 6: Conclusion

MS is a complex autoimmune disease considered to be the consequence of an environmental pressure in genetically susceptible individuals. MS causative agents remain to be determined, despite the fact that a number of studies have been set up in order to discover a putative etiological agent. To date, MAP is one of the candidates that best suits the role of the environmental trigger. The foremost evidence supporting a link between MAP and MS comes from the higher detection rates of MAP DNA in samples from MS patients compared to HCs and from the demonstration of a serological and T-cell response toward specific MAP antigens. Anyway, it is still premature to conclude whether cross-recognition between MAP proteins/peptides and homologous human proteins involves an epitopes mimicry phenomenon starting or precipitating MS. Of further note, to definitely prove that MAP infection plays a causal role in MS, animal model studies should be performed.

Definitive evidence proving a causal relationship between MAP and MS is not available at present - perhaps it may never be. However, we demonstrated a clear, positive association of MAP with MS in Sardinia. MAP is most likely one of the environmental factors that trigger the autoimmune process in genetically predisposed subjects after infection through milk ingestion in early life, but other roles cannot be excluded. Genetic susceptibility may be important for explaining the progression of infected patients to a chronic autoimmune stage and the containment of the process in HCs. This finding could partially change the therapeutic approach to the disease, so good diagnostic tests for MAP presence will be important.

In fact, all the drugs actually in use for MS work by suppressing, or altering, the activity of the body's immune system. Thus, these therapies are based on the theory that MS is a result of an abnormal response of the body's immune system that causes it to attack the myelin sheaths. It can be reasonably assumed that during the next 5-10 years, immunodominant peptides of MAP will be determined, helping in turn to discover the specific regions targeted by the immune system. In the future, new vaccines based on these regions may be used to protect susceptible individuals. This approach is an advance to "personalized medicine". This alternative method of treatment is an extension of traditional approaches and better takes into account the specific need of each patient. This is of fundamental importance given the unique background of the Sardinian population. It is therefore necessary to obtain extra data supporting a direct involvement of MAP in triggering the disease, and the association must be confirmed outside Sardinia.

Methods

MAP culture

The MAP ATTC 43015 (Linda) strain of MAP was cultured in Middlebrook 7H9 medium enriched with 10% Middlebrook OADC (oleic acid-albumin-dextrose-NaCl) and complemented with 2 mg/L of Mycobactin J. The cultures were maintained at 37°C without shaking until they reached an optical density of approximately 0.7 at 600 nm. MAP was harvested from liquid culture at stationary phase, which contained approximately 5-10 mg/ml of bacteria, and centrifuged at 2,600 g for 10 min. The resultant pellet was used for DNA isolation and extraction.

Extraction of MAP DNA

Total cellular DNA was extracted by using CTAB methodology according with the protocol of Van Sooligen *et al.* [99]. To check the purity of the extracted DNA, a sample is run on a 1% agarose gel, and stained with ethidium bromide for band characterization via ultraviolet transillumination. The concentration of DNA was determined with NanoDrop® 2000 spectrophotometer (Thermo scientific).

Detection of MAP DNA

The presence of MAP-specific DNA in blood samples was detected by PCR amplification of *IS900* sequences. Briefly: Two rounds of amplification were undertaken. Firstly primers Liz1 and Liz2, secondly primers Av1 and Av2, were used to amplify a 298-bp fragment specific for MAP.

The reaction mixture (final volume of 50 µl) comprised primers at a concentration of 0.5 µM, Expand High Fidelity reaction buffer (1µ), 200 µM (each) dNTPs, and 3 Uof Expand High-Fidelity Taq polymerase (Expand High-fidelity PCR system; Roche, Lewes, United Kingdom). Amplified fragments were visualized with ethidium bromide on 2 % agarose- 1000 gel (Life Technologies, Grand Island, NY) and purified with a QIAquick gel extraction kit (QIAGEN, Crawley, United Kingdom). Each amplicon was then sequenced in both directions by using Av1 and Av2 primers. *IS900* PCR primers Cycling conditions Liz1: 5‘-CTTTCTTGAAGGGTGTTCGG-3‘ 94°C for 2 min and 36 cycles of 94°C Liz2: 5‘-ACGTGACCTCGCCTCCAT-3‘ for 40 s, 58°C for 40 s, and 72°C for 40 s (First amplification, amplicon length 298bp) final step 72°C for 5 min Av1: 5‘-ATGTGGTTGCTGTGTTGGATGG-3‘ 94°C for 2 min and 36 cycles of 94°C Av2: 5‘- CCGCCGCAATCAACTCCAG-3‘ for 40 s, 62°C for 40 s, and 72°C for 40 s (Second amplification, amplicon length 298bp) final step 72°C for 5 min

Construction of the pMAL-MAP_2694 and protein expression

DNA was amplified using a Phusion® High-Fidelity DNA Polymerase (Finnzymes) according for the manufacturer’s instructions. The sample reagent mixtures of 30µl were heated in a thermal cycler at 99°C for 5 minutes, then cycled 35 times of denaturation at 98°C for 10 seconds, annealing at 67°C for 30 seconds, extension at 72°C for 35 seconds and a final extension step at 72°C for ten minutes.

According to the DNA sequence (GenBank accession number: NC_002944.2) forward primer MAP2694-BamHI Fw (GCGCGGATCCGTGACGGCGGTTGATGACAGTA) was designed with the *BamH*I restriction site whereas the reverse primer MAP2694-PstI Rv (GCGCCTGCAGTCATTAGGACTTCGCGACCTTCAG) with the *Pst*I restriction site. The PCR products were analyzed with 1.5% agarose gel electrophoresis, and recovered with a QIAquick® PCR purification kit.

The purified PCR product was cloned into the prokaryotic expression vector pMAL-c2x (NewEngland Biolabs, USA). This plasmid has an inducible lacZ promoter, an exact deletion of the mal*E* signal sequence and *BamH*I and *Pst*I restriction recognition sequences within the MCS. Expression vector pMAL-c2x and the PCR products were double digested with *BamH*I and *Pst*I enzymes. The fragment of interest were recovered from 1% agarose gel, purified using QIAquick®PCR Purification Kit (QIAGEN) and ligated by the Quick Ligation Kit (Biolabs).

The ligation mixtures was used to transform *E. coli* K12 TB1cells (New England Biolab). Competent cells of *E.coli* TB1 were prepared for electroporation with the linearized plasmid pMAL-c2x. The electroporation was performed in a 2mm gap cuvette at 2.0 kV, 25 µF, and 200Ω using a gene-pulser (Bio-Rad). Positives clones were previously confirmed by both the blue white screening on Xgal plates and Colony PCR (fig), the positive recombinant products were plated on rich medium (10g/liter tryptone, 5g/liter yeast extract, 5g/literNaCl, 2g/liter glucose) supplemented with ampicillin (100 µg/ml) and further confirmed by both *BamHI/PstI* restriction analysis and DNA sequencing.

E. coli TB1 harboring the expression plasmid were grown at 37°C by shaking in 1L Rich medium supplemented with ampicillin (100 µg/ml).

When the OD_{600} measured approximately 0.5 protein expression was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich). After a 1,5-hr induction at 37°C, cultured cells were harvested by centrifugation at 4000 x g for 20min, the pellet was resuspended in 20ml ice-cold cells lysis buffer (20 mM Tris, 200 mM NaCl, 1mM EDTA) containing 1:100 protease inhibitor cocktail (Sigma-Aldrich), and stored at -20°C overnight.

The next day the sample was sonicated on ice in short pulses of 15s (70w) for about 3 min at 20 s intervals. The induced lysate was clarified by centrifugation at 9000 x g for 25 min and the fusion protein was then affinity purified using an amylose resin (New England Biolabs) according to the manufacturer's protocol. Amylose resin was used for purification of the fusion protein, and equilibrated with the running buffer. Previously the lysate was decanted and eluted 1:5 with column buffer, then was loaded into the column at a flow rate. The fusion protein was eluted from column with a buffer containing 10 mM maltose. Collected fractions were pooled and concentrated by using Centricon centrifugal filter devices (Millipore).

A specific fraction corresponding to our recombinant/fusion protein(s), at the level of 78.5 kDa, was observed on the Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) on a 12% Bis/Tris (MOPS) polyacrylamide gel. This was in agreement with the expected collective molecular mass of the fusion proteins (maltose binding protein of 42.5 kDa and MAP_2694 of 36 kDa). Western blotting confirmed that the protein was recognized by human sera indicating that the purified protein could be used as an antigen for immuno-diagnostic tests.

Construction of the pET-28a(+)-MAP_FprB and protein expression

DNA was amplified using a Phusion® High-Fidelity DNA Polymerase (Finnzymes) according for the manifacturer's instructions. Total volume of each PCR reactions was 30µl. PCR condition consisted of initial denaturing at 99°C for 5 minutes, then cycled 35 times of denaturation at 98°C for 10 seconds, annealing at 69°C for 30 seconds, extension at 72°C for 35 seconds and a final extension step at 72°C for ten minutes.

The region encoding the MAP FprB gene was amplified with the primers forward (GCGCCATATGATGCCGCACGTTATTACCCAGTCG) and reverse Rv (GCGCCTCGAGCTAGCCGCGCCCGGACGGCAC) and then cloned into pET-28a+ expression vector. The sequence of forward primer was designed with an endonuclease site of *NdeI* and reverse primer with an endonuclease site of *XhoI*. The PCR products were analyzed with 1.5% agarose gel electrophoresis, and recovered with a QIAquick® PCR purification kit.

The pET-28a+ vector carry an N-terminal His•Tag plus an optional C-terminal His•Tag sequence. PCR products and the plasmid were double digested with *NdeI* and *XhoI* enzymes. The fragment of interest were recovered from 1% agarose gel, purified using QIAquick®PCR Purification Kit (QIAGEN) and ligated by the Quick Ligation Kit (New England Biolabs). The ligated product was then amplified in *E.*coli strain DH5α (Invitrogen) and then extracted by plasmid midi kit (QIAGEN). Recombinant pET-28a(+)-MAP Fprb was transformed into competent *E. coli* BL-21 (DE3) (Novagen). The electroporation was performed in the same condition as described before. Positive clones were plated on rich medium supplemented with kanamycin (30 µg/ml) and further confirmed by both NdeI/XhoI restriction analysis and DNA sequencing.

E. coli BL-21 (DE3) harboring the expression were grown at 37°C by shaking in 1L Rich medium supplemented with kanamycin (30 μg/ml). When the O.D.600nm measured approximately 0.5 protein expression was induced by addition of 2 mM IPTG. After a 3-hr induction at 37°C, cultured cells were harvested by centrifugation at 4000 x g for 20min and resuspended in Native Binding Buffer with imidazole containing lysozime. The bacterial cell suspension was sonicated for 3 min with a pulse interval of 10 s. The sonicated extract was centrifuged at 5000 rpm for 30 min at 4 °C.

The supernatant and cell pellet were analyzed by 12 % SDS-PAGE stored at -80°C overnight. The next day, after confirmation of the solubility, the protein was purified by His-tag binding affinity to Ni-NTA agarose. Purification of the cell lysate was carried out using a Qiagen Ni-NTA spin column with a native purification protocol as specified by the manufacturer. Purification was achieved under native conditions; recombinant protein that bound to the Ni-NTA agarose was eluted using a pH gradient. The different washes and eluates were analyzed by SDS-PAGE. MAP_FprB was detected upon Western blotting and was quantified utilizing NanoDrop®.

Construction of the pET-28a(+)-MAP_HSP70

The dnaK gene encoding for MAP_3840 (HSP70) protein (UniProtKB/Swiss-Prot: Q00488) was cloned and expressed in *E.coli* BL21(DE3) codon plus (Stratagene, La Jolla, CA, USA). The gene was PCR amplified using MAP genomic DNA extracted by CTAB methodology.

Forward primer NdeI -MAP HSP70-Fw (5'-GCGCCATATGCCAGGAGGAATCACTATGGCT -3') and reverse primer HindIII –MAP HSP70-Rv (5'- GCGCAAGCTTGGTTGCCTTCCGTCTTCTGAT -3'), were used to amplify MAP dnaK gene by proofreading Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The PCR amplification product obtained was inserted into the pET-28a+ expression vector, in order to generate a construct that carried the MAP HSP70 with upstream codons for a 6xHis tag. The recombinant protein was purified by both Ni-NTA affinity chromatography under native conditions and by automated chromatography systems Profinia®. The specific fraction corresponding to MAP HSP70 recombinant protein, at the level of 70 kDa, was observed on the SDS-PAGE gel.

Peptides

A peptide library spanning the entire aminoacidic sequence of MAP_2694 protein (58 overlapped 9-mers) was synthesized at 85% purity (LifeTein, South Plainfield, NJ 07080 U.S.). Stock solutions were stored in single-use aliquots at -80°C **(Table 3)**.

TABLE 3. Sequence of the 58 peptides spanning the entire MAP2694 protein sequence.

Peptide_n residue	Sequence	Peptide_n residue	Sequence	Peptide_n residue	Sequence
Pep_1 1-9	MTAVDDSKD	MAP2694 127-135	GLVVLAVIA	MAP2694 253-261	AQATKLMQC
MAP2694_2 7-15	SKDGFSMTA	MAP2694 133-141	VIALVATLV	MAP2694 259-267	MQCLANGPG
MAP2694 13-21	MTAPPGGIY	MAP2694 139-147	TLVVMKGGH	MAP2694 265-273	GPGYANAMP
MAP2694 19-27	GIYGPGSYG	MAP2694 145-153	GGHGSKPSG	MAP2694 271-279	AMPTLGPIK
MAP2694 25-33	SYGSNPYGQ	MAP2694 151-159	PSGATPSST	MAP2694 277-285	PIKTSPITV
MAP2694 31-39	YGQEPNWGG	MAP2694 157-165	SSSTSTSVSQ	MAP2694 283-291	ITVDGTKAV
MAP2694 37-45	WGGQPPGGQ	MAP2694 163-171	VSQPKNSAQ	MAP2694 289-297	KAVRADADV
MAP2694 43-51	GGQPPGGQP	MAP2694 169-177	SAQNATDCT	MAP2694 295-303	ADVTIADPT
MAP2694 49-57	GQPQGGPYP	MAP2694 175-183	DCTPNVSGG	MAP2694 301-309	DPTRNVKGD
MAP2694_10 55-63	PYPQPGQYP	MAP2694 181-189	SGGDMPRSD	MAP2694 307-315	KGDSVTIIA
MAP2694 61-69	QYPAGGPYP	MAP2694 187-195	RSDSIAAGK	MAP2694 313-321	IIAVDTKPV
MAP2694 67-75	PYPYPPPGG	MAP2694 193-201	AGKLSFPAN	MAP2694 319-327	KPVSVFIGS
MAP2694 73-81	PGGGYPYPG	MAP2694 199-207	PANAAPSGW	MAP2694 325-333	IGSTPIGDS
MAP2694 79-87	YPGGPYPGG	MAP2694 205-213	SGWTVFSDD	MAP2694 331-339	GDSASAGLI
MAP2694 85-93	PGGPYPGAP	MAP2694 211-219	SDDQGPNLI	MAP2694 337-345	GLIGKIIAA
MAP2694 91-99	GAPYPGPGQ	MAP2694 217-225	NLIGALGVA	MAP2694 343-351	IAALKVAKS
MAP2694 97-105	PGQPFGPGG	MAP2694 223-231	GVAQDVPGA		
MAP2694 103-111	PGGPYSPGP	MAP2694 229-237	PGANQWMMT		
MAP2694 109-117	PGPPPGGPG	MAP2694 235-243	MMTAEVGVT		
MAP2694 115-123	GPGSKLPWL	MAP2694 241-249	GVTNFVPSM		
MAP2694 121-129	PWLIVAGLV	MAP2694 247-255	PSMDLTAQA		

EBNA1$_{400-413}$ [PGRRPFFHPVGEAD]; MAP$_{121-132}$ [PGRRPFTRKELQ]; MBP$_{85-98}$ [ENPVVNFFKNIVTP]. In addition, MMP25$_{252-263}$ [RPFYQGPVGDPD], a peptide belonging to human Metallo proteinase 25 protein (with a mild homology to EBNA1$_{400-413}$), and an irrelevant mycobacterial peptide MAP$_{38-46}$ [GGQPPGGQP] (Cossu et al., 2014) were chosen as negative controls. All peptides were synthesized at >90% purity (LifeTein, South Plainfield, NJ 07080 USA) **(Table 4)**.

Peptide name	Sequence	Protein name	Organism
MBP$_{85–98}$	ENPVVNFFKNIVTP	Myelin Basic Protein	Human
EBNA1$_{400-413}$	PGRRPFFHPVGEAD	Epstein–Barr nuclear antigen-1	*Epstein–Barr* Virus
MAP$_{121-132}$	PGRRPFTRKELQ	MAP_0106c	*Mycobacterium paratuberculosis*
MMP25$_{252-263}$	RPFYQGPVGDPD	Matrix metalloproteinase-25	Human
MAP2694$_{38-46}$	GGQPPGGQP	MAP_2694	*Mycobacterium paratuberculosis*

The 21-mer MOG$_{35–55}$ [MEVGWYRPPFSRVVHLYRNGK] derived from MOG_HUMAN protein (UniProtKB accession number: Q16653); the 10-mer MAP_2619c$_{352-361}$ / BCG_1224$_{355-364}$ [WYIPPLSPVV] derived from MAP_2619c protein (UniProtKB accession number: Q73WP1) and from BCG_1224 protein (UniProtKB accession number: W8SWZ3); the 11-mer BCG_3329c$_{64-74}$ [PPGSVVHLYRD] derived from BCG_3329c protein (UniProtKB accession number: A1KNV2). All peptides were synthesized at >90% purity commercially (LifeTein, South Plainfield, NJ 07080 USA) **(Table 5)**.

Synthetic peptides BOLF1$_{305-320}$ [AAVPVLAFDAARLRLLE] from BOLF1 protein (UniProt accession no. I1YP08), MAP_4027$_{18-32}$ [AVVPVLAYAAARLLL] from MAP_4027 protein (Uniprot accession no. Q73SP6) and IRF5$_{424-434}$ [VVPVAARLLLE] from IRF5 protein (UniProt accession no.Q13568), were synthesized with purity greater than 90% and purchased from LifeTein (South Plainfield, NJ 07080, USA) **(Table 6)**.

Table 5-6 shown the peptides alignment obtained using Clustal W 2 software. Result shows region of identity with a star (*), strong similar amino acid with a dot (.) and a missing region in dashes (-).

Table 5

Peptide	Aminoacidic sequence
MOG$_{35-55}$	**MEVGWYRPPFSRVVHLYRNGK**
MAP_2619$_{352-361}$	**----WYIPPLSPVV-------**
BCG_3329$_{64-74}$	**-------PPGS-VVHLYRD--**
	**** * **

Table 6

Peptide name	Sequence
BOLF1$_{305-320}$	**AAVPVLAFDAARLRLLE**
MAP_4027$_{18-32}$	**AVVPVLAYAAARL-LL-**
IRF5$_{424-434}$	**-VVPV----AARL-LLE**
	.* **** **

Enzyme-linked immunosorbent assays (ELISAs)

All serum samples were assessed by indirect ELISA for measuring levels of total IgG [13, 14, 36, 42, 43, 57] and specific IgG [28]. The optimal dilutions of antigens, serum sample and secondary Ab were determined by check board titration. Then, each well of 96-well Nunc immune-plate was coated (depending on the type of experiment) with 5 μg/ml of proteins or 10 μg/ml of peptides in in 0.05 M carbonate–bicarbonate buffer pH 9.5, overnight at 4°C.

The following day, 300 μl of blocking solution containing 5% non-fat dried milk were added to each well and the plate was further incubated at room temperature for 1h. After rinsing with 200 μl of PBST (10 mM phosphate-buffered saline, pH 7.0, containing 0.5% Tween 80), serum samples were added at 1:100 dilutions in PBST for 2h at room temperature. After the wells were washed four time with PBST, alkaline phosphatase conjugated secondary antibody (Sigma-Aldrich) diluted 1:100 was added for 1h at room temperature. After four washing with PBST, 200 ml of paranitrophenylphosphate (Sigma-Aldrich) as the substrate were added to each well and the plates were incubated at room temperature to develop color reactions according to the manufacturer's instructions. The optical densities (OD) were read at 405nm on a VERSA Tunable Max Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Negative control wells were obtained by incubation of immobilized protein or peptides with secondary Ab alone, and their mean values subtracted from all samples. Positive control sera were also included in all experiments. Results are expressed as means of triplicate 405 nm OD values. Data was also normalized to a positive control serum included in all essays run, whose Ab- reactivity was set at 10.000 arbitrary units (AU)/ml.

Competitive inhibition assay

Sera from MS patients and from HCs were subjected to ELISA on plates coated with $EBNA1_{400-413}$ or MBP_{85-98} respectively. Competitive assays were performed by pre-incubating the sera overnight at 4°C with saturating concentrations of peptides [10μM]; a sero-negative peptides was used as negative controls and, depending on the assay, was added a positive control.

Subjects

A total of 436 MS patients (302 females and 134 males) and 264 HCs (130 females and 134 males) were enrolled [13-15, 36, 42, 43, 57]. The mean age in the MS group and in HCs was 41 (SD ± 11 years) and 36 years (SD ± 12), respectively. The clinical course was relapsing–remitting in 367 (84%), primary progressive in 18 (4%), and 51 patients (12%) were secondary progressive. The median age at onset was 28 years (25th percentile 22 years, 75th percentile 36 years), the median duration of the disease was 10 years (25th percentile five years, 75th percentile 17 years) and the median EDSS at the study time was 2 (minimum 0, maximum 8.5). CSF data were available for only 353 patients: 293 were OB positive and 60 were negative.

The first 50 patients were drug free [13] while all the other patients were selected randomly among those who came for follow-up visits [14, 36, 42, 43, 57].

Most of the MS subjects (294) had been treated with disease-modifying therapies for MS for at least three months at the time of the study: 277 patients took immunomodulant drugs (beta interferon, glatiramer acetate or natalizumab); 17 took immunosuppressive treatments (mitoxantrone or azathioprine).

The numbers of patients who had used some immunomodulant or immunosuppressive drugs in their life before the study were 356 and 124, respectively. Only 60 subjects were free from any MS treatment. We recorded a clinical relapse at the sample collection time in 39 patients, and 29 individuals had taken steroids in the previous 30 days.

Human PBMCs

PBMC were isolated by Ficoll Hypaque density gradient centrifugation from heparinized peripheral blood of MS patients and HCs. Five ml of fresh blood was carefully layered on top of 10 ml Ficoll in 50 ml falcon tubes. Following centrifugation at 2000rpm for 30 min without break at room temperature, the PBMC-containing interphase was carefully collected, transferred to another tube containing PBS, and centrifuged for 10 min at 1300rpm. The pellet was resuspended in PBS and the wash step was repeated once. The cell pellet was then resuspended in complete RPMI medium for cell counting.

Extracellular cytokine expression assay

PBMCs isolated from 4 MS (MS#1-4) fresh blood samples (1 male SP and 3 female RR) and 2 HCs (1 male and 1 female) were cultured with single peptides (25µM) and cytokines secreted during the 48 h culture were measured by a Multi-Analyte ELISArray Kit (QIAGEN). PBMCs without peptide stimulation were used as negative control.

PBMCs were re-suspended in complete medium (CM); RPMI 1640 medium (Sigma-Aldrich) and 10% heat-inactivated human serum (Sigma-Aldrich). The cell viability was examined with 0.4% trypanblue solution (Life Technologies) and was always >95%.

One million cells per well were cultured in a 24-well flat bottom plate. On day 2 of culture, the single identified peptides (25 μM each peptide) and a negative control were added in the presence of CD28/CD49d co-stimulatory antibodies (BD Biosciences) and 50 U/ml recombinant human IL-2 (Sigma-Aldrich), in order to expand the antigen-specific T-cells. On day 4 of culture, 1 ml of culture supernatant was harvested for the cytokine secretion assay. Secreted cytokine levels (IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IFN-γ, and TNF-α) were measured by a Multi-Analyte ELISArray Kit (QIAGEN) according to the manufacturer's protocol.

Peptide-specific cytokine expression

The stimulation of samples was performed on PBMC from patients MS#1-4 and HCs#1-2 as follows. Briefly, 6 ml of peripheral blood was collected in Na^+ heparin tubes. Samples were divided into four 0.5 ml aliquots. Three were incubated with 25 μM of each peptide, the final aliquot served as a non-stimulated control. Anti-CD28/CD49d co-stimulatory antibodies (BD Biosciences, San Diego, Calif., USA) were added to each aliquot. The blood was incubated at 37°C for 8 hours; brefeldin A (10 μg/ml) was added during the last 6 hours of culture. After extensive wash with cold PBS, the cells were fixed and permeabilized using Cytofix/Cytoperm according to the manufacturer's instructions. The cells were then stained with PE anti-CD3, PerCp anti-CD8, FITC anti-IFN-γ and APC anti-TNF-α for 30 min. To characterize monocytes, the samples were stained with PE conjugated anti-CD14, APC anti-IL-6 and PerCP anti-TNF-α.

Cells were then washed and analyzed on FACS Calibur using Cellquest software (BD Biosciences, CA); 10^5 events for each sample were acquired.

SLC11A1 SNP selection and genotyping

Genomic DNA was purified from whole blood according to standard procedures [7]. Eight polymorphisms (rs2276631, rs3731865, rs3731864, rs17221959, rs2695342, rs2279015, rs17235409, rs17235416) were genotyped by PCR restriction fragment length polymorphism (RFLP) analysis, and the set of specific primers were designed based on the GenBank reference sequence (accession no. NT_005403).

In detail, the following primer sequences used were: rs2276631 forward (5'-TGCCACCATCCCTATACCCAG-3') and rs2276631 reverse (3'-TCTCGAAAGTGTCCCACTCAG-5'); rs3731865 forward (5'- TCTCTGGCTGAAGGCTCTCC-3') and rs3731865 reverse (3'-TGTGCTATCAGTTGAGCCTC-5'); rs3731864 forward (5'- CTGGACCAGGCTGGGCTGAC-3') and rs3731864 reverse (3'-CCACCACTCCCCTATGACGTG-5'); rs17221959 forward (5'-CTTGTCCTGACCAGGCTCCT-3') and rs17221959 reverse (3'-CATGGCTCCGACTGAGTGAG-5'); rs2695342 forward (5'- TCCTTGATCTTCGTAGTCTC-3') and rs2695342 reverse (3'-GGCTTACAGGACATGAGTAC-5'); rs2279015 forward (5'- GCAAGTTGAGGAGCCAAGAC-3') and rs2279015 reverse (3'-ACCTGCATCAACTCCTCTTC-5'); rs17235409/rs17235416 forward (5'-GCATCTCCCCAATTCATGGT-3') and rs17235409/rs17235416 reverse (3'-AACTGTCCCACTCTATCCTG-5').

Statistical analyses

BLAST searches were performed using GenBank non-redundant proteins database with low complexity region filter. Statistical analysis of data was evaluated using GraphPad Prism 6.0 software (San Diego, CA, USA). ELISA values were compared by the $\chi 2$ test of significance with Yate's corrections.

Receiver operator characteristic (ROC) curves was utilized to evaluate the accuracy of the experiments. The cut-off for positivity in each assay was set at 95% specificity and the corresponding sensitivity calculated accordingly. The median ± the interquartile range was also calculated. Logistic regression was used to calculate odds ratios (OR). Data is presented as mean ± standard deviation, median and interquartile range; with *p* values below 0.05 indicating a statistical significance. Comparisons between MS patients and HC groups were analyzed by Mann-Whitney U-test.

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List of papers done during the PhD (01-11-2012 / 31-10-2015)

First year (01-11-2012 / 31-10-2013)

1. Masala S, **Cossu D**, Pacifico A, Molicotti P, Sechi LA. Sardinian Type 1 diabetes patients, Transthyretin and *Mycobacterium avium* subspecies *paratuberculosis* infection. Gut Pathog, 4(1), 24, December 2012.

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