SAPIENZA
UNIVERSITÀ DI ROMA

DOTTORATO DI RICERCA IN BIOCHIMICA

XXVI CICLO (A.A. 2010-2013)

"Non heritable risk factors for Multiple Sclerosis: Epstein Barr virus and elements influencing intestinal permeability"

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Dicembre 2013

Summary

Chapter 1: Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic immune-mediated inflammatory demyelinating disease of the central nervous system (CNS) initially identified in 1868 by Jean-Martin Charcot. Morphologically, it is characterized by inflammation, demyelination, axonal injury and gliosis [1]. Its name is due at the presence of multiple discrete sclerotic plaques in white matter of the brain and spinal cord; however, it's also known as "disseminated sclerosis" or "encephalomyelitis disseminata". Plaques are the most recognized pathologic hallmarks of MS and consist in areas of white matter demyelination, often inflammatory. From a biological point of view active plaques are characterized by massive perivascular spaces infiltration of mononuclear cells (T cells, B lymphocytes, plasma cells, macrophages) and soluble mediators of the immune system including a broad range of cytokines, chemokines, antibodies, complement and other toxic substances. Both the myelin sheath and the oligodendrocyte itself are destroyed within plaques, following attack by cells of the immune system that react with myelin-related epitopes, such as myelin basic protein (MBP) [1].

MS represents the major cause of nervous system disability affecting about 2.5 million people worldwide, with a 2-3:1 female to male ratio. Disease onset is usually in the third or fourth decade, but 2% of patients with MS present it before age 10 years, and 5% before age 16 years [2]. The estimated

incidence of the disease is greatest at the extremes of latitude in both the northern and southern hemisphere and it's of about seven per 100,000 every year. Moreover the prevalence is of around 120 per 100,000 and the lifetime risk is of one in 400 [2].

1.1.1 Symptoms and signs of MS

The CNS controls much of the body's functioning and much of this activity passes through the white matter at some point. It is not surprising, therefore, that a disease like MS which damages white matter can produce a very wide range of symptoms. Symptoms of MS usually appear in episodic acute periods of worsening (called relapses, exacerbations, bouts, attacks, or "flare-ups"), in a gradually progressive deterioration of neurologic function, or in a combination of both. The relapses are believed to be the consequences of focal inflammatory demyelinating lesions, the histopathological hallmark of MS. Relapses are often unpredictable, occurring without warning and without obvious inciting factors with a rate rarely above one and a half per year. Some attacks, however, are preceded by common triggers: for example spring and summer, viral infections (such as the common cold, influenza, or gastroenteritis), stress and pregnancy increase the risk of relapse [3]. There are no clinical symptoms that are unique to MS, but some are highly characteristic of the disease. Some of the major MS symptoms include:

- visual impairment
- motor and sensory deficits
- difficulties with coordination and balance

- bowel and bladder incontinence
- sexual dysfunction
- cognitive dysfunction
- depression
- severe fatigue
- pain

However, there are more than 50 symptoms characterizing the intermittent episodes of disease relapse, and each one can vary in severity, duration and associated disability. MS affects the ability of nerve cells in the brain and spinal cord to communicate with each other effectively. Nerve cells communicate by sending electrical signals called “action potentials” down long fibers called axons, which are contained within an insulating substance called myelin (Fig. 1).

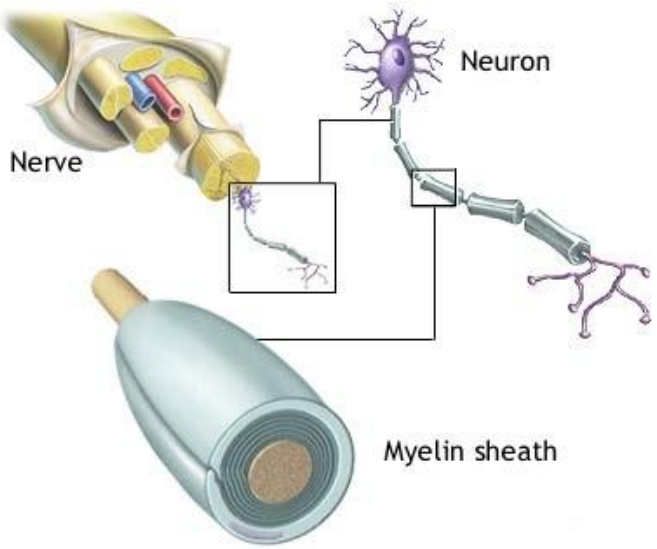

Figure 1 Conduction of the action potential along a myelinated nerve fiber. (Photo by A.D.A.M Medical Illustration Team)

In MS, the recurrent immune system's inflammatory attacks on the white matter of the brain and spinal cord damage the myelin. Hence the neurologic manifestations of the disease, that arise from the immune-mediated demyelination which impairs the neuronal transmission and results in axonal degeneration, reflect the functional anatomy of impaired salutatory conduction at affected sites [4]. In particular, lesions of the brain stem and cerebellar pathways typically disrupt the coordinated movement of the eyes, limbs, bulbar musculature, and axial muscles; while spinal cord lesions lead to alterations in motor, sensory, and autonomic functions. The prognosis is relatively good when sensory or visual symptoms dominate the course of multiple sclerosis in adults, and there is complete recovery from individual episodes. This pattern is most common in young women. Conversely, motor involvement, especially when coordination or balance are disturbed, has a less positive prognosis. The outlook is also poor in older men who develop the disease. Frequent and prolonged relapses with incomplete recovery at onset and a short interval between the initial episode and first relapse are adverse prognostic features, but the main determinant of disability is onset of the progressive phase [2].

1.1.2 MS subtypes and diagnostic criteria

In 1996 the United States National Multiple Sclerosis Society standardized four clinical MS courses (Fig. 2), named:

- relapsing remitting

- secondary progressive
- primary progressive
- progressive relapsing

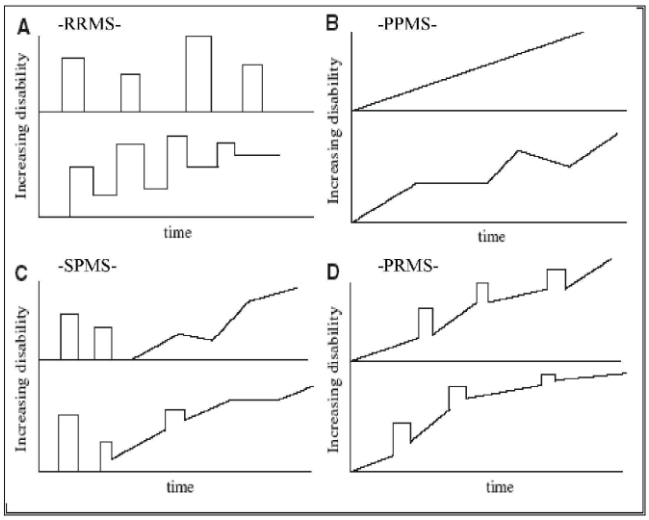

Figure 2 Graphical summary of MS subtype. A. Relapsing-remitting MS (RRMS); B. Primary- Progressive MS (PPMS); C. Secondary-Progressive MS (SPMS); D. Progressive-Relapsing MS (PRMS) [5].

MS subtypes use the past course of the disease in an attempt to predict the future course. In fact, clinical course and disease onset of MS are highly variable and mostly unpredictable. However, MS subtypes are important not only for prognosis but also for therapeutic decisions.

The most common MS subtype shows a waxing and waning of signs and symptoms to produce a "relapsing-remitting" clinical presentation. The

disease often begins in early adulthood with intermittent episodes of neurological dysfunction. “Relapsing remitting MS” (RRMS) affects ~85% of patients and, typically, the illness passes through phases of relapse with full recovery, relapse with persistent deficit, and secondary progression [2].

In general within a decade, approximately 50–60% of RRMS patients will suffer from “secondary progressive MS” (SPMS), a form that shows less inflammation and more neurodegeneration. The progressive phase is dominated by diffuse white matter abnormalities, atrophy and cortical demyelination but not new gadolinium-enhancing lesions.

Conversely, about 15% of patients develop a “primary progressive MS” (PPMS), a distinct form of MS that it’s not clearly associated with relapses and shows neurodegeneration with a relative lack of inflammatory change. The onset of PPMS is typically about 10 years later than RRMS but at a similar age when the conversion to SPMS occurs [6].

A less common form of MS, progressive relapsing MS (PRMS), is progressive from the onset but it also presents acute relapses with or without full recovery. This MS subtype develops in 5% of diagnosed MS cases [6].

Although many persons lead full and rewarding lives, MS can also result in longterm disability. In particular in about a quarter of patients, MS never affects activities of daily living; conversely, up to 15% become severely disabled within a short time. Fixed disability in MS is acquired through two distinct mechanisms: incomplete recovery from relapse and disease progression. Patients with RRMS accumulate disability from disease onset more slowly than those with PPMS [2]. The main clinical measure of disability progression and symptom severity is the Expanded Disability Status Scale or EDSS. In detail, the EDSS is considered the gold standard for assessing the level of physical disability in MS: it’s an ordinal scale with 19

disease steps between 0 and 10, that measures impairment or activity limitation based on the examination of eight functional systems plus ambulation [7].

There are several reasons why MS diagnosis is extremely difficult, including the fact that: more than 50 symptoms are linked to MS, and each person develops symptoms differently; many of the symptoms mimic problems that occur with other diseases; there is no blood test for MS yet; symptoms usually come and go; many symptoms are vague and hard to quantify, such as fatigue, sexual dysfunction, depression and cognitive difficulties.

Medical organizations have created diagnostic criteria to ease and standardize the diagnostic process especially in the first stages of the disease. Historically, the Schumacher and Poser criteria were both popular; currently, McDonald criteria are regarded as the gold standard for non-invasive MS diagnosis. McDonald criteria, that are recommended in the guidelines of the International Panel on MS Diagnosis, imply the objective evidence of dissemination in time and space of lesions typical of MS and the exclusion of other, better explanations for the clinical features [8]. Therefore individuals must have a minimum of two attacks, affecting more than one anatomical site; but, assuming an initial presentation suggestive of MS, the second lesion need not necessarily be clinically express [2]. The most commonly used diagnostic tools are neuroimaging, analysis of cerebrospinal fluid and evoked potentials. The diagnosis of MS has been revolutionized with the introduction of the magnetic resonance imaging (MRI) technology. MRIs use magnetic waves to produce images of the brain and spinal cord; at the same time, a special contrast material (called gadolinium) reacts to areas of inflammation allowing to detect the multiple gadolinium-enhancing lesions, distributed throughout the white matter of the CNS. This technology is considered the

best test for diagnosing MS, as abnormal lesions appear on MRIs in over 95% of people with MS. However, 5% of people with MS do not have abnormalities that can be detected on an MRI (producing a false negative), and some age-related damage looks like MS lesions (producing a false positive). Hence, MRI is essential but not sufficient for a correct MS diagnosis, requiring to be integrated with clinical and other paraclinical diagnostic methods. Studies of surrogate markers of disease activity revealed another diagnostic important immunopathological feature in MS, that is the presence of oligoclonal bands (OCBs) in cerebrospinal fluid (CSF). The CSF can be considered a 'window to the brain'; moreover, OCBs that arise from the continuous intrathecal synthesis of immunoglobulins (predominantly oligoclonal IgG's) are present in more than 90% of patients with MS. Testing of CSF obtained from a lumbar puncture can provide evidence of chronic inflammation of the central nervous system. Currently, the presence of these bands on isoelectric focusing (IEF) gels is one of the reliable laboratory tests for the diagnosis of inflammatory disorders of the nervous system, such as MS (Fig. 3) [9]. In patients with a single demyelinating episode (clinically isolated syndrome (CIS)), detection of intrathecal immunoglobulin synthesis may predict progression to clinically definite MS (CDMS), and OCBs in CSF during the early phase of disease are associated with a worse outcome [10].

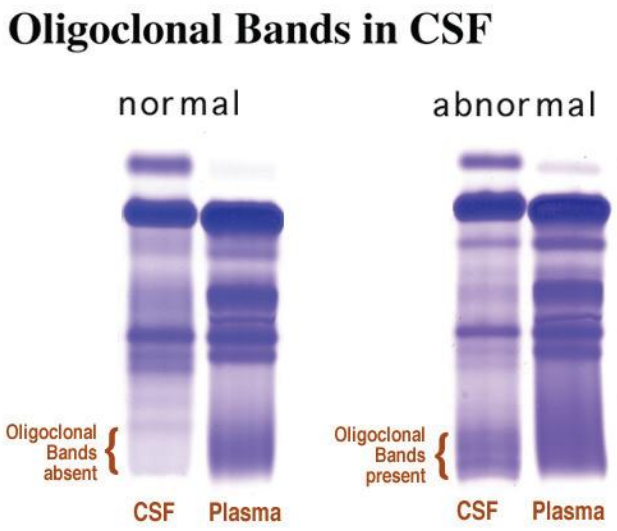

Figure 3 Oligoclonal IgG are the products of oligoclonal plasma cells that secrete immunoglobulins, which migrate as discrete bands after electrophoretic separation. These bands can be detected in the CSF of most (>95%) patients with MS, persist throughout the course of the disease, and are a diagnostic hallmark of MS; however, their antigenic specificity is largely unknown [11].

The nervous system of a person with MS responds less actively to stimulation of the optic nerve and sensory nerves due to demyelination of such pathways. As an additional control in MS diagnosis, these brain responses can be examined using visual and sensory evoked potentials.

1.1.3 Therapeutic approaches

Over the last two decades, therapeutic approaches to MS have emerged to reduce the relapse frequency and the rate of disability progression. First-line therapies include interferon-β formulations (Avonex, Biogen-Idec and Rebif, Merck-Serono)and glatiramer acetate (Copaxone, Teva). Second-line therapies include humanized antibodies, e.g. natalizumab (Tysabri, Elan/Biogen-Idec) that recognizes the α4 subunit of α4β1 integrin, a cell adhesion molecule expressed on lymphocytes. All of these therapies target the immune system and require parenteral delivery through injection or infusion, which can be associated with both patient compliance issues as well as deleterious side effects. Interferons are often associated with injection-site reactions and flu-like symptoms, and other less commonly reported events including liver dysfunction and cytopenias. Of greater concern with the efficacious agent natalizumab is the increasing incidence of progressive multifocal leukoencephalopathy (PML),

a rare but serious infection associated with immunosuppression that has resulted in multiple fatalities in MS patients receiving this therapy. Another example of therapy is the cytostatic agent mitoxantrone, that has a cumulative dose-dependent cardiac toxicity with additional risk of leukemia, both of which limit its long-term use. Drugs under development for MS include the monoclonal antibodies named rituximab, ocrelizumab, and ofatumumab (which target CD20 to deplete B cells); alemtuzumab/ Campath-1H (which targets CD52 to deplete T and B cells and some monocytederived dendritic cells); and small molecules including the oral agents. Among others, cladribine (a cytotoxic adenosine deaminase-resistant purine nucleoside,

recently withdrawn from commercial development), dimethylfumarate BG-12 (an activator of the nuclear factor-E2-related factor 2 (Nrf2) transcriptional pathway that alters glutathione levels, but is also associated with tumorigenesis and immunosuppression), laquinimod (unknown cellular target), teriflunomide (an inhibitor of dihydroorotate dehydrogenase, which catalyzes the rate-limiting step in the de novo synthesis of pyrimidines) and FTY720/fingolimod (commercial name Gilenya, Novartis; it's the first oral MS therapy approved by the United States Food and Drug Administration (FDA), the European Union, and several other countries, which interacts with the sphingosine 1-phosphate (S1P)). All of these agents target lymphocytes as well as other immune-related cells, and currently remain subject to additional clinical and regulatory evaluation before possible entry into the therapeutic armamentarium [12].

1.2 Etiology and pathogenesis of MS

The total number of people living with MS worldwide is estimated to be 2–2.5 million. The disease is unevenly distributed throughout the world: its prevalence varies between < 5 cases per 100,000 people in tropical areas or Asia and >100–200 cases per 100,000 in temperate areas, especially those with large populations of Northern European origin, including the United States, Canada, New Zealand and parts of Australia (Fig. 4) [13]. It is a widely held notion that MS frequency increases progressively with geographic latitude in an incomplete distribution model. Nonetheless, there are pockets of high MS frequency such as Sardinia in the warm, southerly Mediterranean region [14], while among Inuit people living in Canada's cold, far north, MS frequency is low [15], contrary to what would be predicted from a simple ambient temperature/latitudinal geographic model of MS distribution. The irregular geographic distribution of MS and ethnic differences in MS frequency have attracted the interest of medical investigators for almost a century, reasons for its irregularity have remained elusive. Nonetheless, a general latitudinal gradient in MS frequency persists, even in recent observations [16]. The large range of incidence and prevalence seen in different regions in some countries does not necessarily follow latitudinal or other geographic gradient, and may imply the influence of both environmental and genetic factors [17].

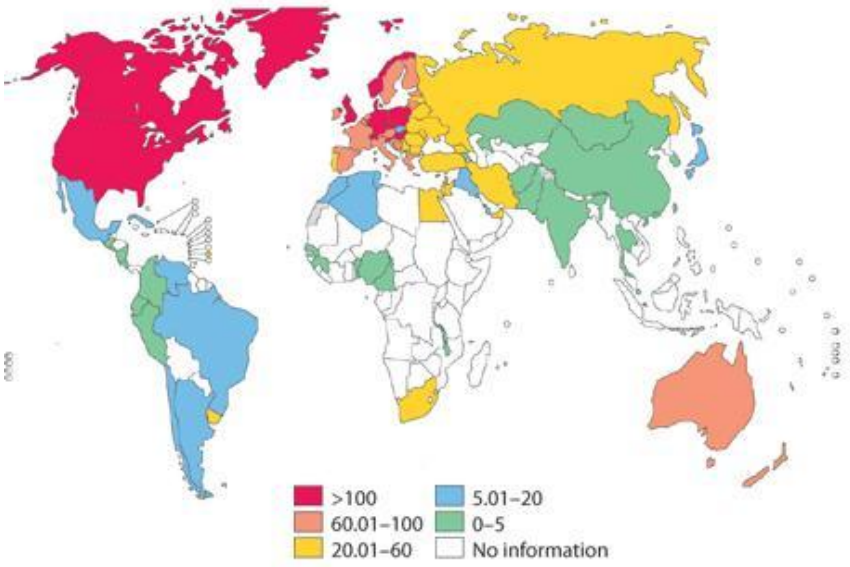

Figure 4 Prevalence of multiple sclerosis (x 100.000)

1.2.1 Immune genetic background and immune pathogenesis

The etiology of MS is unknown, but many findings indicate a central role of the immune system in the disease pathogenesis, and both genes and environmental factors influence the risk of developing disease. The disease predominantly affects northern Europeans and there is a familial recurrence rate of about 15%. The age-adjusted risk is higher for siblings (3%), parents

(2%), and children (2%) than for second-degree and third-degree relatives. Recurrence in monozygotic twins is around 35%. The risk for half-siblings is less than for full siblings. Recurrence is higher in the children of conjugal pairs with multiple sclerosis (20%) than in the offspring of single affected (2%) (Fig. 5) [2].

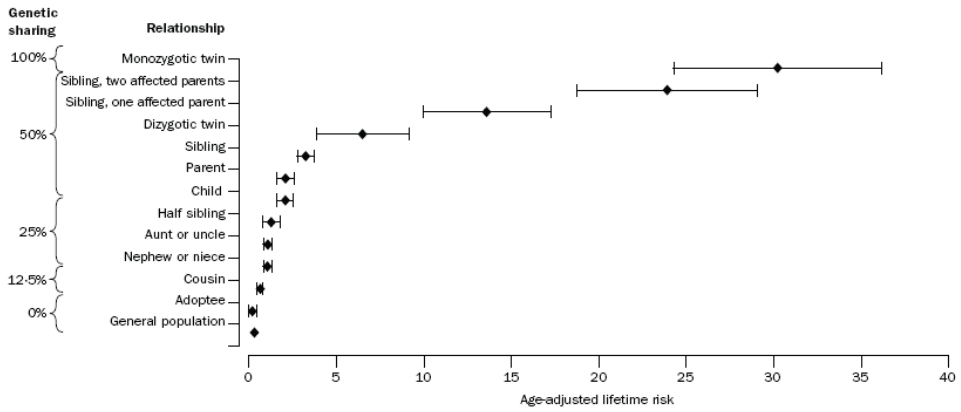

Figure 5 Age adjusted recurrence risks for different relatives of probands with multiple sclerosis [2].

Family and genetic studies indicate a highly polygenetic mode of inheritance in MS. Genes responsible for complex traits are not mutations coding for aberrant gene products but normal polymorphisms that exert a small contributory effect on some as yet undefined structure or physiological function. Extensive searches suggested that the human leukocyte antigen (HLA) region is the major gene locus associated with MS disease. The HLA-DR1501 and HLA-DQ0601 alleles, which encode restriction elements of T cells, are associated with a 2–4-fold increased risk of developing MS in white

populations. In detail, the presence of HLA-DR1501 allele in homozygosity increases the risk of MS more than 6-fold [18]. For almost forty years, HLA has been the only genetic locus consistently linked to the disease; this changed in 2007 when for the first time was identified a single-nucleotide polymorphism (SNP) within a nonmajor histocompatibility complex (MHC) gene. Specifically, this gene encodes for the interleukin 7 receptor alpha chain (IL7R) and its non-synonymous SNP rs6897932 (T→C, T244I) is independent of the known HLA effect [19]. To date, Genome-Wide Association Studies (GWAS) have identified more than 20 additional non-MHC risk loci containing associated SNPs and involved in disease susceptibility [20].

Altought etiology and pathogenesis of MS are still not resolved, it's widely accepted that MS is a T cell-dependent autoimmune disease, with activation and entry into the CNS of T cells specific for myelin antigens. This view is based on the cellular composition of the brain, on the CSF-infiltrating cells and on data from the most commonly and accepted studied model of MS, that is EAE. Healthy individuals harbour autoreactive myelin T cells, normally kept in check by regulatory T cells. Failure of this regulation leads to proliferation, activation, and entry into the circulation of autoreactive T cells; they express adhesion molecules and induce reciprocal changes in endothelia, allowing access across the blood-brain barrier into the CNS. There, activated T cells re-encounter antigen and activate microglia (the CNS macrophage); they, in turn, express class II molecules, re-present antigen to T cells, and set up a proinflammatory loop. One hypothesis to explain the breakdown of immuneregulation in MS is the molecular mimicry, which suggests that peptide (the environmental factor), presented in the groove of specific class II molecules (one component of inherited risk), is immunologically

indistinguishable from self-antigen and, hence, an appropriate response to infection generates inappropriate inflammation against some component of the oligodendrocyte-myelin unit [2]. To date it's known that immune attack to the nervous system by autoreactive lymphocytes that penetrate the blood-brain barrier involves not only the cellular immunity, with T cells direct against myelin and oligodendrocytes, but also the humoral immunity, with the secretion of anti-myelin antibodies from B cells and subsequent fixation of complement and opsonization of the myelin sheath and the oligodendrocyte by macrophages (Fig. 6) [1].

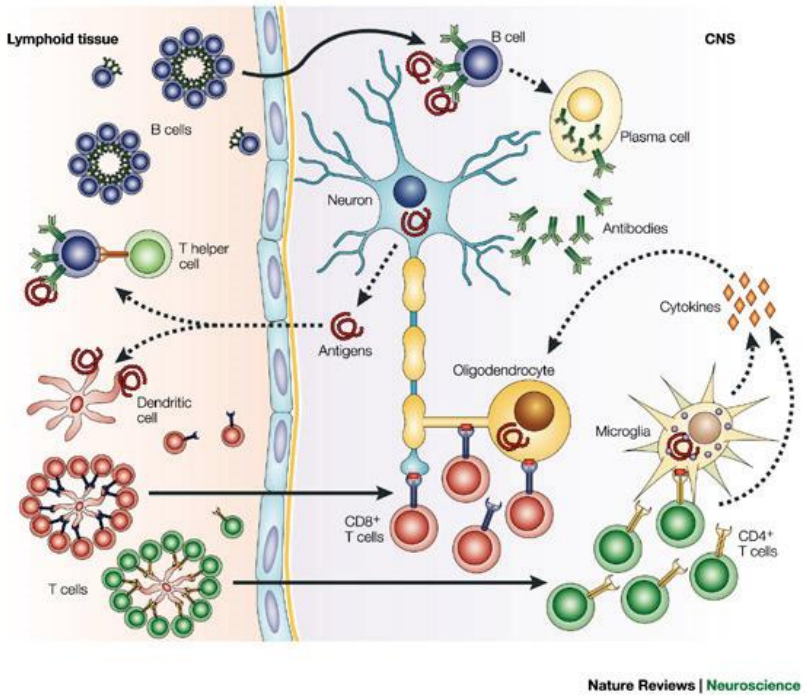

Figure 6. Immunopathogenesis of multiple sclerosis. Activated antigen-specific T cells and B cells cross the blood–brain barrier and target self antigens expressed by oligodendrocytes and neurons [21].

Although all the major immune cell types have been implicated in the pathogenesis of MS, CD4+ and CD8+ T cells being the most commonly and historically studied. In general, CD4+ T helper cells (Th) recognize peptides that are presented by MHC class II molecules on specialized antigen-presenting cells (APC) and are usually derived from antigens ingested by the APC. Once activated, naive Th cells differentiate into functional subsets: the so-called pro-inflammatory Th1 cells, which predominantly produce interferon (IFN)-g and tumour necrosis factor (TNF)-a, protect against intracellular pathogens; and Th2 cells, which produce interleukin (IL)-4, IL-5 and IL- 13, and help to combat extra cellular infections. By contrast, CD8+ T cells mostly recognize peptides from the endogenously synthesized antigens presented by MHC class I molecules which, in general, are expressed by all nucleated cells. They mediate effector functions through production of cytokines, such as IFN-g and TNF-a, and/or by direct cytotoxicity [3].

CD4+ T cells have long been presumed to play an almost exclusive role throughout the disease. This hypothesis was supported by many parallels with EAE, that can be adoptively transferred to mice by the injection of encephalitogenic myelin-specific Th cells, and also by the fact that certain HLA class II molecules represent the strongest genetic risk factor for MS, presumably via their role as antigen-presenting molecules to pathogenic CD4+ T cells [3]. The necessity to re-evaluate the role of different cell types in MS was suggested when a phase II clinical trial testing the effect of Ustekinumab, an antibody directed against the common subunit of IL-12 and IL-23, relevant for Th1 and Th17 cell differentiation respectively, reported no

benefit in RRMS [22]. In confirmation, another antibody directed against CD4, which successfully depleted CD4+ T cells, was also found to have no effect on disease activity or course in patients with MS [23]. In clear contrast, analogous antibodies prevented the development of EAE in animals [24]. These trials even demonstrate why, although animal models represent useful and essential tools in modern research, one needs to be aware of their distance from the human disease. Recently, the interest toward CD8+ T lymphocytes has been strengthened by several reports showing their contribution to CNS tissue damage and the correlation with progression and severity of disease [25; 26]. In demyelinating plaques, CD8+ usually outnumber CD4+T cells and undergo local clonal expansions more frequently than CD4+T cells. Moreover, specific enrichment and oligoclonal expansions of memory CD8+ T cells were found in CSF of MS patients [27; 28]. Based on the fact that the model of MS as a disorder mediated only by T cells is overly simplistic, further studies have proposed an important role even for B cells in the propagation of the disease. For this reason, over the past two decades B cells have increasingly moved into the spotlight in MS research. Growing understanding and acceptance of pathological involvement of B cells and antibodies in MS derived from data in animal models of MS, human histopathological studies, and analyses of B cells in the peripheral blood and CSF [29]. In particular has been noticed that most active MS lesions from autopsy are composed of a mixed population of T and B cells and characterized by the deposition of immunoglobulins, Fcγ receptors and terminally activated complement [30; 31]. Furthermore, B lymphocyte infiltration into the CNS and levels of intrathecal IgG synthesis correlate with disease severity and parenchymal inflammation [32], and anti-B cell therapies are the only cell-specific treatments efficacious in RRMS patients

[33]. Lastly, environmental factors associated with MS susceptibility (vitamin D and the EBV) influence B cell proliferation and function.

Specifically, vitamin D plays an important role in B cell homeostasis by decreasing cell proliferation, inducing apoptosis, inhibiting plasma cell differentiation, and regulating the expression of genes associated with MS susceptibility in B cells (including HLADRB1, CD40, CXCR4, and CXCR5); EBV establishes a latent infection in B cells, promoting their proliferation and activation [34].

Although the precise role of B cells in disease is presently unknown, it could involve antigen presentation to pathogenic T cells, the production of proinflammatory cytokines, or the synthesis of antibodies to unknown viruses or autoantigens that promote oligodendrocyte loss or tissue destruction.

A growing number of recent publications have emphasized the potential importance of B cells in the pathology of MS. The renaissance of this relatively neglected area reflects an increasing appreciation of the failings of standard T cell dominated animal models of CNS demyelination fully to represent the human disease and its response to treatment [35]. In fact EAE, induced in animals by intradermal injection with whole or parts of various proteins that make up myelin or directly with myelin-specific Th cells, only resembles some forms and stages of MS; hence, a number of significant assumptions made when proposing EAE as MS may only be partially true [36]. In parallel, there has been a progress in our knowledge of the basic immunology of the B cell lineage in terms of both antibody-dependent and antibody-independent roles. Currently it’s well-known that B cells may be involved at many levels in the pathogenesis of MS, starting from the earliest stage of antigen capture, continuing through the processes of tissue damage and even extending through to remyelination and repair [35].

1.3 Environmental factors: non infectious factors

Epidemiological and clinical studies have shown that environmental factors such as infections, smoking and vitamin D are associated with the risk of developing multiple sclerosis (MS). Furthermore, the high discordance rate among monozygotic twins as well as the multitude of migration and epidemiological studies that show latitude-dependent north to south gradient of increasing disease incidence (in the northern hemisphere and the opposite in the southern) and remarkable differences in risk among people of common ancestry who migrate to areas of low or high MS prevalence [37], indicate that MS is a complex multifactorial disease that results from the intricate, yet not well-understood, interaction of both genetic and environmental factors. The quest for identification of the potential environmental triggers of MS has been ongoing for decades. Both infectious and noninfectious agents have been implicated as potential culprits. Yet, the plethora of controversy in the field makes it difficult to discern one single environmental factor as a sole trigger of MS. Rather it is more likely that MS susceptibility is dependent on the elaborate interaction of a variety of low penetrance genetic factors and environmental agents conveying by themselves low risk, but when combined together resulting in disease initiation.

Recent epidemiological studies have identified new risk factors, not only associated with the risk of developing MS, but also, and perhaps more immediately important, with the MS disease course. While previous factors associated with MS disease course, such as gender or the age at disease onset, are not modifiable, the newly identified factors such as smoking and vitamin D levels are modifiable through individual and public health measures.

Future studies on risk factors in MS will need to build upon the classical epidemiological studies and integrate the new factors into the "big picture". Such studies will need to unravel how the whole set of classic and newly identified factors interact with each other, how they are modulated by immunomodulatory drugs, genetic factors, gender and hormones, and at which stages of disease they have their greatest impact [38].

1.3.1 Vitamin D

Among the non-infectious environmental triggers, there is a recent upsurge in studies investigating the effects of vitamin D levels on MS pathogenesis. Vitamin D is a potent immunomodulator affecting proinflammatory pathways as well as the number and activity of regulatory T cells [39]. Epidemiological studies have shown an increase in MS frequency with increasing distance from the equator, which inversely correlates with duration and intensity of sunlight. Interestingly, populations situated at high latitudes but having high consumption of vitamin D rich food were observed to have reduced MS prevalence while the risk of MS incidence decreased with movement from high to low altitudes [37]. Timing of birth has also been implicated as a predictor of MS susceptibility. A study including a large data set of more than 40000 MS patients from Sweden, Denmark, Canada and Great Britain revealed that significantly fewer individuals with MS were born in November, while the incidence of MS was significantly higher for people born in May, indicating an association between prenatal sunlight and risk of

MS. Interestingly, the effect of month of birth was more evident for familial cases, suggesting a potential gene/ environment interaction [40]. There is also mounting evidence that vitamin D influences the disease course of MS. Cross-sectional studies showed that low levels of vitamin D and lower bone mineral density, are common in MS, and lower vitamin D levels are associated with higher levels of disability [41]. Although other immune modulatory mechanisms potentially exist for ultraviolet radiation [42], the association of sunlight with MS is most likely due to the ultraviolet B radiation of sunlight (290–320 nm) converting cutaneous 7-dehydrocholesterol to previtamin D3, which then spontaneously forms vitamin D3. The latter then undergoes 2 hydroxylations, most notably in the liver and kidney by D-25-hydroxylase (CYP2R1) and 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1), to produce the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3 (Fig. 7). Notably, variants of the CYP27B1 gene have been found to be associated with increased risk of MS [43]. Another study found association of MS with CYP27B1 and a second region containing another vitamin D associated gene, CYP24A1 [20], a vitamin D response element lies close to the promoter region of HLA-DRB1, the main risk allele for MS [44].

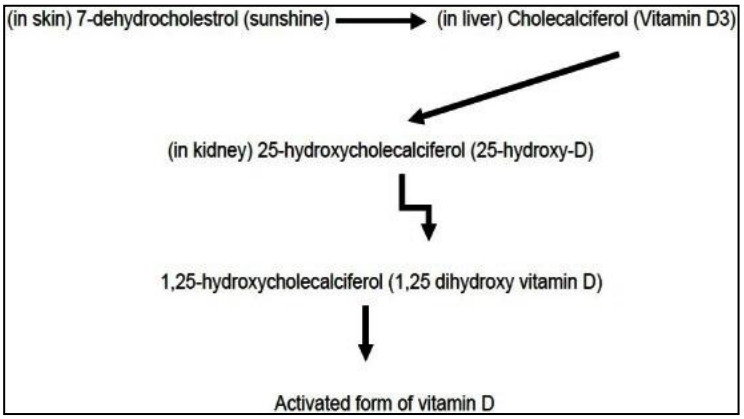

Figure 7 Fate of vitamin D from skin to blood circulation [48]

The utility of vitamin D in MS is almost certainly due to its multiple immune-regulating properties that impact on the majority of the immunopathogenic steps outlined in figure 8. Vitamin D suppresses the maturation and activity of APCs, including dendritic cells, or increases their "tolerogenic" phenotype, thereby reducing the probability of presenting self-antigens to generate autoreactive cells, or decreasing the likelihood of expanding autoreactive cells through molecular mimicry.

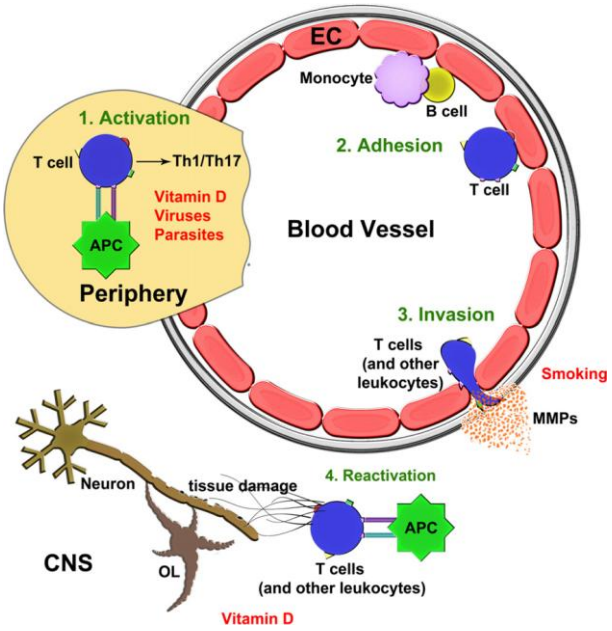

Figure 8 Steps in the immunopathogenesis of MS and modulation by environmental factors [47].

The differentiation of CD4+T helper cells is also affected by vitamin D, with a reduced production of pro-inflammatory Th1 and Th17 cells [45]; concurrently, Th2 cells are increased, thereby helping to reduce the pro-inflammatory state that typifies MS. Other major immunologic consequences of vitamin D treatment are the increased activity of regulatory T cells and the reduction of pro-inflammatory molecules produced by stimulated monocytes. These immunologic changes caused by vitamin D can be detected in culture, in samples obtained from MS patients and in EAE. In EAE, treatment with vitamin D either prevents the development of symptoms when given early in the disease course, or reduces the severity of EAE when treatment is initiated at onset of clinical signs or at peak disease. Vitamin D is known to penetrate into the CNS, and it may potentially have several activities within the CNS

including the reduction of antigen presentation to reduce neuroinflammation. More intriguing is the possibility that vitamin D may be neuro-protective. The enzymes necessary to synthesize the bioactive 1,25-dihydroxyvitamn D3 are present in the brain [46]. Overall, there is substantial evidence that vitamin D corrects many of the immune abnormalities seen in MS. It is not clear which of its multiple mechanisms are most critical to its therapeutic efficacy. Whether some of the benefits of vitamin D may relate to its actions within the CNS remains to be determined [47].

1.3.2 Smoking

A history of cigarette smoking has been almost unanimously associated with increased susceptibility to MS. It remains to be determined whether smoking is indeed an independent risk factor for MS development. The biological mechanisms that would link smoking to the pathogenesis MS are elusive. Nicotine, however, is not be the sole culprit since studies have shown that use of tobacco snuff does not increase the risk of MS [49]. It is not known how smoking increases the risk of MS and although cigarette smoke contains many mutagens which potentially may affect long lasting immunity, a recent review indicates that smoking generally leads to an immunosuppressant state [50]. Nonetheless, aspects of immune functions are promoted by cigarette smoking and genetic studies indicate an interaction between smoking and genes that regulate immune function [51]. However, it is highly improbable that this environmental factor alone account for the worldwide variation in MS prevalence [47].

Observational studies suggest that cigarette smoking may exert an influence on the course of established MS. Outcomes that have been linked to smoking effects include measures of disease activity (clinical relapses and development of new lesions visible on MRI), more rapid conversion from a first ever demyelinating clinical event (also known as a clinically isolated syndrome (CIS), typically optic neuritis or partial myelitis) to confirmed MS, the rate of conversion from relapsing–remitting to secondary progressive MS, and the rate of neurological deterioration once progressive MS has been established. Evaluation of the magnitude of the effect of smoking on the course of established MS may shed light on underlying disease mechanisms and is important because more than half of MS patients smoke at some time [52]. The epidemiological evidence reported to date suggests that smoking is probably associated with a greater risk of conversion from relapsing–remitting to secondary progressive MS. Early and heavier cigarette consumption may increase the risk of primary progressive MS compared with a relapsing–remitting onset. Smoking may increase the rate of conversion from CIS to confirmed MS and increase the rate of accumulation of disability in established progressive forms of MS. The effects of smoking on the response to disease-modifying therapy are not known [53].

1.3.3 Diet

It is commonly accepted that nutrition is one of the possible environmental factors involved in the pathogenesis of multiple sclerosis (MS), but its role as

complementary MS treatment is unclear and largely disregarded. At present, MS therapy is not associated to a particular diet, probably due to lack of information on the effects of nutrition on the disease. To overcome the distrust of the usefulness of dietary control in MS and to encourage nutritional interventions in the course of the disease, it is necessary to assess the nature and the role of bioactive dietary molecules and their targets, and establish how a dietary control can influence cell metabolism and improve the wellness of MS patients [54]. Studies on the relationship between diet and MS are very few, and diets available to the MS patient are often lacking a scientific basis. There is, however, an apparently great need to apply non-conventional therapies, and the majority of MS patients often use complementary or alternative treatments without informing the physician [55]. The most intriguing aspects of MS are perhaps its uneven geographical distribution and the influence of migration in young age on disease course [56]. To understand how diet can exacerbate or ameliorate MS symptoms, it is necessary to identify the dietary molecules, their targets and the molecular mechanisms involved in the control of the disease [54]. Cells possess specific sensors to adapt themselves to changes in their environment, and changes in content and type of food molecules are the most common over time. Dietary molecules exert their direct influence on metabolism by modulating the expression and the activity of enzymes, hormones, transcription factors, and nuclear receptors [57]. The influence of dietary molecules and some common drugs on metabolism and inflammation is sketched in figure 9.

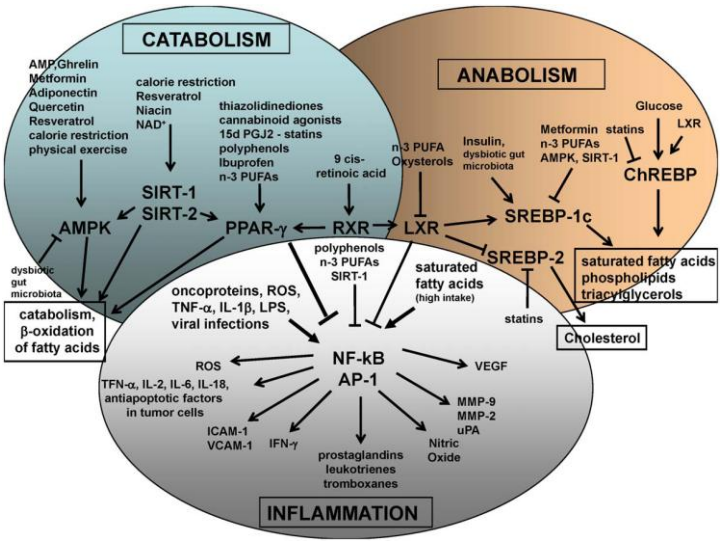

Figure 9 Schematic representation of the influence of some natural dietary compounds and some common drugs on cell transcriptional activity in metabolism and inflammation [54].

In MS, the transcription factors involved in inflammation and autoimmunity, the activator protein (AP-1) and the nuclear transcription factor kB (NF-kB), are activated and induce the expression of several pro-inflammatory genes and the production of pro-inflammatory molecules. The peroxisome proliferator-activated receptors (PPAR/RXR) complex exerts a tight control over the expression of inflammatory genes by inhibiting NF-kB and AP-1, thus integrating metabolic and inflammatory signaling by inhibiting inflammatory gene expression. Thus, through their binding to PPARs or directly by their binding to NF-kB and AP-1, dietary molecules can inhibit the inflammatory process [58].

Among the dietary factors that have been considered most frequently for their deleterious influence on MS are saturated fatty acids of animal origin. In

1950, Swank suggested that the consumption of saturated animal fat is directly related to the frequency of MS, and in 2003 Swank and Goodwin reported that restriction of saturated fat induces remission of the disease. These effects were ascribed to the formation of large fat aggregates and obstruction of small capillaries. However, the influence of fat on cells is certainly more complex, since it is directed towards gene expression and cell growth and differentiation and is controlled at transcriptional level [57; 59]. Moreover, animal fats lead to the synthesis of cholesterol and decrease membrane fluidity, activate the CD14/TLR4 receptor complex, increase blood levels of C-reactive protein, and lead to the formation of TNF-alpha and to dysbiotic gut microbiota.

Environmental (microbial or dietary) factors can be associated with autoimmunity by molecular mimicry [60] and epidemiological studies have shown that some proteins from cow's milk could be responsible for the link between milk consumption and MS [61] Abnormal T cell immunity to several cow milk proteins was observed in MS patients and in children with autoimmune disease and CNS injury. However, the milk proteins that appear to be more detrimental in MS are the proteins of the milk fat globule membrane (MFGM proteins) and in particular butyrophilin (BTN). BTN is very similar to myelin oligodendrocyte glycoprotein (MOG), the MS candidate autoantigen, and induces experimental inflammatory responses in the CNS. MOG/BTN cross-reactive antibodies have been found in MS [62]. Specific dietary molecules might be able to counteract inflammation, autoimmunity, oxidative stress, and angiogenesis associated with MS, and for this reason they are considered as ''healthy'' molecules that can improve the wellness of MS patients. Polyphenols are bioactive molecules found in vegetables, fruits, spices, herbs, soy, tea, wine and other fruit-based

beverages [63]. These dietary molecules are well known for their antioxidant activity, but recent findings indicate that they have additional neuroprotective properties, independent of their roles as antioxidants and radical scavengers. Polyphenols include well-known flavonoids (quercetin, catechins, genistein) and non-flavonoids (resveratrol, hydroxytyrosol and curcumin) molecules. Most polyphenols contain the catechol group, which has been recently associated with anti-inflammatory activity in stimulated microglia and neutrophils [64]. In general, polyphenols have antioxidant, antibacterial, antifungal, antiviral, anti-aging, anti-angiogenic, anti-carcinogenic, anti-proliferative and anti-inflammatory effects, and have been found to prevent neurodegenerative, cardiovascular, metabolic, autoimmune, and neoplastic diseases. They pass the blood brain barrier and ameliorate experimental allergic encephalomyelitis (EAE) [65; 66; 67; 68; 69].

Unsaturated vegetable oils are the alternative to saturated animal fat. They contain the essential fatty acids linoleic acid (n-6) e linolenic acid (n-3). The n-6 and n-3 fatty acids have opposing effects and their intake in the diet should be equivalent in healthy individuals. However, in Western diets, the n-6/n-3 ratio is largely increased and this causes a greater incidence of cardiovascular and inflammatory diseases. Few clinical trials indicate that n-3 PUFA may represent a good complementary treatment in the course of MS [70; 71]. Fish oil may be useful also in healthy conditions to improve motor performances. Seed oils, from sunflower, corn, soybean, and sesame, contain more n-6 than n-3 fatty acids and their consumption should be limited in MS. Coconut oil has a high content of saturated fatty acids and is not indicated for MS patients. Olive oil is a good alternative to the oils reported above. Hydrogenated fatty acids are found in margarine and in other treated fat, or are formed in frying. Differently from the natural ones, they are trans fatty

acids (TFAs) and interfere with PUFA metabolism. The TFAs show pro-inflammatory effects and increase the levels of IL-6, TNF-□ and C-reactive protein [72].

As discussed above, vitamin D plays an important role in the pathogenesis of MS linked to geographic latitude and exposure to the sun. Also, nicotinic acid and vitamin B12, vitamin E and vitamin C should be taken into consideration [73; 74]. In particular, niconitic acid activates the PPARs (indirectly) and the sirtuins (directly), stimulates the synthesis of prostaglandins 15d-PGJ2 and PGE2, raises HDL levels, corrects dyslipidemia, and in the form of nicotinamide protects the CNS from demyelination. Oligoelements and other useful compounds with antioxidant activity that can be associated to the MS diet are: selenium, alpha-lipoic acid (ALA), N-acetylcysteine94 (NAC) and glutathione. ALA has immunomodulating effects, inhibits T cell migration, stimulates cAMP production, inhibits IFN-gamma synthesis, and is effective in the treatment of EAE. NAC, a precursor of reduced glutathione, is beneficial for antioxidant protection and energy metabolism [75; 76].

Recent research data from in vivo and in vitro experiments appear to substantiate at molecular level a clear anti-inflammatory role of hypocaloric, low-fat diets and bioactive dietary supplements, and suggest that they might slow down the occurrence of relapses in relapsing-remitting MS. These evidences open new perspectives for further clinical trials based on the complementary treatment of chronic inflammatory diseases such as MS. However, the most important challenges in the future are represented by clinical studies. Clinical trials should be done by teams of neurologists, nutritionists, gastroenterologists and physiotherapists or exercise trainers to assess the impact of a nutritional intervention and exercise on the frequency of relapses and the wellness of patients [54].

1.4 Infectious environmental factors

The hypothesis of an infectious etiology of MS was advanced many years ago, at the time that the disease was described by JM Charcot. His student, Pierre Marie, in 1884 asserted an infectious etiology for MS, with the possibility of the development of a vaccine against the disease. Several studies implicate infectious and non-infectious environmental factors present during childhood and young adulthood as strong determinants of MS risk, although their nature remains unidentified [77]. Microbial infections can act as triggers inducing or promoting autoimmunity, resulting in clinical disease manifestations in genetically predisposed individuals. Alternatively, infections might accelerate sub-clinical autoimmune processes [78]. Many hypotheses have been proposed to help explain to what extent an infectious agent might affect MS epidemiology. The 'polio' hypothesis, postulates viral infections acquired in late childhood or adulthood increase MS risk, but cause little harm and confer protective immunity when acquired in infancy [79]. Another theory, proposed by Kurtzke, is the prevalence hypothesis, based on investigations conducted during the Faroe Islands epidemic. It postulates MS is caused by a pathogen, common in regions of high disease prevalence, a widespread agent causing asymptomatic and persistent infection in most individuals which may generate neurological symptoms years later [80]. As experience with infectious pathogens accumulated, particularly viruses, the possibility of a viral etiology of MS became more acceptable (Table 1). With the demonstration of a viral etiology of progressive multifocal leukoencephalopathy and subacute sclerosing panencephalitis, interest in viruses increased considerably. The biologic evidence for an infectious

etiology for MS includes the association of MS with infectious mononucleosis, the presence of pleocytosis, and the intrathecal synthesis of antibodies and oligoclonal bands detectable in the cerebrospinal fluid (CSF), high immune reactivity to particular viruses, and the example of other human and animal viral demyelinating diseases [81].

Table 1

Rabies virus	1946
Herpes simplex virus	1964
Scrapie agent (prion)	1965
MS-associated agent	1972
Parainfluenza virus 1	1972
Measles virus	1972
Simian virus 5	1978
Chimpanzee cytomegalovirus	1979
Coronavirus	1980
Epstein-Barr virus	1981
SMON-like virus	1982
Tick-borne encephalitis virus	1982
HTLV-1	1986
LM7 (retrovirus)	1989
HSV-1	1989
VZV	
HHV-6	1997

Table 1 Viral detection from MS brain or CSF [81]

The hypothesis of "bystander activation" postulates that autoreactive T cells are activated by nonspecific inflammatory molecules occurring during infections, such as cytokines, superantigens and toll-like receptor ligands [3]. Yet another hypothesis is that viruses activate immune cells through the process of "molecular mimicry" [82]. The hypothesis postulates that upon exposure to a pathogen, the pathogen/MHC conformation on an APC bears

molecular similarity to that of a endogenous peptide, such as a fragment from myelin basic protein (MBP), presented within an MHC. If co-stimulatory molecules are properly engaged, the result could be the expansion and differentiation of not only the pathogen-reactive T cells, a properly directed immune response, but also the expansion of MBP-reactive T cells, an improper outcome. If both pools differentiate into Th1 or Th17 pro-inflammatory subsets these can become reactivated within the CNS to promote pathology. In support of this idea, T cell lines isolated from MS patients demonstrate cross-reactivity between MBP and corona virus or Epstein-Barr virus (EBV) antigens [83]. Overall, given that, there are several pathogens with molecular similarity to a number of myelin peptides and other molecules within the CNS, the probability is high that several viruses can lead to the improper expansion of CNS-reactive T cells that can promote pathology within the CNS. For this reason it will be difficult to pinpoint a single infectious pathogen uniquely associated with MS [47].

1.4.1 Viral infections: EBV

The Epstein–Barr virus (EBV), also called human herpesvirus 4 (HHV-4), is a virus of the herpes family, and is one of the most common viruses in humans. EBV has the unique ability to infect, activate and latently persist in B lymphocytes for the lifetime of the infected individual [84]. It is transmitted by oral contact (‘kissing disease’) and readily infects the recipient. On the basis of careful epidemiological studies, Niederman et al. showed that of all acute EBV infections, there were two who were

symptomatic infectious mononucleosis to each asymptomatic infection [85]. In children, acute EBV is generally asymptomatic, whereas in young adults, the clinical illness known as infectious mononucleosis (IM) usually occurs. In the higher latitudes, infection is acquired later in life and is, therefore, much more common than near the equator [86].

During primary infection, EBV transmitted via saliva infects naive B cells in the tonsil through binding of the viral surface glycoprotein gp350 to complement receptor 2 (CR2; also known as CD21), which is expressed by mature B cells and follicular dendritic cells [87]. EBV drives the infected B cell out of the resting state to become an activated B blast and then exploits the normal pathways of B cell differentiation so that the B blast can become a latently infected resting memory B cell (Fig. 10) [88].

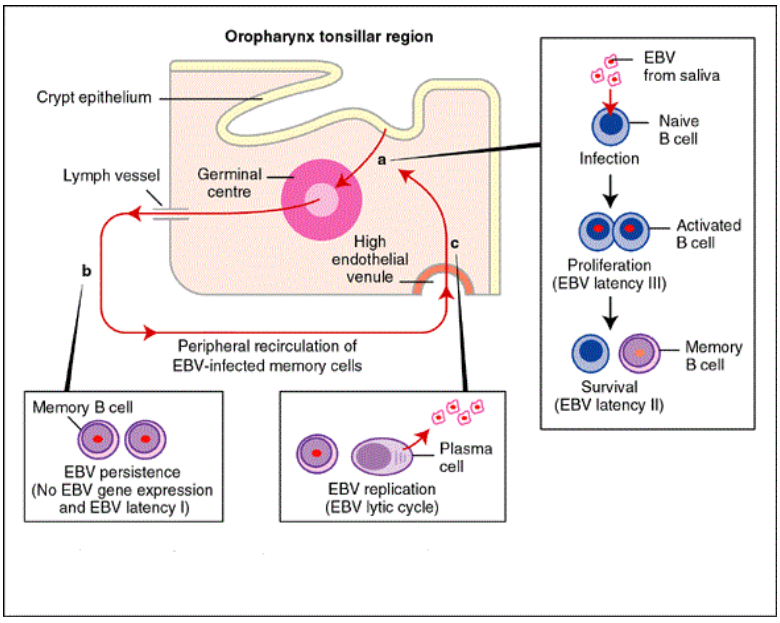

Figure 10 A model for Epstein–Barr virus (EBV) infection and persistence. This model is derived from work published by Thorley-Lawson and colleagues [88].

To achieve this, the virus deploys a series of different latency transcription programs. On initial infection of naive B cells, EBV uses the latency III or "growth" program in which all viral latent proteins are expressed—namely, the Epstein-Barr nuclear antigens (EBNA) 1, 2, 3A, 3B, 3C, and LP and the latent membrane proteins (LMP) 1, 2A, and 2B. This activates the B cell to become a B blast, which then enters a germinal center in the tonsil where it down-regulates the expression of the EBNA proteins 2, 3A, 3B, 3C, and LP. The continuing expression of EBNA1, LMP1, and LMP2 (latency II or "default" program) allows the infected B cell to progress through a germinal center reaction to become a memory B cell, which exits from the germinal center and circulates in the blood. The EBV-infected memory B cell expresses no viral proteins except during cell division, when it expresses only EBNA1 (latency I), which engages the host cell DNA polymerase and ensures transmission of the viral genome to the daughter cells. The lack of viral protein expression allows the virus to persist in memory B cells despite a healthy immune response [88]. Latently infected memory B cells returning to the tonsil and they can terminally differentiate into plasma cells, which initiates the lytic (replicative) transcription program with the production of infectious virus. The resulting free virions infect tonsil epithelial cells, where the virus replicates at a high rate and is continuously shed into saliva for transmission to new hosts [89]. Newly formed virus can also infect additional naive B cells in the same host. Latently infected memory B cells display the molecular hallmarks of classical antigen-selected memory B cells namely, somatic hypermutation and class-switch recombination of their immunoglobulin (Ig) genes [90]. In normal B cell differentiation, naïve B

cells are activated by antigen through the B cell receptor (BCR) and by T cell help through the CD40 receptor so that they proliferate and progress through a germinal center reaction. Remarkably, LMP2A and LMP1, which are expressed by EBV during latency II and latency III, mimic the antigen-activated BCR and the activated CD40 receptor, respectively. In vitro LMP2A can mimic and replace constitutive BCR signalling and thereby support an activated, proliferative state in B cells, which it renders resistant to apoptosis [91]. EBV infection is normally kept under tight control by EBV-specific immune responses, especially by cytotoxic CD8+ T cells, which eliminate proliferating and lytically infected B cells [92]. Even if, during the incubation period, the cycle of infection, B cell activation, germinal center reaction, lytic replication, and reinfection initially goes unchecked by cytotoxic CD8+ T cells because of the delay in mounting an adaptive immune response. As a result, the number of latently infected memory B cells during infectious mononucleosis can rise to half and perhaps even higher of the peripheral memory B cell compartment. Eventually, the infection evokes a massive expansion of activated EBV specific CD8+ T cells, which rapidly control the infection by killing a high proportion of the EBV-infected B cells. With the rapid decline in the EBV viral load, the number of EBV-specific CD8+ T cells also rapidly declines toward the levels found in persistently infected healthy virus carriers. It has been suggested that the difference between asymptomatic primary EBV infection and infectious mononucleosis is the higher number of EBV-infected B cells in the latter, with the symptoms being due to the massive destruction of virus-infected B cells by cytotoxic CD8+ T cells [93]. Why a higher proportion of B cells should be infected when primary infection is delayed beyond childhood to adolescence or later is unclear. Possible explanations include a higher dose of

viral inoculum acquired by intimate oral contact and a reduced capacity to mount a rapid effective CD8+ T cell response in adolescents/adults compared with young children. The importance of CD8+ T cells in controlling primary EBV infection is demonstrated by the development of potentially fatal B cell lymphoproliferative disease in immunosuppressed transplant recipients with low numbers of EBV-specific T cells and high viral loads [94].

1.4.2 Role of Epstein-Barr virus in the risk of MS

There is now substantial evidence that infection with the EBV plays a major role in the development of MS. It is important to distinguish the role of EBV from that of other infectious agents in MS. It is well established that clinical exacerbations of MS are three times more likely to occur at the time of acute systemic infection with a wide variety of viruses and bacteria than at other times [95]. Several reviews as well as ecological studies suggest that the similar latitudinal distributions of infectious mononucleosis (IM) and MS are evidence of an association [96; 97; 98] . Warner et al. noted that classical IM and MS were both uncommon in African, Oriental and Polynesian populations, with the high incidence of IM “a characteristic of affluent Caucasians’ ” that is, socioeconomic status was considered the important risk factor [99]. A later age of EBV infection has been suggested as a key MS risk factor that could explain persisting latitudinal gradients in incidence of CNS demyelination or loss of a previously observed latitude gradient. Certainly, the prevalence of seropositivity in childhood appears to be higher in less developed countries compared with more developed regions but no study has

yet shown whether the age specific prevalence of EBV seropositivity varies by latitude after the level of development is considered that is, within a single country with relative homogeneity of socioeconomic status [100; 101; 102]. An associated risk for MS following EBV infection, but not cytomegalovirus- or HSV1-infection, was also found in cases of pediatric MS [108]. Individuals who acquire EBV during adolescence or later, as manifested by infectious mononucleosis, are at an additional 2–3-fold higher risk to develop MS than children infected early in life [104]. Heightened humoral immune responses to EBV are found in individuals with MS. Notable differences in antibody titers to EBV antigens, particularly EBNA-1 and the nuclear EBNA complex, are detected in MS patients up to five years before the diagnosis of disease. Compared with matched controls, increased EBV Antibodies (Ab) titers indicate an increased risk for developing MS disease and are suggested to be an early event in the disease process [105; 106]. The association between enhanced EBV immunity and the role of EBV as a causative agent is complicated by elevated Ab titers in MS serum and cerebrospinal fluid (CSF) to many common viruses and infections prevalent within the human population, which may be more indicative of an overall immune dysregulation of B cell memory responses [107]. Cellular immunity is also somewhat altered in MS patients relative to healthy controls. Generally, there are increased numbers of circulating EBV-specific CD8+ cells but not EBV-specific CD4+ T cells, the exception being EBNA-1 [108]. EBNA-1-specific memory CD4+ T cells are elevated in MS blood, show increased proliferative capacity and possess higher avidity and broadened peptide specificity compared with demographically and HLA-DR matched controls. Although the epidemiologic and immunologic data provide a compelling connection between exposure to EBV and risk of MS, alternative

explanations that do not necessitate a direct causal link cannot be excluded, the most likely being a shared susceptibility for MS and EBV infection [109]. Important clues are emerging from studies that are beginning to delineate specificities of the humoral immune response to Epstein–Barr nuclear antigen-1 (EBNA-1) [110,111]. The combination of DRB1*1501 haplotype with high-titer responses to the whole EBNA-1 protein or to a particular sequence domain markedly increased the risk of MS [112]. Mechelli and co-workers analyzed the fine specificity of the humoral immune response to EBNA-1 in MS-discordant monozygotic (MZ) twins, a setting that considerably decreases the influence of heritable and non-heritable confounders [113; 114; 115]. They observed that the humoral immune response to EBNA-1 in MS frequently comprises an epitope that overlaps with a portion of EBNA-1 which is targeted during IM [116]. This specificity wanes over time in asymptomatic virus carriers. This epitope narrows down other EBNA-1 specificity domains 3,4 that had been described as associated with an increased risk of MS. These findings may provide an interpretative key for two consistent epidemiological observations: the association of MS with EBV serology and with a history of IM. IM is an immunopathologic disease ‘per se’ since its symptoms correlate, also temporally, with the immunologic alterations and not necessarily with virologic changes. It has a profound impact on the immune system not only during the acute phase. The enduring presence of a B-cell response that normally wanes after IM may increase and prolong the risk of immunopathology [113]. In addition to anti-EBV antibodies being increased in the serum in MS, they are also elevated in the CSF, which could indicate specific synthesis of antibody against EBV or simply reflect polyspecific antibody synthesis. The corrected Antibody Index makes this distinction and reveals specific anti-EBV antibody synthesis in the

CNS, which is also demonstrated by the reactivity of some of the characteristic CSF oligoclonal IgG bands to EBV proteins. Indeed, the anti-EBV response is the dominant antibody response in the CSF in MS, as revealed by Cepok and others, who screened CSF IgG for reactivity against 37,000 proteins and found the two most frequent reactivities to be against the EBV proteins, EBNA1 and BRRF2. Further indirect evidence that EBV is present in the CNS in MS is provided by the finding that EBV-specific CD8+ T cells are enriched in the CSF, compared with the blood, in early MS but not in patients with other inflammatory neurological diseases [117; 118; 119; 120]. In a recent work Angelini et al. show that MS patients with inactive disease exhibit a lower CD8+ T-cell response to EBV when compared to healthy donors and active MS patients while the latter have a higher frequency of CD8+ T cells specific for EBV lytic antigens. Therapy with interferon-b and natalizumab, two treatments for relapsing remitting MS, was associated with marked changes in the EBV specific CD8+ T cell response. They also demonstrate that one of the EBV lytic antigens, recognized by CD8+ T cells expanding in the blood during active MS is expressed in the inflamed MS brain [121]. The recruitment of CD8+ T cells was selective for EBV specific T cells because T cells reactive to cytomegalovirus, another herpesvirus, were not enriched in the CSF. Given that antigen-specific CD8+ T cells infiltrate the brain only when their cognate antigen is present, these findings suggest that EBV is present in the CNS in MS [122]. An alternative but less likely explanation is that EBV-specific CD8+ T cells in the CSF are recognizing not EBV but a CNS autoantigen with which they cross-react. Recently, Serafini and others reported that a substantial proportion of the B cells and plasma cells in 21 of 22 brains of MS patients were infected with EBV [123]. They identified EBV-infected cells using in situ hybridization for

EBV-encoded small RNA (EBER-ISH), the gold standard for detection of EBV-infected B cells in histological material and by immunohistochemistry with antibodies specific for EBV proteins [124]. EBV-infected B cells were not detected in the brains of patients with other inflammatory CNS diseases. The major sites of EBV persistence in the MS brain were meningeal structures resembling B cell lymphoid follicles with germinal centers, which the authors had previously demonstrated to be present in patients with secondary progressive MS (Fig. 11) [125]. B cells expressing EBV latent proteins were regularly found in the brains of patients with MS, whereas B cells expressing the EBV early lytic cycle protein, BFRF1, were restricted to acute lesions and meningeal B cell follicles. Their finding that EBV-infected B cells in meningeal follicles express LMP1 and LMP2A but not EBNA2 indicates that here EBV deploys the latency II ("default") transcription program which allows infected B cells to progress through a germinal center reaction to become latently infected memory B cells [88; 123; 126]. It also accords with the observation that, in the tonsils of healthy EBV carriers, EBV-infected B cells expressing this viral transcription program not only are functionally indistinguishable from classical germinal center B cells but also are physically located in germinal centers [127]. In contrast, Willis and others found EBV in only 2 of 24 brains of MS patients. They did not detect EBV-infected B cells by EBER-ISH or immunohistochemistry using antibodies against LMP1 or EBNA2 in any of the specimens but did detect low levels of EBV DNA in two samples by realtime polymerase chain reaction. They concluded that EBV infection is rare in MS brain tissue and unlikely to contribute directly to the pathogenesis [128]. Possible explanations for the discrepancy between these findings and those of Serafini and others, are differences in experimental design and interpretation, tissue selection and

processing, the degree of CNS B cell infiltration, and the sensitivities and specificities of the techniques used. It has been suggested that the inability of Willis and others to detect any EBV-infected B cells by EBER-ISH in their MS brain specimens might have been due to degradation of EBER RNA by long tissue fixation times in formalin [129].

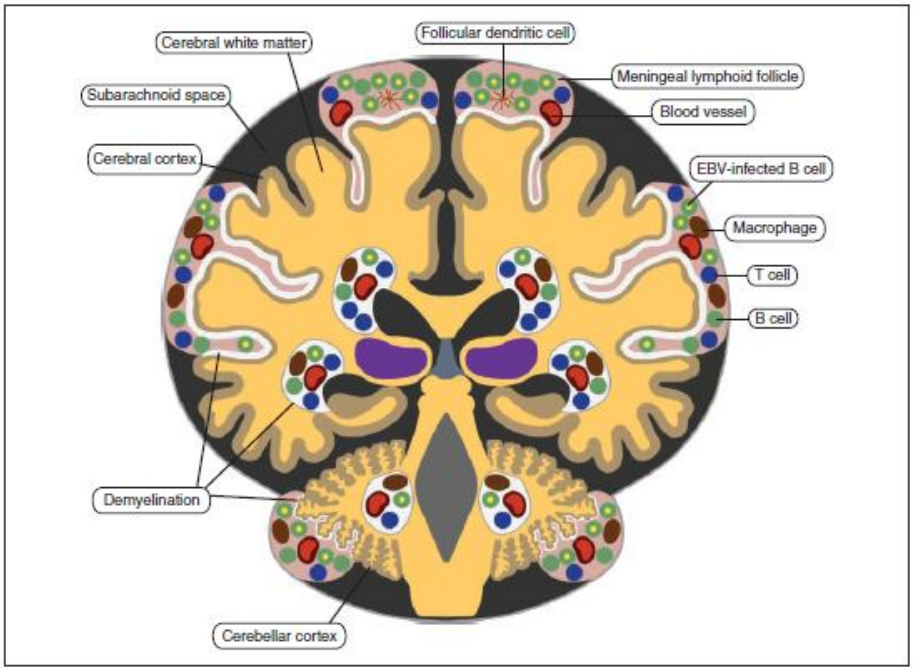

Figure 11 Epstein-Barr virus (EBV)–infected B cells in the brain in secondary progressive multiple sclerosis (MS). This distribution of EBV-infected B cells in the MS brain is based on the study of Serafini and others [123].

Lünemann and others found the EBV DNA load in peripheral blood mononuclear cells (PBMCs) to be moderately increased in patients with clinically isolated syndromes (first episodes of the type seen in MS) compared with healthy subjects [108]. Using the same assay in an earlier

study, the same group concluded that the EBV viral load is not increased in the PBMCs of patients with relapsing–remitting MS; however, the viral load was substantially higher in the MS patients than in the healthy controls, although the difference was not statistically significant, possibly because of the small numbers of subjects [130]. There is strong evidence that EBV infection precedes MS onset and there is a dose dependent relationship between MS risk and the level of EBV specific antibodies, particularly EBNA-1 IgG titres. A key emerging feature is that it is important to examine not only the antibody titre but also the specificity of the humoral immune response to EBV and better understand what determines this. The evidence in relation to the presence or amount of EBV DNA in blood, CSF or brain is less clear, with studies presenting directly conflicting results. CD8 cytotoxic T cells maintain immunosurveillance and there is some evidence that there is dysfunction of these cells in MS. Such dysfunction should be accompanied by evidence of reactivation or higher EBV DNA load in cellular compartments but this has not been consistently shown. Table 2 summarizes the evidence on the role of EBV infection in the development of MS [102].

Measure of EBV infection (and relevant references)	Level of evidence for an association with MS risk*
Latitudinal gradient in EBV infection	+
History of infectious mononucleosis	+++
EBV antibodies in serum	+++
EBV DNA load in the peripheral blood	+
EBV DNA in the CNS	+
EBV specific T cell responses	++
Interaction with other viruses	+

*Level of evidence is based on the quantity and quality of the research evidence currently available, including strength of associations, consistency across studies and locations, temporality of exposure and outcome, and a dose–response effect (where relevant).
EBV, Epstein–Barr virus; MS, multiple sclerosis.

Table 2 Summary of the level of evidence in relation to measures of EBV infection and MS risk (adapted from Lucas et al., *J Neurol Neurosurg Psychiatry* 2011).

1.4.3 Viral infections: other viruses

Other viruses have been implicated as factors that may influence MS risk. These include, in addition to EBV, principally, human herpesvirus 6, Torque teno virus, varicella-zoster virus. The evidence for and against a role of most of these viruses in MS is inconsistent, except for that supporting EBV, as discussed above.

Human herpesvirus-6

Another ubiquitous herpesvirus that has gained relative popularity as potentially associated with MS is the human herpesvirus- 6 (HHV-6). It is characterized by phases of latency and reactivation that bear similarity to relapsing-remitting MS. The virus has neurotropic potential and was reported to infect oligodendrocytes and microglia cells, as well as to establish latency in CNS tissue which serves as a reservoir of persistent infection [131]. Another aspect that strengthens the hypothesized involvement of the virus in MS is the observation that primary HHV-6 infection may lead to severe neurological complications such as encephalitis and epilepsy. Besides being able to infect neural cell, HHV-6 most commonly establishes infection in T lymphocytes and supposedly exerts modulatory effects on the immune system [35]. Various studies in the past 15 years have investigated HHV-6 association with MS. HHV-6 has been detected in serum, CSF as well as in brain tissue and more predominantly in MS lesions as reported by some studies [132; 133]. Besides direct cytopathic effect, other putative mechanisms of HHV-6 involvement in MS pathology include bystander

activation, and molecular mimicry. The later of which has gained compelling evidence. Myelin basic protein (MBP), a suspected target of autoreactivity in MS, has been determined to share sequence homology with HHV-6-encoded U24 protein. Cross-reactive T cells responding both to MBP and U24 peptides were elevated in MS patients compared to controls [134]. So far, there is no epidemiological evidence that symptomatic HHV-6 infection increases the risk for development of MS and there is no data that would unequivocally support an etiologic role of HHV-6 in the pathogenesis of MS. However, the neuro- and lymphotropic potential of virus suggests that bystander reactivation of HHV-6 could potentially augment CNS inflammation [135].

Varicella zoster virus

Varicella-zoster virus (VZV) is a ubiquitous pathogen that establishes latency in the dorsal ganglia in about 95% of adults. It is the causative agent of varicella (chickenpox), and can occasionally lead to symptomatic reactivation known as zoster [136]. Numerous epidemiological studies have investigated the link between VZV and MS, nevertheless, a meta-analysis including 40 reports conducted between 1965-1999 revealed that there is insufficient evidence to support such association [137]. Presence of VZV DNA, however, has been reported in blood cells and CSF from relapsing MS patients [138]. Furthermore, Sotelo et al. observed electron microscopically abundance of viral particles identical to VZV in the CSF of MS patients during the initial days of disease relapse [139]. However, when Burgoon et al. used identical electron microscopic and PCR techniques they failed to identify presence of viral particles or DNA in MS CSF or acute MS plaques [140]. The weak

epidemiological data and the inconsistency in the observations render the link between VZV and MS elusive [38].

Torque teno virus

Sospedra et al. determined the specificity of clonally expanded T cells from CSF of MS patients during exacerbation and observed that these T cell clones recognized evolutionary conserved arginine- rich motifs of functional protein domains of the orphan virus Torque teno virus (TTV) [141]. T cell clones resounded to TTV peptides as well as to peptides from other common viruses and selfantigens. Therefore, the authors speculated that repeated encounter, both of pathogenic or non-pathogenic infectious agents can lead to expansion of T cells responsive to conserved protein domains that are able to cross-react with ubiquitous self-antigens. These observations allude to the conclusion that recurrent infections with multitude of ubiquitous pathogens rather than one particular infectious agent could be implicated in the initiation of reactivity towards self-antigens that may trigger autoimmune pathologies. However, due to the paucity of data, the relation of TTV infection and MS remains ill defined [38].

1.4.4 Parasitic infections

Not only viruses and bacteria have been linked to MS, but also parasites. But helminths are thought to have a protective role in MS among other

autoimmune diseases [142]. The prevalence of helminth infections in countries with high sanitation standards has lead to the proposition of the hygiene hypothesis stating that regular exposure to various pathogens is required for the development of appropriate immune responses. Administration of helminths has been shown to ameliorate disease in a mouse model of MS [143]; while one clinical study has shown that when MS patients developed asymptomatic gastrointestinal infection, they experienced reduction of clinical and radiological MS activity, characterized by downmodulation of proinflammatory cytokines and increase in IL-10 and TGF-b production [144]. The long life span of helminths is evidence enough that they are well accomplished at immune evasion. Parasite infections are often asymptomatic, and most hosts tolerate the presence of parasites considered pathogens for considerable time periods without ill effects. Correale and collegues identified 12 parasite-infected MS patients that showed significantly lower number of relapses, minimal changes in disability scores and significantly lower MRI activity compared with uninfected MS individuals [144]. Parasite-driven protection was associated with induction of regulatory T cells secreting suppressive cytokines IL-10 and TGF-b, as well as CD4þ CD25þ FoxP3þ T cells displaying significant suppressive function. These findings provide evidence to support autoimmune down-regulation secondary to parasite infections in MS patients through regulatory T cell action, with effects extending beyond response to an invading agent. In addition to the development of regulatory T cells, helminth infections also induce regulatory B cells in MS patients, capable of dampening the immune response through production of IL-10 [145]. Interestingly, production of IL-10 by B cells is restricted to helminth-infected individuals exclusively. B cells from patients with infections of other parasites such as Trypanosoma

cruzi exhibit IL-10 production levels similar to those observed in uninfected MS patients, indicating intracellular parasites are unable to down-modulate harmful autoimmune responses the way helminths do. Infectious agents may also down-modulate immune response through mechanisms not involving specific immune response induction against constituent antigens. Dendritic cells (DCs) express a range of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide-binding oligomerization domainlike receptors (NLRs), retinoic acid-inducible gene-like receptors (RIG-like receptors) and the C-type lectin receptors (CLRs). Upon engagement, signaling cascades are initiated, inducing gene expression involved in DC maturation and in the ability to prime and skew T cell regulatory immune responses [146]. Another mechanism through which parasites affect host immunity, and ultimately concomitant disease also, is signaling-independent, and mediated by enzymatic activities of helminth-derived products, which play an important role in establishing and maintaining infection through impairment of innate immune cell function [147]. Furthermore, a number of studies have documented the potent effects of cystatins, a class of molecules expressed by filarial nematodes, interfering with DC antigen presentation. Finally, helminth pathogens express cysteine proteases, termed cathepsines, which lead to immune deviation by suppressing Th1 immunity. Figure 12 summarizes all the potential mechanisms for immune regulation discussed above.

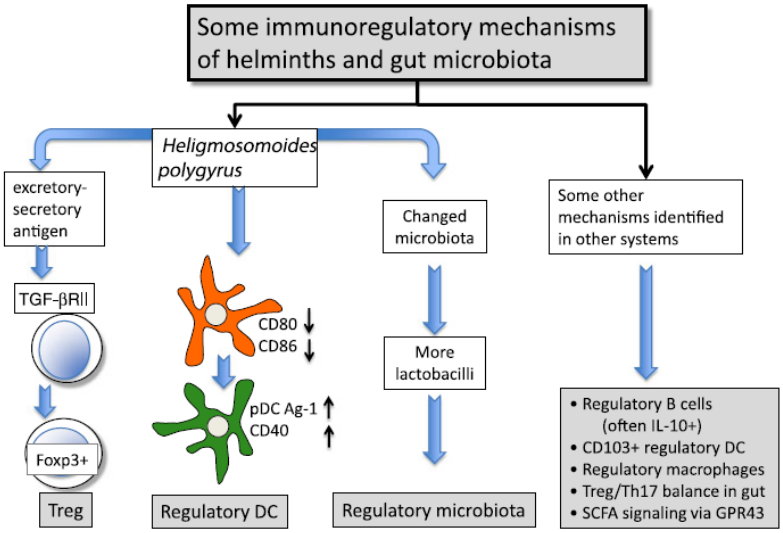

Figure 12 Summary of putative mechanisms through which helminths could modulate MS [149].

Each species can develop very different infection dynamics over time, and/or during peak infection intensity. Moreover, the same species may exert opposite effects under varying conditions. Finally, if susceptibility to autoimmune diseases is genetically influenced, so too must be the propensity for infections to modulate disease immunopathology, making particular infections protective only in certain genotypes, rather than in the whole population [148].

1.5 Intestinal permeability and microbiota

1.5.1 Hygiene hypothesis and gut microbiota

Throughout the twentieth century, there were striking increases in the incidences of many chronic inflammatory disorders in the rich developed countries. These included autoimmune disorders such as Type 1 diabetes and multiple sclerosis. Although genetics and specific triggering mechanisms such as molecular mimicry and viruses are involved, a series of environmental factors, most of them microbial, have led to a decrease in the efficiency of our immunoregulatory mechanisms because we are in a state of evolved dependence on organisms with which we co-evolved as inducers of immunoregulatory circuits. The mammalian intestine hosts a large microbiota, which probably includes more than 103 different species. An equilibrium has been established, during the course of millennia of evolution, between the microbiota and the immune system, which is essential for immune homeostasis. However, epidemiological and clinical data describe a marked increase in the incidence of autoimmune and allergic disorders in western countries, where the reduced exposure to microbes, the wide-spread use of antibiotics and the major improvements in sanitation in general seem to correlate with a higher incidence of chronic autoimmune or allergic disorders. It is in fact possible that the delicate equilibrium between the microbiota and the immune system is perturbed by modern lifestyle, resulting in an imbalance between harmful and protective bacteria known as dysbiosis. All of these microrganisms will exacerbate the immunoregulatory deficit [149]. Figure 13 lists some relevant factors,

and also emphasizes the immunoregulatory role of the gut. One of the most important discoveries in recent years is the fact that manipulations of the immune system may act indirectly via changes in the gut flora. Consequent changes in the composition of the microbiota were responsible for the immunoregulatory effect that blocked the autoimmune process. Thus changes in the microbiota, which is profoundly different in Europeans from that of people living in a traditional rural African village must be regarded as part of the hygiene hypothesis[150].

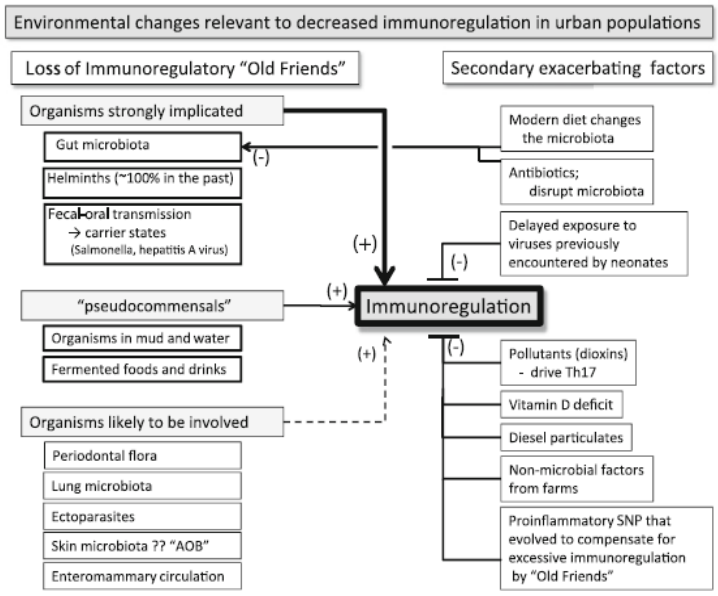

Figure 13 Aspects of man's microbiological history that are most relevant to the hygiene hypothesis. Epidemiological data, laboratory and animal models, and preliminary clinical trials investigating the hygiene hypothesis implicate organisms that are thought to have accompanied mammalian and human evolution.

The hygiene hypothesis, or the "Old Friends" hypothesis, can be traced back to the 1870s when Charles Harrison Blackley noticed that aristocrats and

citydwellers were more likely to get hayfever than were farmers. Similarly, in 1966, Leibowitz and colleagues noted that the incidence of multiple sclerosis in Israel was positively related to levels of sanitation [151]. The hygiene hypothesis suggests that one reason for the increasing incidences of chronic inflammatory disorders, (both Th2-mediated and Th1/Th17-mediated [152]), in developed countries since the mid-nineteenth century is the depletion from the urban environment of organisms that accompanied mammalian evolution and had to be tolerated. Some of these organisms had to be tolerated because they were essential symbiotic intestinal microbiota. Others, like helminths, had to be tolerated because once established they cannot be removed by the immune system, which is therefore downregulated to avoid pointless immunopathology. Other relevant organisms were species that established carrier states soon after birth. Because these all had to be tolerated, co-evolutionary forces ensured that they came to play essential roles in the priming and optimal functioning of immunoregulatory pathways that are involved in tolerance [153]. Our knowledge of species and functional composition of the human gut microbiome is rapidly increasing, but it is still based on very few cohorts and little is known about variation across the world. Arumugam and coworkers identified three robust clusters or enterotypes, each identifiable by the variation in the levels of one of three genera: Bacteroides (enterotype 1), Prevotella (enterotype 2) and Ruminococcus (enterotype 3) (Fig. 14). Moreover they are not nation or continent specific demonstrating that intestinal microbiota variation is generally stratified and not continuous. The robustness and predictability of the enterotypes in different cohorts and at multiple phylogenetic and functional levels indicates that they are the result of well-balanced, defined microbial community compositions of which only a limited number exist

across individuals. These enterotypes are not as sharply delimited as, for example, human blood groups; they are, in contrast, densely populated areas in a multidimensional space of community composition. They are nevertheless likely to characterize individuals, in line with previous reports that gut microbiota are quite stable in individuals and can even be restored after perturbation. The enterotypes are mostly driven by species composition, but abundant molecular functions are not necessarily provided by abundant species [154; 155; 156].

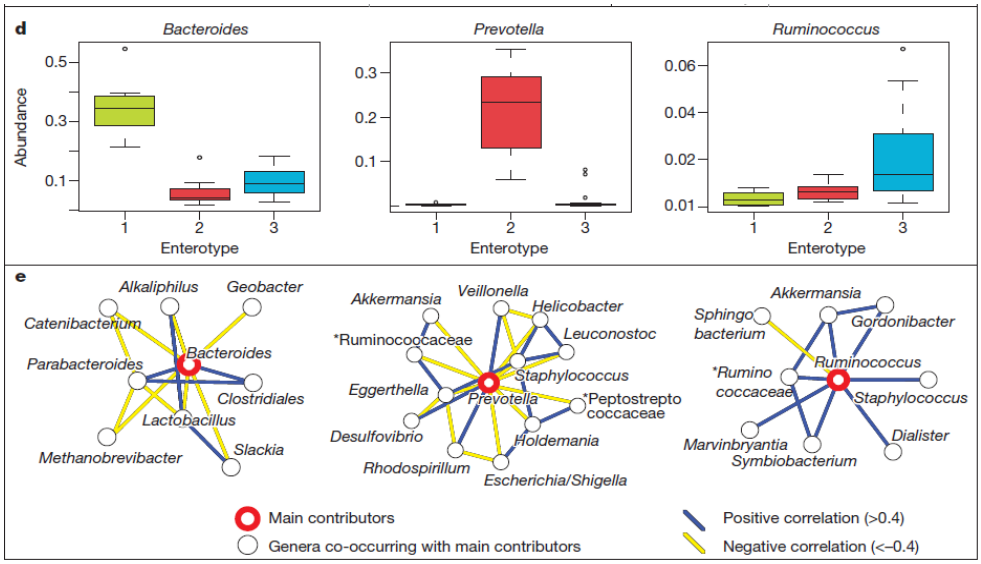

Figure 14 Phylogenetic differences between enterotypes (adapted from Arumugam et al., Nature 2011)

The influence of the gut microbiota on human health is continuous from birth to old age. The maternal microbiota may influence both the intrauterine environment and the postnatal health of the fetus. At birth, about 100

microbial species populate the colon. Early environmental factors (e.g., method of delivery), nutritional factors (e.g., breast or bottle-feeding), and epigenetic factors have been implicated in the development of a healthy gut and its microbial symbionts. Changes in gut microbial composition in early life can influence risk for developing disease later in life. During suckling, the microbial community develops rapidly; shifts in microbial diversity occur throughout childhood and adult life; and in old age, there is a decrease in the Bacteroidetes and an increase in Firmicutes species. The gut microbiota is important for maintaining normal physiology and energy production throughout life. Body temperature regulation, reproduction, and tissue growth are energy-dependent processes thatmay rely in part on gut microbial energy production. Extrinsic environmental factors (such as antibiotic use, diet, stress, disease, and injury) and the mammalian host genome continually influence the diversity and function of the gut microbiota with implications for human health (Fig. 15) [157].

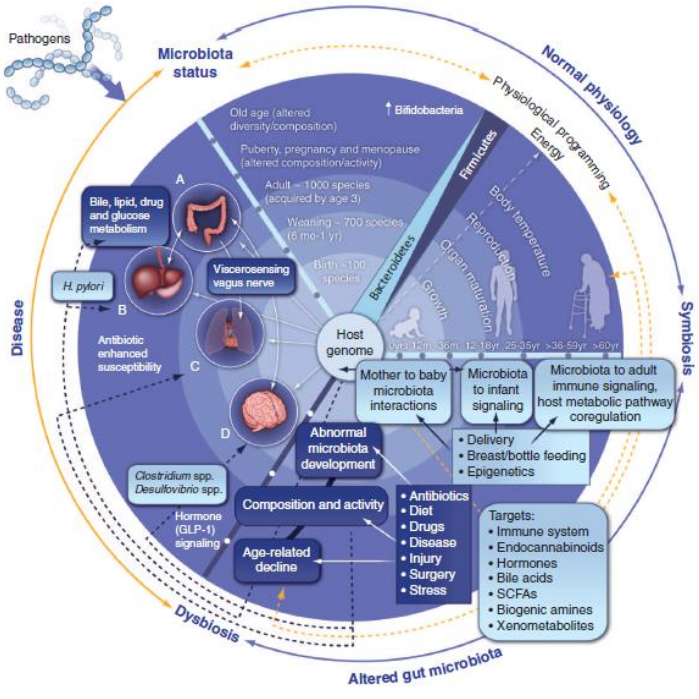

Figure 15 The gut microbiota in development and disease [157]

Additionally, the use of gnotobiotic mice, in which the presence and composition of the microbial composition is experimentally manipulated, has allowed for evaluation of specific microorganisms that may affect immune development. In particular, germ-free (GF) mouse experiments have led to great discoveries in autoimmune disease pathogenesis [158; 159]. The host mucosal immune system, particularly in the gut, works to protect the host from commensals and influences the make-up of the microbiome. After birth, the neonatal gut is colonized with maternal bacteria and ultimately forms a stable microbiotic profile. The mucosal immune system inhibits the entrance of intestinal bacteria through multiple mechanisms that include the epithelial barrier (e.g. mucus, tight junctions), antimicrobial peptides, mucosal IgA and innate and adaptive immunity. However, the containment of intestinal bacteria can be perturbed by environmental factors such as chemicals, tissue

injury (e.g. ischemia), and pathogenic infection [158; 159; 160; 161]. Defects in mucosal immunity allow bacteria to penetrate the epithelial barrier and induce inflammatory responses. If these defects or immune responses are not regulated, chronic inflammation can occur leading to a shift in the gut microbiome, often to a less diverse population of colonizing bacteria. Conversely, microbiota directly influence normal mucosal immune system development. GF mice have underdeveloped Peyer's patches, mesenteric lymph nodes, and splenic white pulp as well as defective formation of intestinal lymphoid follicles and decreased numbers of intestinal lymphocytes. These deficiencies can largely be corrected with the introduction of specific bacterial mixes, some single species, or bacterial products [158; 159; 161; 162; 163]. Investigation of MS immunopathogenesis has been greatly enabled by animal models, in particular that of the mouse experimental autoimmune encephalomyelitis (EAE) model. Berer and co-workers using the relapsing–remitting mouse model of spontaneously developing EAE, showed that the commensal gut flora—in the absence of pathogenic agents—is essential in triggering immune processes, leading to a relapsing–remitting autoimmune disease driven by myelin-specific CD4+ T cells. They showed further that recruitment and activation of autoantibody-producing B cells from the endogenous immune repertoire depends on availability of the target autoantigen, myelin oligodendrocyte glycoprotein (MOG), and commensal microbiota [164].

Microbiota also influence T cell subpopulations at steady state. Th17 cells, usually abundant in the gut, are absent in GF mice but can be restored with introduction of segmented filamentous bacteria (SFB) [158; 161; 163]. Regarding multiple sclerosis, although $CD4^{+}$T lymphocytes are traditionally

considered the main actors in MS immunopathology, multiple evidences suggest that CD8+T lymphocytes are also implicated in the pathogenesis [165]. Recently, a significant expansion of $CD161^{hi}CD8^{+}$ T cells in the peripheral blood of MS patients was described [166]. This subset includes $CCR6^{+}$, cytokine-producing, effector-memory T cells with pro-inflammatory profiles. It also contained all circulating $IL\text{-}17^{+}CD8^{+}$ T cells. Moreover, $CD161^{+}CD8^{+}CD3^{+}$ T cells producing IFN-γ were part of intralesional immune infiltrates and ectopic B cell follicles in autopsy multiple sclerosis brains [167]. Finally, the $CD161^{hi}CD8^{+}$ T subset proved to be positive for iVα7.2 TCR usage, thus overlapping with the mucosal-associated invariant T (MAIT) cells. Human MAIT cells express the semi-invariant TCR iVα7.2-Jα33 and are selected by the MHC class Ib molecule MR1 on hematopoietic cells. They are specifically activated by antigen presenting cells (APC) or epithelial cell lines co-cultured with bacteria or yeasts but not viruses. Moreover, MAIT cells include all the multidrug resistance transporter (ABCB1) $^{+}CD161^{hi}CD8^{+}$ T cells, being probably resistant to the xenobiotics secreted by the gut bacteria. These features, together with their already known antimicrobial specificity, the gut-liver homing characteristics, suggest a prevalent role in interaction with intestinal microbiota [168]. Several reports indeed have started to point to dysbiosis as a major factor which influences the generation of proinflammatory rather than tolerogenic responses, with certain members of the microbiota such as Segmented Filamentous Bacteria inducing prominent pro-inflammatory Th17-mediated T cell responses, and other bacterial strains such as Clostridium facilitating the generation of immunosuppressive T regulatory lymphocytes [169; 170]. Interestingly, it was found that MAIT cells are in much lower numbers in subjects of rural

Zimbabwe in comparison with french blood donors [150]. At this stage it is not clear whether this decrease is genetically or environmental determined. The high number of MAIT cells found in MS patients would be a marker of low infection burden that would have favored autoimmunity in the context of the hygiene hypothesis. Alternatively, MAIT cells could be mechanistically implicated in MS pathogenesis, as suggested by recent works on the role of gut microbiota in neuroinflammation. Stimulation of the immune system by bacteria can alter the outcome of demyelinating disorders in murine EAE, the widely accepted experimental model for human MS. Oral immunization with an attenuated Salmonella strain conferred protection against EAE as did the modification of bacterial populations in the gut. Bacteroides fragilis producing a bacterial capsular polysaccharide (PSA) can protect against EAE, via the induction of tolerogenic $CD103^{+}$ dendritic cells, reduction of Th1 and Th17 responses, and enhancement of IL-10 production[171; 172]. The impact of microbiota on the balance between pro- and anti-inflammatory immune responses during EAE has been investigated by Mazmanian and colleagues: germ-free animals were colonized with segmented filamentous bacteria, known to induce Th17 cells in the gut, and augmented Th17 responses could be measured not only in the colon and small intestine, but also in the spinal cord, underscoring the notion that the microbiota contains organisms that can direct proinflammatory immune responses in the CNS [173]. Bacteroides fragilis and PSA can also induce T regulatory cells (Tregs), that are decreased in the large intestine of GF mice [174]. These cells contribute significantly to the maintenance of peripheral tolerance, but they ultimately fail in autoimmune diseases. The events that lead to Treg failure in controlling autoreactive effector T cells (Teffs) during autoimmunity are not

completely understood. Many autoimmune diseases manifest themselves in an oscillating fashion, which is likely due to the aforementioned struggle between the regulatory and inflammatory arms of the immune system, suggesting that Tregs are still actively involved in regulating this self-reactive response. There are three mechanisms that might contribute to a breakdown in Treg control: (a) Treg numbers are reduced and/or Tregs are dysfunctional due to inherent deficiencies in autoimmune susceptible individuals, (b) Treg suppressive function is inhibited, diverted or converted by the chronic inflammation that occurs in autoimmunity, and/or (c) self-reactive effector T cells (Teff) become unusually aggressive and are refractory to regulation by otherwise functional Tregs because they either overwhelm regulatory control or express molecules that render them resistant. In EAE, Foxp3+ Tregs accumulate in the central nervous system (CNS) as the disease progresses and in some instances aid in natural recovery from EAE [175; 176; 177]. Moreover, $CD4^{+}FOXP3^{+}$ Treg cells are present most abundantly in the intestinal mucosa at steady state, and contribute to intestinal and systemic immune homeostasis. In germ-free mice, the frequency of colonic Treg cells and levels of IL-10 expression by Treg cells are markedly reduced [178; 179; 180; 181; 182]. Atarashi et al. showed that a combination of Clostridia strains isolated from conventionally reared mice potently affect the number and function of CD41FOXP31 Treg cells in mouse colonic lamina propria4. Although a recent study showed that the human microbiota had no impact on the immune responses in the mouse small intestine, these findings suggest that the human intestinal microbiota contains Treg-cell-inducing bacteria [183].

Altogether these data suggests the potential importance of intestinal microbiota on mucosal and intestinal immunity in the immunopathogenesis of autoimmune disease such as MS.

1.5.2 Intestinal permeability and the zonulin system

Regarding the immune system and in particular the mucosal immunity, gut-associated lymphoid tissue (GALT) , the neuroendocrine network and the intestinal epithelial barrier, with its intercellular tight junctions, controls the equilibrium between tolerance and immunity to non-self antigens. As mentioned in the previous paragraphs, the development of autoimmune diseases, was believed that their was associated with viral and bacterial infections. The connection between infection and autoimmune disease is often explained by a mechanism known as "molecular mimicry," whereby microbial antigens are postulated to resemble self-antigens . The induction of an immune response to the microbial antigens results in a cross-reaction with the self-antigens and the induction of autoimmunity. According to this theory, once the autoimmune process is activated, it becomes independent of continuous exposure to the environmental trigger and is therefore self-perpetuating and irreversible [184]. Epitope-specific cross-reactivity between microbial antigens and self-antigens has been shown in some animal models to initiate autoimmunity. Conversely, in most human autoimmune diseases, molecular mimicry seems to be a factor in the progression of a pre-existing subclinical autoimmune response, rather than in the initiation of

autoimmunity [185]. Another theory suggests that microorganisms expose self-antigens to the immune system by directly damaging tissues during active infection, and that this leads to the development of autoimmunity. This mechanism has been referred to as the "bystander effect," and it occurs only when the new antigen is presented with the orally administered triggering antigen. Whether pathogens mimic selfantigens, release sequestered self-antigens, or both, however, remains to be elucidated. A common denominator in autoimmune diseases is the presence of several pre-existing conditions that lead to an autoimmune process [186]. The first of these conditions is the genetic susceptibility of the host immune system to recognize, and potentially misinterpret, an environmental antigen presented within the gastrointestinal tract. The second is that the host must be exposed to the antigen. Finally, the antigen must be presented to the gastrointestinal mucosal immune system following its paracellular passage from the intestinal lumen to the gut submucosa; this process is normally prevented by competent TJ . In many cases, increased intestinal permeability seems to precede disease and causes an abnormality in antigen delivery that triggers the multiorgan process leading to the autoimmune response [187; 188]. Epithelial surfaces are immediately after birth coming into contact with numbers of micro-organisms. These surfaces therefore evolved a number of protective mechanisms to resist the invasion of micro-organisms. In particular, gastrointestinal surface is mostly covered with a single-layered epithelium and require, therefore, a more extensive protection: this is represented by a complex of mechanical and chemical agents responsible for effective degradation and removal of heterogeneous substances [189]. The primary functions of the gastrointestinal tract have traditionally been perceived to be limited to the

digestion and absorption of nutrients and to electrolytes and water homeostasis. A more attentive analysis of the anatomic and functional arrangement of the gastrointestinal tract, however, suggests that another extremely important function of this organ is its ability to regulate the trafficking of macromolecules between the environment and the host through a barrier mechanism. Together with the gut-associated lymphoid tissue and the neuroendocrine network, the intestinal epithelial barrier, with its intercellular tight junctions, controls the equilibrium between tolerance and immunity to non-self antigens [190]. The intestinal epithelium consists of a layer of interconnected, polarised epithelial cells separated by a basal membrane from the connective and supporting tissue surrounding various types of cells present in the lamina propria. Normally, antigens are selectively sampled by intestinal M cells which are modified epithelial cells specially designed for the endocytosis of luminal antigens [191]. Than, the antigens are displayed to antigen presenting cells, such as macrophages, where they are degraded and incorporated into an antigen presenting protein complex termed the major histocompatability complex (MHC) or human leukocyte antigen (HLA). From here, down stream T and B cell activation will occur, which will ultimately result in the production of chemical mediators to elicit an immune response. Despite this, some macromolecules have shown the ability to cross the intestinal epithelium without M cell passage. This can occur transcellularly, where antigens are absorbed directly through epithelial cells or through (Fig. 16) [192].

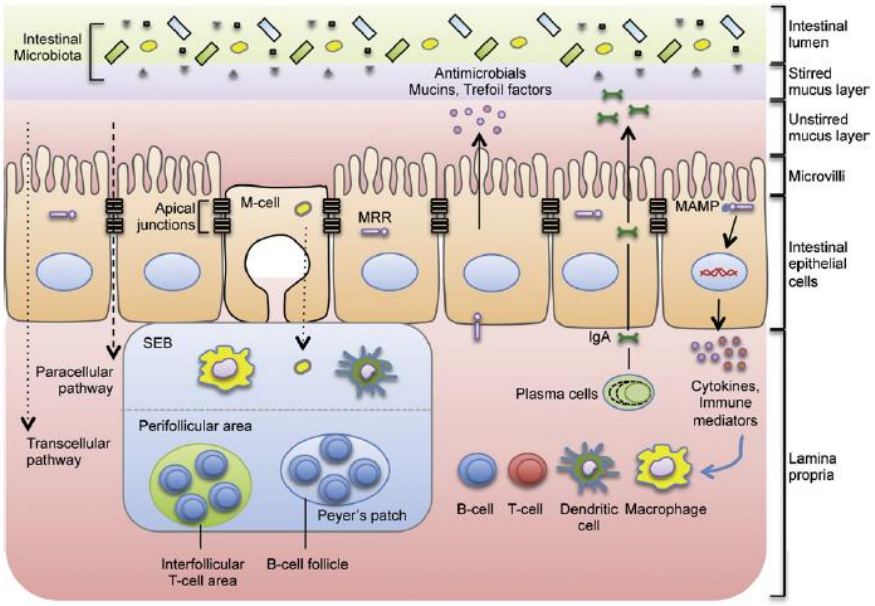

Figure 16 Intestinal barrierer as modulator of intestinal homeostasis (Natividad&Verdu, *Pharm. Res*., 2012)

The paracellular route that is the dominant pathway for passive solute flow across the intestinal epithelial barrier, and its functional state depends on the regulation of the intercellular TJ [193]. The TJ is one of the hallmarks of absorptive and secretory epithelia. As a barrier between apical and basolateral compartments, it selectively regulates the passive diffusion of ions and small water-soluble solutes through the paracellular pathway, thereby compensating for any gradients generated by transcellular pathways [194]. TJ are structures dynamic and readily adapt to a variety of developmental [195], physiological [196], and pathological [197; 198] circumstances. To meet the diverse physiological challenges to which the intestinal epithelial barrier is subjected, TJ must be capable of rapid and coordinated responses. This requires the presence of a complex regulatory system that orchestrates the state of assembly of the TJ multiprotein network. The molecules forming tight junctions (principally occludins and

claudins) are connected to the cytoskeleton of epithelial cells. In addition to laterally situated tight junctions present in zonula occludens, there are intermediary junctions and desmosomes in the so-called zonula adherens (Fig. 17). While knowledge about TJ ultrastructure and intracellular signaling events has progressed significantly during the past decade, only recently we have seen effort focused on elucidating the link between TJs and many pathophysiological states [186; 199]

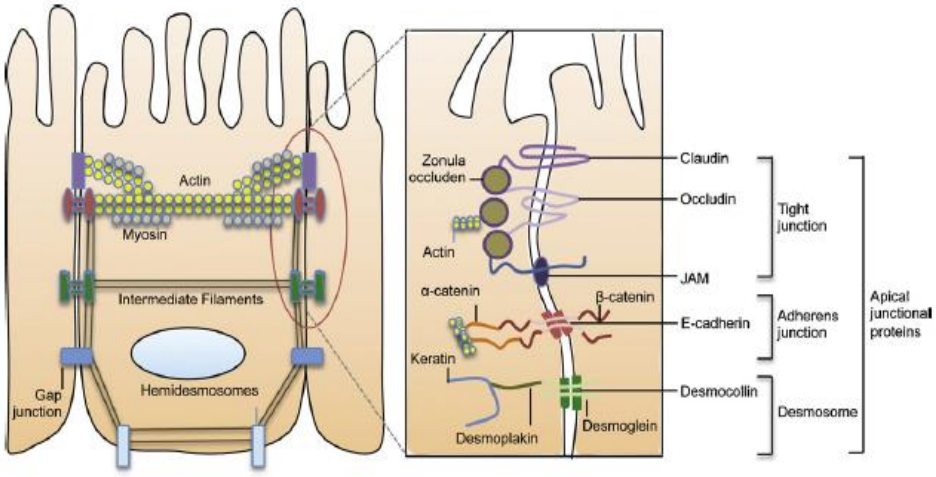

Figure 17 Structure of intestinal epithelial barrier ((Natividad&Verdu, *Pharm. Res*., 2012).

Zonulin is the only physiological modulator of intercellular tight junctions described so far that is involved in trafficking of macromolecules and, therefore, in tolerance/immune response balance. When the finely tuned zonulin pathway is deregulated in genetically susceptible individuals, both intestinal and extraintestinal autoimmune, inflammatory, and neoplastic disorders can occur. This new paradigm subverts traditional theories underlying the development of these diseases and suggests that these

processes can be arrested if the interplay between genes and environmental triggers is prevented by reestablishing the zonulin-dependent intestinal barrier function. Zonulin is an human homolog of Zot (zonula occludens toxin), an enterotoxin elaborated by Vibrio cholerae that affects the TJ competency, has shed light on the intricate mechanisms involved in the modulation of the intestinal paracellular pathway [190]. Zonulin expression is augmented in autoimmune conditions associated with TJ dysfunction, including celiac disease (CD) and T1D. Both animal studies and human trials using the zonulin synthetic peptide inhibitor AT1001 (now named Larazotide acetate) have established that zonulin is integrally involved in the pathogenesis of autoimmune diseases. Zonulin can be used as a biomarker of impaired gut barrier function for several autoimmune, neurodegenerative, and tumoral diseases and can be a potential therapeutic target for the treatment of these devastating conditions [200; 201; 202; 203].

Zonulin was recently identified, through proteomic analysis of human sera, as pre-haptoglobin (HP) 2, a molecule that, to date, has only been regarded as the inactive precursor for HP2, one of the two genetic variants (together with HP1) of human HPs. Mature human HPs are heterodimeric plasma glycoproteins that form stable complexes with haemoglobin (HB) preventing Hb-induced oxidative tissue damage [204; 205].

Conversely, zonulin is detectable in an uncleaved form in human serum, adding another extremely intriguing aspect of the multifunctional characteristics of HPs. HPs are unusual secretory proteins in that they are proteolytically processed in the endoplasmic reticulum, the subcellular fraction in which we detected the highest zonulin concentration [206]. Wicher and Fries found that the complement C1r-like protein mediates this cleavage in a specific manner, since the enzyme did not cleave the proform

of complement C1s, a protein similar to Pre-HP2. Therefore, it is conceivable to hypothesize that the activity of Cr-like protein modulates the zonulin pool [206]. Zonulin interacts with a specific surface receptor whose distribution within the intestine varies. The protein then activates phospholipase C that hydrolyzes phosphatidyl inositol to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG). PKCα is then activated, either directly (via DAG) [4] or through the release of intracellular Ca2+ (via PPI-3) [185; 187; 188]. Membrane-associated, activated PKCα catalyzes the phosphorylation of target protein(s), with subsequent polymerization of soluble G-actin in F-actin. This polymerization causes the rearrangement of the filaments of actin and the subsequent displacement of proteins (including ZO-1) from the junctional complex [206]. As a result, intestinal TJ becomes looser. Once the zonulin signaling is over, the TJs resume their baseline steady state (Fig. 18)

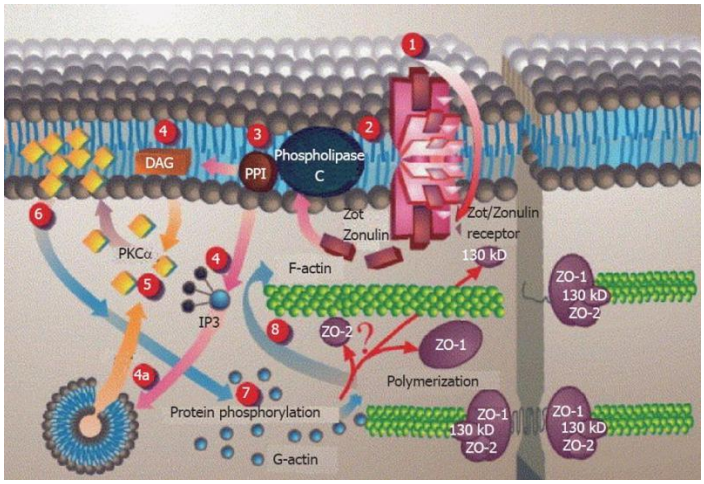

Figure 18 The zonulin signalling [184].

Among the several potential intestinal luminal stimuli that can trigger zonulin release, we identified small intestinal exposure to bacteria and

gluten as the two more powerful triggers. Enteric infections have been implicated in the pathogenesis of several pathological conditions, including allergic, autoimmune, and inflammatory diseases, by causing impairment of the intestinal barrier. We have generated evidence that small intestines exposed to enteric bacteria secreted zonulin. This secretion was independent of either the animal species from which the small intestines were isolated or the virulence of the microorganisms tested, occurred only on the luminal aspect of the bacteria-exposed small intestinal mucosa, and was followed by an increase in intestinal permeability coincident with the disengagement of the protein ZO-1 from the tight junctional complex [208]. This zonulin-driven opening of the paracellular pathway may represent a defensive mechanism which flushes out microorganisms so contributing to the innate immune response of the host against bacterial colonization of the small intestine. In addition to bacterial exposure, it was shown that gliadin also affects the intestinal barrier function by releasing zonulin. This effect of gliadin is polarized, i.e., gliadin increases intestinal permeability only when administered on the luminal side of the intestinal tissue [209]. Concerning multiple sclerosis, in addition to an increase in blood-brain barrier permeability in multiple sclerosis (MS) patients may also experience an increased permeability of intestinal TJ [210; 211]. Yacyshyn et al. have demonstrated that 25% of MS patients studied had an increased intestinal permeability. The fact that patients with MS (and Crohn's disease both present an increased number of peripheral B cells exhibiting CD45RO, a marker of antigen exposure, further supports the concept of preexisting, genetically determined small intestinal permeability abnormalities with subsequent altered antigen exposure as a pathogenic factor common to these diseases [212; 213]. To challenge this hypothesis,

Fasano e coworkers measured serum levels of zonulin in MS patients with different subtypes—relapsing–remitting [RRMS] vs. secondary–progressive [SPMS]—and activities to ascertain whether expression of zonulin into peripheral circulation can differentiate these two groups. Approximately 29% of patients with either RRMS or SPMS had elevated serum zonulin levels with overall average serum levels ~2.0-fold higher than in controls. Interestingly, patients with RRMS in remission showed serum zonulin levels comparable to controls [190].

1.5.3 MRP8/14

Calprotectin (MRP8/14, S100A8/S100A9, 27E10 antigen) is a heterodimer of two calcium-binding proteins present in the cytoplasm of neutrophils and expressed on the membrane of monocytes. Upon neutrophil activation or endothelial adhesion of monocytes, calprotectin is released and may be detected in serum or body fluids as potentially useful clinical inflammatory marker. The soluble form of calprotectin provides both bacteriostatic and cytokine-like effects in the local environment. Calprotectin was originally discovered as an antimicrobial protein that was present in the cytoplasm of neutrophil granulocytes but it is absent in mature limphocytes [214; 215]. Subsequently, it has been recognized as a promising marker of inflammation, or rather a trace of the antagonism going on inside the organism [216]. Furthermore, the molecule is involved in the recruitment of inflammatory cells by interactions with endothelial cells and its zinc capturing function may affect physiological homeostasis [217]. The pleiotropic functions of calprotectin are associated mostly with active

inflammatory processes including antibacterial defense mechanisms or with Th1-mediated responses such as allograft rejection or in autoimmune reactions. It is a 24 kD heterodimer composed of light (MRP8) and heavy (MRP14) chains (8 and 14 kDa), members of the S-100 family of calcium-binding proteins [218; 219]. The binding of calcium induces conformational changes, the calcium-saturated state, which allows binding of other proteins. In the presence of calcium, MRP8/14 heterodimeric complexes may tetramerize into heterotetramers [220]. Calprotectin also contains zinc-binding domains, which have a zinc-binding capacity higher than other S100 proteins, and are not affected by the binding of calcium. Both MRP8 and MRP14 contain histidine-based zincbinding sequences (His-X-X-X-His motif), which are involved in the antibacterial activity of calprotectin [221]. Calprotectin was originally found in neutrophils and a subpopulation of mononuclear phagocytes. Its concentration is abundant and constitutes about half of total cytosolic protein. Calprotectin is secreted extracellularly from stimulated neutrophils and monocytes, or is released as a result of cell disruption or death [222; 223]. After cell death, calprotectin is released into pus or abscess fluid together with microbicidal nucleohistones. The soluble form of calprotectin is found in serum, urine, body secretions (higher calprotectin levels were found in saliva of subjects with candidiasis, and the calprotectin concentration correlated positively with the severity of candidal infection), intestinal fluid and feces [224].

It has been shown that the concentration of the heterodimer MRP8/14 in serum is a marker for acute inflammation and it has been related to disease activity in inflammatory diseases like colitis ulcerosa and rheumatoid arthritis [225]. Regarding multiple sclerosis, as described previously, this disease is characterized by the presence of sclerotic lesions or plaques,

scattered throughout the brain [226]. Early events in the development of these lesions are the increase of blood-brain barrier (BBB) permeability and the formation of cellular infiltrates (monocytes and lymphocytes) [227]. Demyelination is characterized by the presence of myelin degradation products within macrophages and by the expression of macrophage differentiation markers (MRPs) such as MRP8 that is equally distributed throughout the lesion whereas MRP14 and the heterodimer MRP8/14 are mainly present in active demyelinating lesions. Indeed, Bogumil and collegues found elevated serum levels of MRP8/14 in MS patients during a relapse compared to patients in remission [228].

Furthermore, in vitro studies suggested an important role of calprotectin in extravasation of leukocytes by the attachment to endothelial cells via the MRP-14 subunit interacting mainly with endothelial heparan sulfate proteoglycans [229]. Calprotectin binding to microvascular endothelial cells may also be induced by arachidonic acid [230]. The signalling pathways of calprotectin are not fully elucidated, but involve MAP kinase cascade activation [231]. The interaction of monocytic calprotectin with activated endothelium leads to its release, which may account for the high calprotectin concentrations in the body fluids of patients with acute or chronic inflammatory diseases [232]. Released calprotectin may be involved in inflammation by enhancing CD11b expression in human monocytes and by participating in the transendothelial migration mechanism [233]. Calprotectin also inhibits the microbial growth through competition for zinc. Zinc chelation that is mediated by histidine-rich regions of calprotectin represents an important antimicrobial mechanism in host defense [221]. The upregulation of serum calprotectin levels may occur in different immune and immunopathological reactions, especially in

acute inflammation or Th1- mediated responses. In some respects, the sensitivity and dynamics of calprotectin seem to overcome traditional inflammatory markers such as C-reactive protein (CRP). There are several lines of evidence showing the importance of calprotectin in defense mechanisms and physiological functions of the immune system. The clinical usefulness of calprotectin as an inflammatory marker has been shown not only in gastroenterology, where determination of the protein in feces is a noninvasive parameter reflecting pathological processes going on in the mucosa. The serum level of calprotectin may be a very sensitive non-specific flamnmatory marker in various clinical settings [234].

Chapter 2: Aim of the study

Multiple sclerosis (MS) is an inflammatory, autoimmune disease of the central nervous system (CNS) in which an interplay of genetic and environmental factors leads to the chronic activation of immune cells and to neuronal injury. Epidemiological studies have identified several non-heritable risk factors in MS, including viruses, vitamin D and sun exposure, smoke, diet and many others. All these factors may be associated with both disease susceptibility and disease course [38; 45; 53; 84]. To explain the significant increase of autoimmune diseases in western societies in recent decades, the 'hygiene hypothesis' speculates that modern life styles and widespread use of antibiotics have reduced the exposure of humans to microbes. This decreased microbial load contrasts with the situation in developing countries where exposure to microbes is high and prevalence of autoimmune disease is low [149]. This work is in the context of our long-lasting in-depth study of the relationship between microbes and MS. In particular this thesis is focused on the study of Epstein Barr virus (EBV) genotypes and the evaluation of gut epithelium permeability in MS.

Among the non-heritable causative factors potentially involved in the MS etiology, EBV received the most convincing evidence. Nonetheless, it is difficult to understand how a virus that ubiquitously infects humans can be associated with a disease of relative limited prevalence. The possibility that only a particular viral genotype associates with the disease is plausible and has been investigated in this project. We studied EBV genomic variants in B

cells of MS patients and healthy controls and tried to disclos whether a single or several genomic variants of EBV are specifically associated to MS and may help to clarify the role of a plausible etiologic factor, such as EBV.

With regard to a possible involvement of the gut flora in MS pathogenesis recent works show that the commensal gut flora, in the absence of pathogenic agents, is essential in triggering immune processes, leading to develope experimental autoimmune encephalomyelitis (EAE) driven by myelin-specific CD4+ T cells in a mouse model. The same work showed further that recruitment and activation of autoantibody-producing B cells from the endogenous immune repertoire depends on availability of the target autoantigen, myelin oligodendrocyte glycoprotein (MOG), and commensal microbiota [164]. Furthermore, Annibali and co-workers recently reported the potential pathogenic relevance of a proinflammatory CD161hiCD8+ T cell subset that was increased in the peripheral blood of patients compared to controls and detectable in post- mortem MS lesions [166]. The same group, by analyzing the T cell receptor (TCR) usage of CD161hiCD8+ T cells, found that this subset largely overlapped with the mucosal-associated invariant T (MAIT) cells, that have been recently characterized as antibacterial, gut-homing CD161hi T cells. The MAIT cells have been shown to contain the entire CD8+ T cell subset able to secrete IL-17. Th17 cells implication has been suggested in the pathogenesis of MS [168].

Alterations in the composition of the gut flora could affect the intestinal mucosa and inappropriately activate the immune system. Thus an altered physiology and/or immune function of gut mucosa may impact intestinal

permeability, especially leading to an increase of epithelial paracellular space. A change in intestinal permeability may be considered a biomarker of local or even distant immune-mediated disorders. In fact increased gut permeability allows the passage, through the intestinal epithelial layer, of macromolecules, toxin, and bacterial species, both pathogenic and commensal, that may trigger immune-mediated illnesses in different systems, even distant from the gastrointestinal tract.

Another aim of this project is verifying the hypothesis that dysfunction of gut permeability and unbalance of intestinal microbiota may contribute to MS immunopathogenesis. For this part of the project, we will study the intestinal permeability in MS patients and healthy donors through the evaluation of lactulose-mannitol ratio in urine. Moreover, intestinal dysfunction will be also studied by different serum biomarkers, such as zonulin and calprotectin, whose role has been recently recognized. Zonulin modulates the tight junctions, multiproteic structures that regulate the para-cellular spaces between epithelial cells [188]. In autoimmune disorders associated with a dysfunction of intestinal permeability, such as celiac disease and type1 diabetes, an higher expression of zonulin correlates with increased levels of intestinal permeability [184]. Being the zonulin system active only in the small intestine, we chose to consider also the calprotectin which is a good marker of altered permeability and inflammation in the colon. Calprotectin is a heterodimer consisting of two calcium binding proteins, MRP8 and MRP14 (myeloid related proteins), that was found to be elevated in active MS lesions [228].

Chapter 3: Materials and methods

3.1 Analysis of EBV genotypes: EBNA2 single nucleotide polimorphisms

Patients and matched (age-sex and geographic origin) healthy donors (HD) were blood sampled and imaged at the same time point with gadolinium-enhanced magnetic resonance imaging (MRI). The local Institutional Review Board approved the study and all participating subjects gave written informed consent. All patients were free from therapy at the time of sampling.

Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation over Ficoll–Hypaque according to standard procedure. The cells were stained with anti-human CD19 antibodies (Miltenyi Biotec) and separated by magnetic beads in accordance with the manufacturer's recommended protocol.

Genomic DNA was extracted from PBMCs, CD19+ B cells and cell lines (B95-8 EBV-positive cell line and BJAB EBV-negative cell line) by commercially available kit (QIAamp DNA mini kit, Qiagen).

All samples were analyzed by a semi-nested PCR approach using EBNA2 type specific primers (ref. thierney). All PCR products were assayed by sequence analysis. The sequences were aligned with ClustalW2 multiple sequence alignment program (www.ebi.ac.uk/Tools/msa/clustalw2/) and the variants were considered when there was a deviation from the published B95.8 (NCBI accession number V01555) EBV prototype.

3.2 Evaluation of intestinal permeability and serological markers of intestinal inflammation

Blood and urine samples were obtained from RRMS patients and healthy matched for age, sex, ethnicity and geographic origin. The patients, followed at Centre for Experimental Neurological Therapies [(CENTERS), S. Andrea Hospital Site, Sapienza University of Rome, Italy], were free of disease-modifying therapies. The local Institutional Review Board approved the study and all participating subjects gave written informed consent.

Intestinal permeability test: intestinal permeability will be evaluated through a test based on the assumption of an oral solution of lactulose and mannitol and subsequent measurement of their intestinal absorption evaluating the urinary excretion in the 5 hours after the start of the test. After collecting a pre-test urine sample, the subjects will take 50ml of a solution of 5 g of lactulose and 2 g of mannitol dissolved in deionized water. After one hour the subjects will be encouraged to drink water. The urine will be collected during the next 5 hours: the total volume of urine will be measured and a sample of 10 ml will be stored at -20 °C until the assay will be performed. The urine concentrations of lactulose and mannitol will be measured using a method based on liquid chromatography combined with mass spectrometry [234].
All participants will follow a lactose-, lactulose-, mannitol- free diet for 72 h before the test. A list of forbidden food will be provided.

Serological dosage of zonulin: In order to obtain the serum, the whole blood sample was centrifuged at 3000rpm for 10 minutes. The dosage of zonulin was performed through the use of a specific Enzyme-linked immunosorbent (ELISA) assay commercially available kit (Zonulin Elisa, Immundiagnostik) according to the protocol of the manufacturer.

MRP8/14 (calprotectin) dosage: In order to obtain the serum, the whole blood sample was centrifuged at 3000rpm for 10 minutes. Serum MRP8/14 was dosed using a specific ELISA commercially available kit (PhiCal Calprotectin (MRP8/14) ELISA, Immundiagnostik), according to the protocol of the manufacturer.

Chapter 4: Results

4.1 Epstein Barr virus genomic variants

We obtained blood samples from 58 patients and 49 matched (age, sex, ethnicity and geographic origin) HD. The rate of successful EBNA2 genotyping was comparable between the two groups: 39/58 (67.2%) in patients and 33/49 in the HD (67.4%). The 39 patients with a successful genotyping had a relapsing-remitting form of disease (7 were under disease modifying therapies and the others were treatment naïve). The mean age was 34.8±8.6 in the patients and 36.52±9.5 in the HD; the male/female ratio was 9/30 in the patients 10/23 in the controls. Seven patients and three HD were analyzed at different time points (from one to twelve months apart): two patients and one HD were infected by different strains (1.2, 1.3B, GD1) at different time points, while the EBV subtype remained the same in the remaining subjects (Table 3). Taking into account each sampling as an independent observation, we analyzed a total of 89 blood samples (52 in MS patients and 37 in HD).

We observed a significant bias in the distribution of EBV subtypes (Table 3A): the 1.2 allele was predominant in patients (29/52 vs 8/37, p=0.002), while the 1.3B allele prevailed in controls (24/37 vs 14/52, p=0.0005). Gender did not appear to modify this distribution (Table 3B). Going beyond the variants that are encompassed by B95.8 coordinates 48959–49208 [235] our analysis identified other previously undetected divergences from the

EBV wt strain at the amino acids 134, 236, 245, 256 and 267 (Table 5; Table 4A). While the change at position 256 (D/N) was present in subtypes 1.2, 1.3B and GD1, without differences between patients and controls, the other four variants proved to be associated with the 1.2 subtype and consequently correlated with MS status (Table 5; Table 4B). Interestingly, when comparing only patients and HD infected by 1.2 EBV subtype (Table 4C) the divergences at position 245 (P/S or P/T) were more frequent in the patients (21/29 [72.4%] vs 3/8 [37.5%], p=0.04); moreover, the co-occurrence of the four 1.2 subtype-related variants was significantly higher in the patients than in the HD (18/29 [62%] vs 2/8 [25%], p=0.04).

We found no correlation between EBV genotypes and clinical characteristics of patients (MRI data, expanded disability status scale, disease duration). When we analyzed samples from single time points in the population with stable EBV infection (37 MS patients and 32 HD), we obtained comparable results in the EBV subtypes and variants distribution, as well as in the potential to predict MS status (not shown).

Table 3: Frequency of subtypes of EBNA2 in the peripheral blood of MS and HD: global data (A) and results separated by gender (B).

A

Type	Subtype	MS (%)	HD (%)	p-value
B95-8	1.1	0	0	/
B95-8	1.2	29/52 (56)	8/37 (22)	0.002
B95-8	1.3B	14/52 (27)	24/37 (65)	0.0005
B95-8	1.3A	4/52 (8)	0/37	n.s.
GD1	/	5/52 (10)	5/37 (13.5)	n.s.

B

Type	Subtype	Females (%)		P-value	Males (%)		p-value
		MS	HD		MS	HD	
B95-8	1.1	0	0	/	0	0	/
B95-8	1.2	17/37 (46)	2/23 (8.7)	0.004	12/15 (80)	6/14 (43)	0.06
B95-8	1.3B	13/37 (35)	18/23 (78)	0.001	1/15 (6.7)	6/14 (43)	0.04
B95-8	1.3A	4/37 (11)	0/23	n.s	0	0	/
GD1	/	3/37 (8)	3/23 (13)	n.s	2/15 (13)	2/14 (14)	n.s

EBNA2 = Epstein Barr Nuclear Antigen 2; aa = aminoacids; MS = Multiple Sclerosis; HD = healthy donors; ns = not significant.

Table 4: Frequency of aa variants of EBNA2 (A) and their different combinations (B) in MS patients and HD; frequency of variants in patients and HD infected by subtype 1.2 (C)

A

Position aa	B95-8	Variant	MS (%) (N = 52)	HD (%) (N = 37)	p-value*
134	L(CTT)	L(CTG)	27 (52)	7 (19)	0.002
236	T(ACC)	T(ACT)	26 (50)	7 (19)	0.004
245	P(CCA)	S(TCA)	17 (33)	1 (3)	0.0004
		T(ACA)	5 (3.8)	2 (5.4)	0.69
256	D(GAC)	N(AAC)	12(23)	10 (22)	0.8
267	T(ACC)	I (ATC)	28 (54)	6 (16)	0.0009

B

Position aa	MS (%) (N = 52)	HD (%) (N = 37)	p-value*
134+236	25 (48)	6 (16)	0.003
134+245	20 (38)	3 (8)	0.001
134+267	27 (52)	5 (13.5)	0.0003
236+245	19 (36.5)	3 (8)	0.002
236+267	26 (50)	6 (16)	0.001
245+267	21 (40)	2 (3.8)	0.0002
134+236+245	18 (35)	3 (8)	0.005
134+236+267	23 (44)	5 (13.5)	0.002
134+245+267	20 (38)	2 (3.8)	0.0003
236+245+267	19 (36.5)	2 (3.8)	0.0007
134+236+245+267	18 (35)	2 (3.8)	0.001

* Fisher exact test

C

Position aa	MS (%) (N = 29)	HD (%) (N = 8)	p-value**
134	27 (93)	7 (87.5)	n.s
236	26 (90)	7 (87.5)	n.s
245	21 (72)	3 (37.5)	0.04
267	28 (96.5)	6 (75)	n.s
134+236+245+267	18 (62)	2 (25)	0.04

** Barnard exact test

EBNA2 = Epstein Barr Nuclear Antigen 2; aa = aminoacids; MS = Multiple Sclerosis; HD = healthy donors; ns = not significant.

Table 5: nucleotide and amino acid substitutions within the sequence EBNA2 compared to the genomes of type 1 B95-8 and GD1

Donatore	F/M	aa134	aa163	aa165	aa185	aa196	aa198	aa204	INS	aa223	aa236	aa245	aa 256	aa267	Variante
	---	L(CTT)	R	V(GTC)	Q	M	P	T	------	L(CTG)	T(ACC)	P	D	T	B95-8
	---	L(CTT)	M	V(GTA)	Q	M	P	T	------	L(CTG)	T(ACC)	P	D	T	GD1
HD1	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD2	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD3-1	M		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD3-2			V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD4	F		M	V(GTA)											GD1
HD5-1	M	L(CTG)	V	V(GTA)			S				T(ACT)		N	I	1.2
HD5-2		L(CTG)	V	V(GTA)			S				T(ACT)		N	I	1.2
HD5-3			V	V(GTA)			S				T(ACT)			I	1.2
HD6	M		M	V(GTA)											GD1
HD7-1	M	L(CTG)	V	V(GTA)			S				T(ACT)	T			1.2
HD7-2			V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD8	F				R	I		S	L	L(TTG)					1.3B
HD9	F		V	V(GTA)	R	I	S	S	L	L(TTG)					1.3B
HD10	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD11	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD12	M		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD13	F	L(CTG)	V	V(GTA)			S				T(ACT)	T	N	I	1.2
HD14	M		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
HD15	F		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
HD16	F		M	V(GTA)									N		GD1
HD17	M	L(CTG)	V	V(GTA)			S								1.2
HD18	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD19	M	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
HD20	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD21	F	L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
HD22	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD23	F		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
HD24	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD25	M		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
HD26	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD27	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD28	F		M	V(GTA)											GD1
HD29	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD30	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD31	F		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
HD32	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
HD33	M		M	V(GTA)									N		GD1
MS1-1	F	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS1-2		L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS1-3			V	V(GTA)			S				T(ACT)	S		I	1.2
MS2	F		V	V(GTA)	R	I		S	L	L(TTG)		T			1.3A
MS3	M		M	V(GTA)									N		GD1
MS4-1	M	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS4-2		L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS4-3		L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS5-1	M	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS5-2		L(CTG)	V	V(GTA)			S				T(ACT)	S	N	I	1.2
MS5-3		L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS6-1	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS6-2			V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS6-3		L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS7-1	F	L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
MS7-2		L(CTG)	V	V(GTA)			S				T(ACT)		N	I	1.2
MS8-1	M	L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
MS8-2		L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
MS8-3		L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
MS9	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS10-1	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS10-2	F		M	V(GTA)											GD1

MS10-3	F		V	V(GTA)			S								1.2
MS11	M	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS12	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS13	M	L(CTG)	V	V(GTA)			S					T		I	1.2
MS14	F		M	V(GTA)									N		GD1
MS15	F	L(CTG)	V	V(GTA)			S			L(TTG)		T	N	I	1.2
MS16	F		V		R	I		S	L						1.3B
MS17	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS18	F	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS19	F	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS20	F	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS21	F		V	V(GTA)	R	I		S					N		1.3B
MS22	F	L(CTG)	V	V(GTA)			S				T(ACT)	S	N	I	1.2
MS23	F		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
MS24	F	L(CTG)	V	V(GTA)			S				T(ACT)	T		I	1.2
MS25	F	L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
MS26	M	L(CTG)	V	V(GTA)			S				T(ACT)	S	N	I	1.2
MS27	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3A
MS28	M		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
MS29	F	L(CTG)	V	V(GTA)			S				T(ACT)			I	1.2
MS30	F	L(CTG)	V	V(GTA)			S				T(ACT)	S		I	1.2
MS31	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS32	F		V	V(GTA)	R	I		S	L	L(TTG)			N		1.3B
MS33	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS34	F	L(CTG)	V	V(GTA)			S				T(ACT)	T	N	I	1.2
MS35	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3B
MS36	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3A
MS37	M		M	V(GTA)											GD1
MS38	F		M	V(GTA)											GD1
MS39	F		V	V(GTA)	R	I		S	L	L(TTG)					1.3A

INS = aminoacid insertion; HD = healthy donors; MS = multiple sclerosis patients.

4.2 Evaluation of intestinal permeability

We obtained urine samples from 18 multiple sclerosis patients and 16 matched (age, sex, ethnicity and geographic origin) HD. All patients had a relapsing-remitting form of disease and they were free from therapy at the moment of the sampling. The mean age was 37.4±10.6 in the patients and 36.8±10.1 in the HD; the male/female ratio was 6/12 in the patients 2/14 in the controls.

We observed that MS patients show an increase of intestinal permeability compared than healthy controls (p value=0.039 Mann Whitney test) (Fig 19).

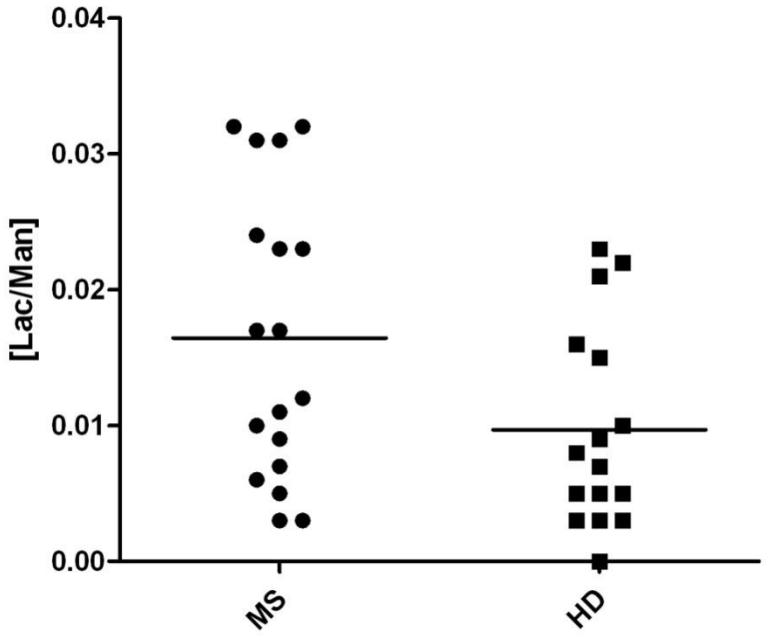

Figure 19 Evaluation of intestinal permeability by lactulose (Lac)/mannitol (Man) concentration in urine in MS patients and healthy controls

Furthermore we observed that MS patients show a lower urinary mannitol concentration (p value=0.033 Mann Whitney test) (Fig 20).
We found no correlation between these alterations and clinical characteristics of patients (MRI data, expanded disability status scale, disease duration).

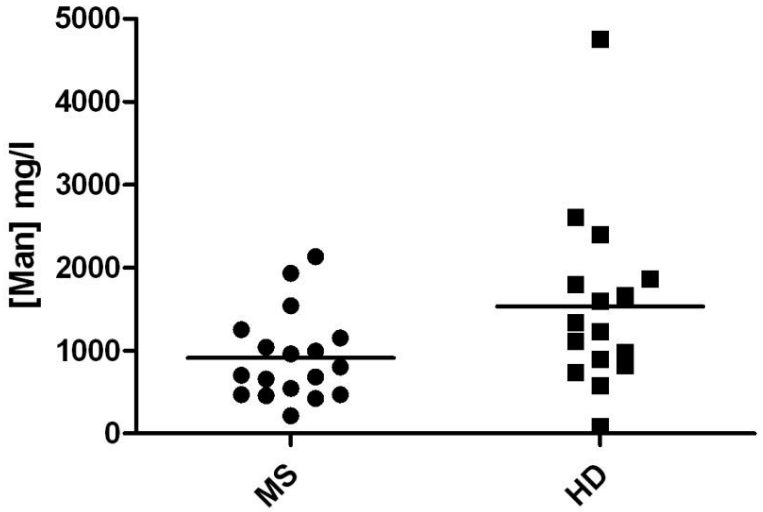

Figure 20 Urinary concentration of mannitol in MS patients and healthy controls

4.3 Evaluation of gastrointestinal inflammation biomarker: zonulin and calprotectin

Concerning the analysis of serum levels of zonulin, we analyzed serum samples from 15 MS patients and 12 matched (age, sex, ethnicity and geographic origin) HD. All patients had a relapsing-remitting form of disease

and they were in remission at the moment of the sampling. Furthermore, all subjects (MS and HD) were free from antibiotics treatment and other therapies at the moment of the sampling. The mean age was 35.5±9.7 in the patients and 35.5±10.7 in the HD; the male/female ratio was 4/11 in the patients 1/11 in the controls.

As shown in the figure, we didn't observe any difference in the serum concentrations of this biomarker between MS patients and HD (p value= 0.102 Mann Whitney test) (Fig.21)

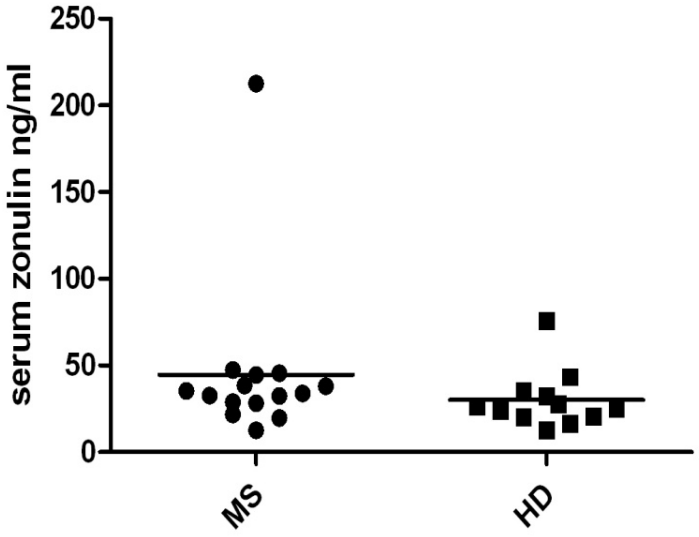

Figure 21 Serum zonulin in MS patients and healrhy donors

With regard to the analysis of the serum calprotectin (MRP8/14) we obtained serum samples from 10 multiple sclerosis patients and 7 matched (age, sex, ethnicity and geographic origin) HD. All patients had a relapsing-remitting form of disease and they were free from therapy at the moment of the

sampling. The mean age was 34.1±8.8 in the patients and 37.5± 11.2 in the HD; the male/female ratio was 3/7 in the patients 1/6 in the controls.

Also in this case we didn't observe any difference in the serum concentrations of the biomarker between patients and healthy controls (p value= 0.845 Mann Whitney test) (Fig. 4).

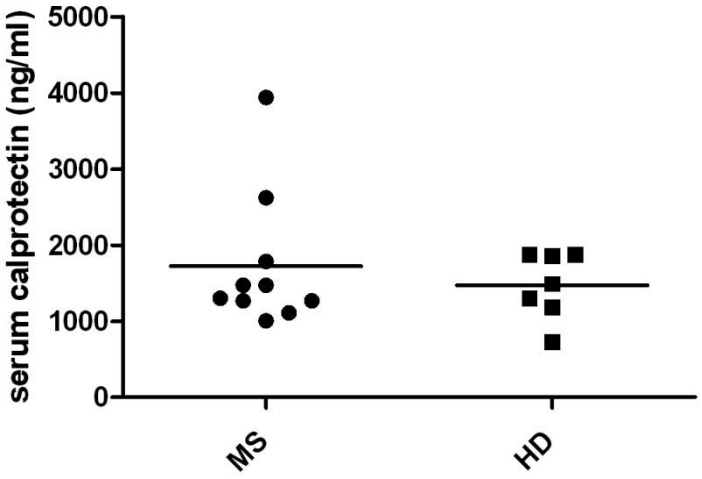

Figure 4 Serum calprotectin (MRP8/14) in MS patients and healrhy donors

Chapter 5: Conclusions, discussions and future perspectives

Similar to other common autoimmune diseases, predisposition to multiple sclerosis (MS) is conveyed by immune system associated genes and by non heritable factors. The role of environmental risk factors such as infections, lack of vitamin D, smoking, diet and their interaction with genetic susceptibility alleles are much less well defined, despite the fact that infections and non-infectious factors have long been associated with MS development.

Concerning the first part of the project, dedicated to the study of EBV, the results obtained show that the MS status correlates with an excess of EBNA2 1.2 and a paucity of 1.3B subtypes. Moreover, patients are more likely to harbor the newly identified 1.2 subtype-related variants (particularly the one at amino acid position 245). Previous studies on a possible association between EBV genotypes and MS gave mixed results: no association with EBNA6, EBNA1 and LMP1, EBNA2; "marginally different frequencies" for BRRF2 and EBNA1; more frequent EBNA2 type 1 and 2 co-infection in patients than in healthy subjects [236; 237; 238; 239; 240; 241]. On a region of EBNA2, that previous studies did not investigate, we find genotypes with associations that are stronger than those previously reported including EBNA2 subtypes and EBNA2 newly detected polymorphisms. Apart from the predictive potential of EBNA2 variants, the likelihood that they may carry functional consequences that underlie contribution to disease etiology deserves further consideration. In a virus not prone to mutations, new variants are more likely to have some

functional impact. There are only two major types of EBV (types 1 and 2), which appear to be identical over the bulk of the genome but show allelic polymorphism in a subset of latent genes: EBNA-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C. In particular, the two EBV types share 64% of the nucleotide and 53% of the predicted amino acid sequences of EBNA2 and EBNA2 sequence mutations are indeed able to affect their interaction with other host proteins [242; 243]. We studied the most polymorphic region of EBNA2, where the sequence divergence between type 1 and type 2 resides. These differences may have functional consequences, also of immunological relevance [244;245]. Moreover this region is involved in interactions with cellular proteins such as Nur77 and SMARCB1 that have been associated with multiple sclerosis and antiviral responses [246; 247; 247; 248; 249; 250]. The association between viral variants and immune-mediated pathology is certainly not new. Our work suggests that this may be the case also for MS, through variants that may have a functional impact on the host. The EBNA2 region that we have investigated is a first objective on which to concentrate further study, and we cannot exclude that additional regions of the complex EBV genome may be relevant as well. Furthermore, these results may help to define the subjects 'at risk' or help clinicians in the diagnosis and prognosis of the disease. In addition, the identified gene variants might be considered as a therapeutic target for possible preventive strategies.

In the light of these findings and those obtained from recent GWAS (Genome Wide Association Studies) studies conducted on MS [20], our research group has proposed a new analysis, a ‘‘candidate interactome’’ (i.e. a group of genes whose products are known to physically interact with environmental factors that may be relevant for disease pathogenesis)

analysis of genome-wide association data in MS, to further understand the gene-environment interaction that is crucial for interpreting the relevance of non-heritable factors and prevents from overlooking genetic associations with small but measurable effects. We elected to center the analysis on viral interactomes but we also analyzed interactomes of other elements that may still be potentially relevant in gene-environment interactions associated with multiple sclerosis (for example human innate immunity interactome for type I interferon and vitamin D receptor). Three interactomes show a statistical enrichment of associations and the EBV interactome is among these. The absence of associations with the interactomes of phylogenetically related viruses (CMV and HHV8, both herpesviruses with the latter that shares the same site of latency as EBV) reinforces the specificity of the EBV result. With regard to EBV, the results obtained from the analysis of the interactome and the study of genotypes suggest a model of the genetic puzzle, so both host and virus polymorphisms influence susceptibility to MS and, through complex epistatic interactions may lead to the development of diseases [251; see Attachments].

The second part of the project focused on the role of intestinal permeability (IP) in MS. We observed that MS patients show altered values of IP compared to healthy controls. The result, though significant, does not allow to say that permeability changes of the intestinal epithelium are involved in MS pathogenesis ..To clarify the possible immunopathogenic role of alterations in intestinal permeability it will be necessary to increase the sample size of the study population and to try to correlate results on IP with those coming from other ongoing studies on gut microbiota and mucosal lymphocyte subsets in MS.

The observation that MS patients show a lower concentration of mannitol in the urine may be indicative of a reduced absorbing surface (mannitol is absorbed through the paracellular intestinal intake, being its urinary concentration proportional to the surface of intestinal absorption). Interestingly, this data is also found in patients who did not show an increase in intestinal permeability (IP is expressed as the ratio of two concentrations, and this value does not take into account the absolute value of the single concentrations of the two sugars). This result might help to understand eventual individual variability in the clinical response to the new oral therapies in MS. Recent clinical research in multiple sclerosis was especially aimed at the development of oral drugs such as fingolimod, teriflunomide, and fumarate. Several clinical trials demonstrated therapeutic efficacy of these drugs in patients with RRMS and an acceptable safety profile. However, common side effects in the gastrointestinal tract occur (and among these are frequent episodes of diarrhoea; suggesting that a better understanding of intestinal dysfunctions in MS may help to deepen mechanisms of action and to increase therapeutic index of the new oral drugs [252].

To complete the study on intestinal dysfunction in MS, we analyzed in the sera of patients and healthy controls, two markers of intestinal inflammation: zonulin, a key regulator of intercellular tight junctions and MRP8/14 (calprotectin), a myeloid related protein expressed by macrophages and a marker of intestinal inflammation at the colon level (ref. calprotectina). Our serological analysis showed no particular increase of these two marker in our patients. Concerning the analysis of the serum levels of zonulin, even if the result does not show differences between patients and controls, must be taken into account that all patients analyzed

were in remission at the time of sampling. In this case, the observed data is in line with the result previously observed in a work conducted by Prof. Fasano and colleagues [190]. The data obtained from the analysis of serum calprotectin is consistent with that observed in the work of Floris and colleagues [253]. Although it is expressed in active MS lesions by macrophages and this expression is a good histopathological marker for monocyte activation, serum levels of MRP8/14 do not correlate with RRMS.

To summarize the results we can conclude that:

1) MS status significantly correlates with a defect of 1.3b and an excess of 1.2 EBNA-2 subtype and with newly identified 1.2 subtype-related variants that could affect the interaction between EBNA-2 and cellular proteins involved in chromatin remodeling and gene expression of B cells.
2) MS patients show altered values of intestinal permeability compared to healthy controls and low urinary concentrations of mannitol, that is indicative of a reduced surface of intestinal absorption.

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Attachments

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A "Candidate-Interactome" Aggregate Analysis of Genome-Wide Association Data in Multiple Sclerosis

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Abstract

Though difficult, the study of gene-environment interactions in multifactorial diseases is crucial for interpreting the relevance of non-heritable factors and prevents from overlooking genetic associations with small but measurable effects. We propose a "candidate interactome" (i.e. a group of genes whose products are known to physically interact with environmental factors that may be relevant for disease pathogenesis) analysis of genome-wide association data in multiple sclerosis. We looked for statistical enrichment of associations among interactomes that, at the current state of knowledge, may be representative of gene-environment interactions of potential, uncertain or unlikely relevance for multiple sclerosis pathogenesis: Epstein-Barr virus, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, HHV8-Kaposi sarcoma, H1N1-influenza, JC virus, human innate immunity interactome for type I interferon, autoimmune regulator, vitamin D receptor, aryl hydrocarbon receptor and a panel of proteins targeted by 70 innate immune-modulating viral open reading frames from 30 viral species. Interactomes were either obtained from the literature or were manually curated. The P values of all single nucleotide polymorphism mapping to a given interactome were obtained from the last genome-wide association study of the International Multiple Sclerosis Genetics Consortium & the Wellcome Trust Case Control Consortium, 2. The interaction between genotype and Epstein Barr virus emerges as relevant for multiple sclerosis etiology. However, in line with recent data on the coexistence of common and unique strategies used by viruses to perturb the human molecular system, also other viruses have a similar potential, though probably less relevant in epidemiological terms.

Citation: Mechelli R, Umeton R, Policano C, Annibali V, Coarelli G, et al. (2013) A "Candidate-Interactome" Aggregate Analysis of Genome-Wide Association Data in Multiple Sclerosis. PLoS ONE 8(5): e63300. doi:10.1371/journal.pone.0063300

Editor: Luwen Zhang, University of Nebraska - Lincoln, United States of America

Received December 12, 2012; **Accepted** March 29, 2013; **Published** May 16, 2013

Funding: This work was funded by Italian Multiple Sclerosis Foundation grants (2007/R/17 and 2011/R/31) to MS. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

As in other multifactorial diseases, genome-wide association studies (GWAS) are providing important data about disease-associated loci in multiple sclerosis (MS) [1]. In parallel, sero-epidemiological studies are reinforcing the evidence that nonheritable factors such as Epstein-Barr virus (EBV) and vitamin D are associated with disease pathogenesis [2].

However, the effect size of the gene variants identified so far in MS appears small. It is therefore important (but difficult: Sawcer and Wason, 2012) [3] to establish if and in which cases (including those gene variants with small but measurable effect size that do not reach the significance threshold of GWAS) the interaction with nonheritable factors may help understand their true impact on disease pathogenesis [4]. Furthermore, as far as the sero-epidemiological associations are concerned, their causal relevance and underlying pathogenetic mechanisms become clearer if interpreted in the light of genetic data.

As an attempt to consider, beyond the statistical paradigms of GWAS analysis, which gene-environment interactions may associate with the development of MS, we performed an interrogation of GWAS data [1] through a "candidate interactome" approach, investigating statistical enrichment of associations in genes whose products "interact" with putative environmental risk factors in MS.

We elected to center the analysis on viral interactomes, based on the classical hypothesis of a viral etiology of MS. Importantly, we examined only direct interactions between viral and human proteins as it has recently been shown that these are the interactions that are more likely to be of primary importance for the phenotypic impact of a virus in "virally implicated diseases" [5]. The chosen interactomes reflect the compromise between

informative size and potential relevance for MS. In detail, EBV was chosen as main association to be verified against phylogenetically related or unrelated viruses. Given the profound influence of EBV on the immune response, and the preponderance of (auto)immune-mediated mechanisms in the pathogenesis of the disease, we added two interactomes of immunological relevance, human innate immunity interactome for type I interferon (hu-IFN) and autoimmune regulator (AIRE). Finally, we included the vitamin D receptor (VDR) and the aryl hydrocarbon receptor (AHR) interactomes to evaluate, on the same grounds, also part of the molecular interactions that compose other established or emerging "environmental" associations.

Methods

Seven interactomes were obtained from the literature: EBV [6], Human Immunodeficiency virus (HIV) [7], Hepatitis C virus (HCV) [8], AIRE [9], hu-IFN [10], Influenza A virus (H1N1) [11], Virus Open Reading Frame (VIRORF) [12]. Four interactomes were manually curated: Human Herpesvirus 8 (HHV8), Cytomegalovirus (CMV), JC virus (JCV), Hepatitis B virus (HBV). VDR and AHR interactomes were extracted from BIOGRID (http: //thebiogrid.org) [13].

As reference to gather gene and single nucleotide polymorphism (SNP) details from their HUGO Gene Nomenclature Committee (HGNC) Ids and rsids, we employed a local copy of the Ensembl Human databases (version 66, databases *core* and *variation*, including SNPs coming from the 1000 Genome project); the annotation adopted for the whole analysis was GRCh37-p6, that includes the release 6 patches (Genome Reference Consortium: human assembly data - GRCh37.p6 - Genome Assembly. http: // www.ncbi.nlm.nih.gov/genome/assembly/304538/).

The genotypic p-values of association for each tested SNP were obtained from the International Multiple Sclerosis Genetics Consortium & Wellcome Trust Case Control Consortium,2 study. All SNPs which did not pass quality checks in the International Multiple Sclerosis Genetics Consortium & the Wellcome Trust Case Control Consortium,2 study were filtered out from the original data. We used ALIGATOR [14,15] to evaluate how single genes get summed to provide total contribution of candidate interactomes (Table S1). The idea behind ALIGATOR's strategy is to evaluate gene category significance by means of an empirical approach, comparing each interactome with the null hypothesis, built using random permutations of the data. Such method begins its analyses by evaluating the Gene Ontology (GO) category association in each interactome provided: (i) each SNP with a p-value stronger than the P-CUT parameter is associated to the gene within 20 kb; then the most representative SNP for each gene is selected; (ii) LD filter of SNPs that have an $r2 \leq 0.2$ and those that are farther than 1000 kb; (iii) count the number of genes significant in each GO category. This is the real observed data.

A non parametric bootstrap approach was used to generate a null hypothesis as follows: (i) build 5000 random interactomes (of the same size of the one under analysis, this procedure is repeated for each interactome); (ii) obtain category-specific p-values by comparing each random interactome with the remaining 4999 built; (iii) elect one of the interactomes in (i) as simulated observed data; (iv) randomly sample interactomes in (i) to generate category-specific p-values; (v) repeat (iv) to simulate 1000 simulated studies. The GO category association distribution in the real observed data is then compared with the null hypothesis: (i) generate an expected number of significant genes in each category, using the simulated studies; (ii) compare the number of significant categories in the real observed data with (i). ALIGATOR parameters that we used are those of its reference paper [14]. p-value cut-off was taken at 0.05, only the SNPs with marginal p-value less than this cut-off were employed (p-value cut-offs were also taken at 0.005 and 0.03 for the re-analysis of interactomes that resulted associated at 0.05, see results). Furthermore, to limit the uncertainties introduced by combined SNP effects in the MHC extended region (that is the haplotype set with the strongest signal in our analysis), we computed two different statistical evaluations for each interactome, one including and the other one excluding SNPs coming from such region (we considered as belonging to the extended MHC region all those SNPs that participate in at least one of the following haplotypes: HSCHR6_MHC_APD, HSCHR6_MHC_COX, HSCHR6_MHC_DBB, HSCHR6_MHC_MANN, HSCHR6_MHC_MCF, HSCHR6_MHC_QBL, HSCHR6_MHC_SSTO according to GRC data). In both cases we used Ensembl API [16] and BioPerl [17] (version 1.2.3) to gather all SNP information, haplotype participation, genes position and size [18]; such annotated information was then fed into ALIGATOR together with the interactomes.

Ingenuity Pathway Analysis (IPA) was employed twice: (i) before the ALIGATOR statistics, to characterize the composition of our interactomes (Table S2), and (ii) on the genes with nominally significant evidence of association [1] that ALIGATOR took as representative of each interactome-SNP relation (Table S3). In both cases we performed the IPA-"core analysis", and we restricted the settings to show only molecular and functional associations. Afterwards, we used IPA-"comparative analysis" to produce the p-value of association between each functional class and all our interactomes. IPA knowledge base (ie, the input data used by IPA) was set to the following criteria in every analyses: consider only molecules and/or relationships where the species in object was human (or it was a chemical), and the datum was experimentally observed. Since IPA-"comparative analysis" provides p-value ranges associated to functional classes, we took as reference the value used by IPA to fill its reports, namely the best p-value for that class.

Results

We performed a "candidate interactome" (i.e. a group of genes whose products are known to physically interact with environmental factors that may be relevant for disease pathogenesis) analysis of genome-wide association data in multiple sclerosis.

We obtained 13 interactomes, 7 from the literature (as such) and 6 by manually selecting those interactions that were reported by two independent sources or were confirmed by the same source with distinct experimental approaches. In all cases we considered only physical-direct interactions (Table S2,Table 1).

Preliminarily to the enrichment of association analysis, we used IPA to obtain a sense of the cellular signaling pathways that are targeted by each interactome. A classification for molecular and cellular functions showed a comparable distribution of components in most interactomes except for VDR, HBV, VIRORF and hu-IFN where a relative enrichment of some functional pathways (cell signaling, cellular growth and proliferation, cellular development, cell cycle, cell death and survival, protein synthesis, RNA post-transcriptional modification, gene expression) was present (Figure 1).

We investigated statistical enrichment of associations within each one of the above interactomes (Table 1). The analyses were performed with and without considering SNPs falling in the MHC extended region. In both cases the interactomes of EBV, HIV and HBV reached significance. To verify the sensitivity of our results

Table 1. Statistical enrichment of MS-associated genes within each interactome.

Interactome	Size	Source	p-value with MHC	p-value without MHC
VIRORF	579	Experimental data [12]	0.0610	0.0632
HIV	446	Experimental data [7]	0.0026	0.0034
HCV	202	Experimental data [8]	0.4244	0.4424
hu-IFN	113	Experimental data [10]	0.2176	0.1838
EBV	110	Experimental data [6]	0.0140	0.0446
H1N1	87	Experimental data [11]	0.9572	0.9648
AIRE	45	Experimental data [9]	0.4322	0.4012
HBV	85	manually curated	0.0124	0.0236
CMV	41	manually curated	0.1156	0.3322
HHV8	40	manually curated	0.1132	0.0920
JCV	10	manually curated	1.0000	1.0000
VDR	78	BioGRID	0.1848	0.1802
AHR	30	BioGRID	0.8752	0.8522

ALIGATOR-obtained interactome p-values (overall contribution given by SNP p-values to each interactome, with and without SNPs falling in the MHC region). The SNPs with marginal p-value less than 0.05 were employed.
MS = multiple sclerosis; ALIGATOR = Association LIst Go AnnoTatOR; SNP = single nucleotide polymorphism; MHC = Major histocompatibility complex; BioGRID = Biological General Repository for Interaction Datasets; VIRORF = Virus Open Reading Frame; HIV = Human Immunodeficiency virus; HCV = Hepatitis C virus; hu-IFN = human innate immunity interactome for type I interferon; EBV = Epstein Barr virus; H1N1 = Influenza A virus; HBV = Hepatitis B virus; VDR = vitamin D receptor; AIRE = autoimmune regulator; CMV = Cytomegalovirus; HHV8 = Human Herpesvirus 8; JCV = JC virus; AHR = Aryl hydrocarbon receptor.
doi:10.1371/journal.pone.0063300.t001

with respect to a choice (SNPs p-value cut-off at 0.05) that is not obvious based on the literature published so far, we evaluated different cut-offs ($p<0.005$ and $p<0.03$) on the three interactomes that were MS-associated at $p<0.05$. These analyses supported the consistency of the results (Table S4).

We then performed the same IPA classification as in Figure 1 (Figure 2) on the MS-associated genes within the EBV, HIV and HBV interactomes (Table S3). The aim was to verify whether the associations emerging from the three interactomes implied new and MS-specific perturbations and whether these perturbations are virus-specific or shared by the three pathogens. The comparison between pre- and post-match distribution of the functional classes (Figure 3) showed that the MS-associated interactomes did not reflect a clear cut involvement of specific pathways though, in the case of EBV, an enrichment of some biological functions (cellular function and maintenance, cell morphology, cellular assembly and organization, energy production) was present. On the other hand the most frequent changes for HBV and HIV could be in accord with the post-match reduction of the interactome sizes.

Discussion

Of the 13 interactomes, 3 show a statistical enrichment of associations. In line with the epidemiological and immunological literature, the EBV interactome is among these. The lack of significant associations with the hu-IFN and AIRE interactomes suggests, though does not exclude, that the result is not an effect of the immunological connotation of the EBV interactome. The absence of associations with the interactomes of phylogenetically related viruses (CMV and HHV8, both herpesviruses with the latter that shares the same site of latency as EBV and belongs to the same subfamily of gamma-herpesviridae) reinforces the specificity of the EBV result. The fact that a portion of the genetic predisposition to MS may be attributable to variants in genes that interact with EBV may be complementary to another our finding showing that EBV genomic variants significantly associate with MS (unpublished data): the two results suggest a model of genetic jigsaw puzzle, whereby both host and virus polymorphisms affect MS susceptibility and, through complex epistatic interactions, eventually lead to disease development.

The associations with the HBV and HIV interactomes were unexpected. Overall, epidemiological data do not support a role of these viruses in the pathogenesis of MS though some controversy still holds concerning the safety of HBV vaccination [19–23]. Interestingly, Gregory et al. (2012) [24] demonstrated that in the TNFRSF1A gene, which is part of the HBV interactome, the MS-associated variant directs increased expression of a soluble tumor necrosis factor receptor 1.

Concerning HIV, the lack of epidemiological association seems more established. However, demyelination is a feature of HIV encephalomyelopathy [25] and cases of difficult differential diagnoses or association between the two conditions are described in the literature [26,27]. All this considered, it might not be surprising that some molecular interactions that take place between HIV and host may predispose to demyelination. Other viruses, sharing homology with HIV may possess better paraphernalia and be more prone to cause MS. The HERV-W family has long been associated with MS [28] and HERV-W/Env, whose expression is associated with MS [29], is able to complement an env-defective HIV strain [30] suggesting a certain degree of functional kinship.

Apart from any conjectures about the data on HBV and HIV interactomes, it remains true, as recently demonstrated by Pichlmair and colleagues (2012) [12], that viruses use unique but also common strategies to perturb the human molecular network. Our pathway analyses do not suggest, in fact, any specific cellular signaling target for the three viruses in MS, perhaps with some exceptions as far as the EBV interactome is concerned. Though preliminary, this acquisition may be in accord with the largely accepted view that, alongside the risk associated with EBV infection, there can be a more general risk of developing MS linked to a variety of other infections [31,32].

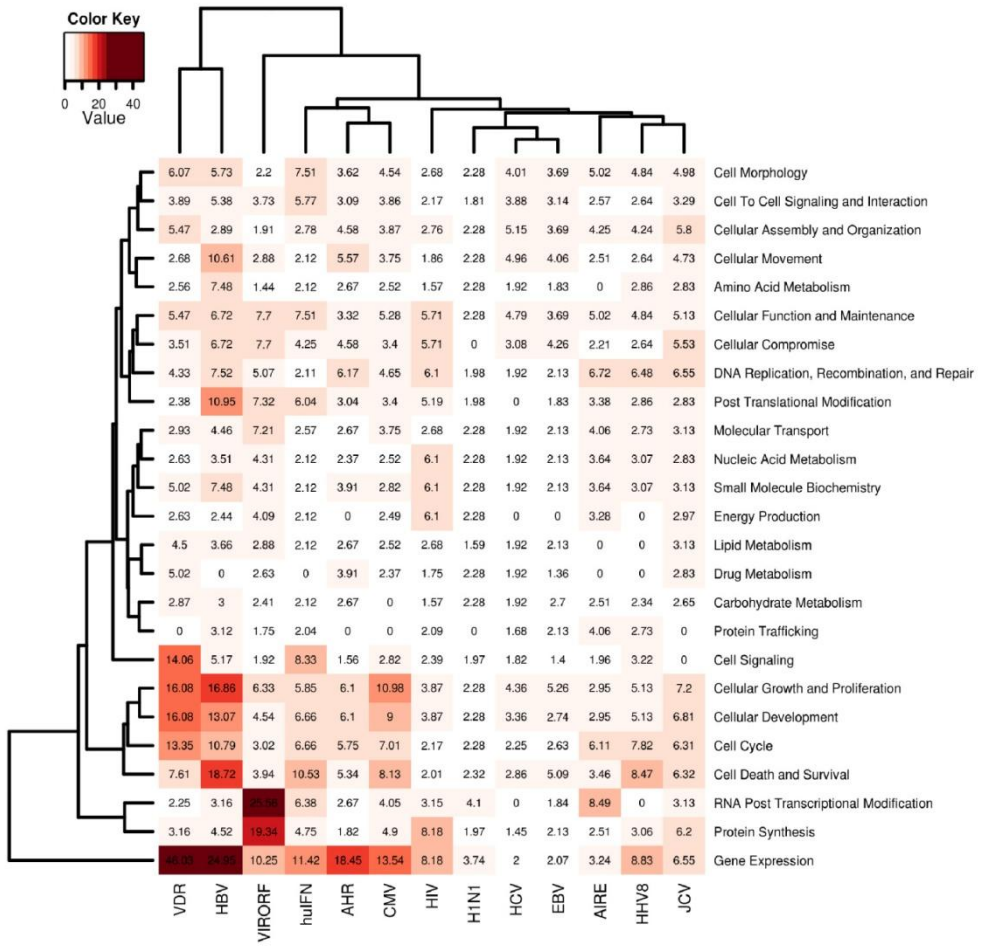

Figure 1. Heatmap from Ingenuity Pathway Analysis of each interactome. Statistical significance (in –log[p-value] notation, where $p<0.05$ corresponds to a –log[p]>1.3) of the functional components in each interactome, as obtained through a Comparative Core-Analysis in IPA (Ingenuity Pathway Analysis). The functional components identified at the molecular and cellular level are presented row-wise (right); the interactomes are presented column-wise (bottom). Each cell in position (*i,j*) contains a number that represents in $-\log$ notation the strength of the association between the functional class *i* and the interactome *j*; this information is also color-matched with a color gradient that moves from white ($-\log[p]=0.0$, $p=1$) to crimson ($-\log[p]=50$, $p<10^{-50}$). Two hierarchical cluster analyses were employed to group functional classes that share similar patterns of associations across all interactomes (left-side clustering), and to group interactomes that share similar functional compositions (top-chart clustering).
doi:10.1371/journal.pone.0063300.g001

The VDR interactome does not show significant enrichment of associations. The result does by no means diminishes the importance of the epidemiological association between vitamin D and MS: its causal relevance is already supported by data that are starting to explain the molecular basis of this association, upstream [33–35,1] and downstream the interactions between the VDR and its protein cofactors [36,37].

Current approaches for gene set analysis are in their early stage of development and there are still potential sources of bias or discrepancy among different methods, including those used in our study. As the reproducibility of the techniques increases, and new facilities [38] and methods become available to identify interactions that still escape detection, new lists will become available for matching with GWAS data. In parallel, also the assessment of human genetic variation will become more comprehensive [39].

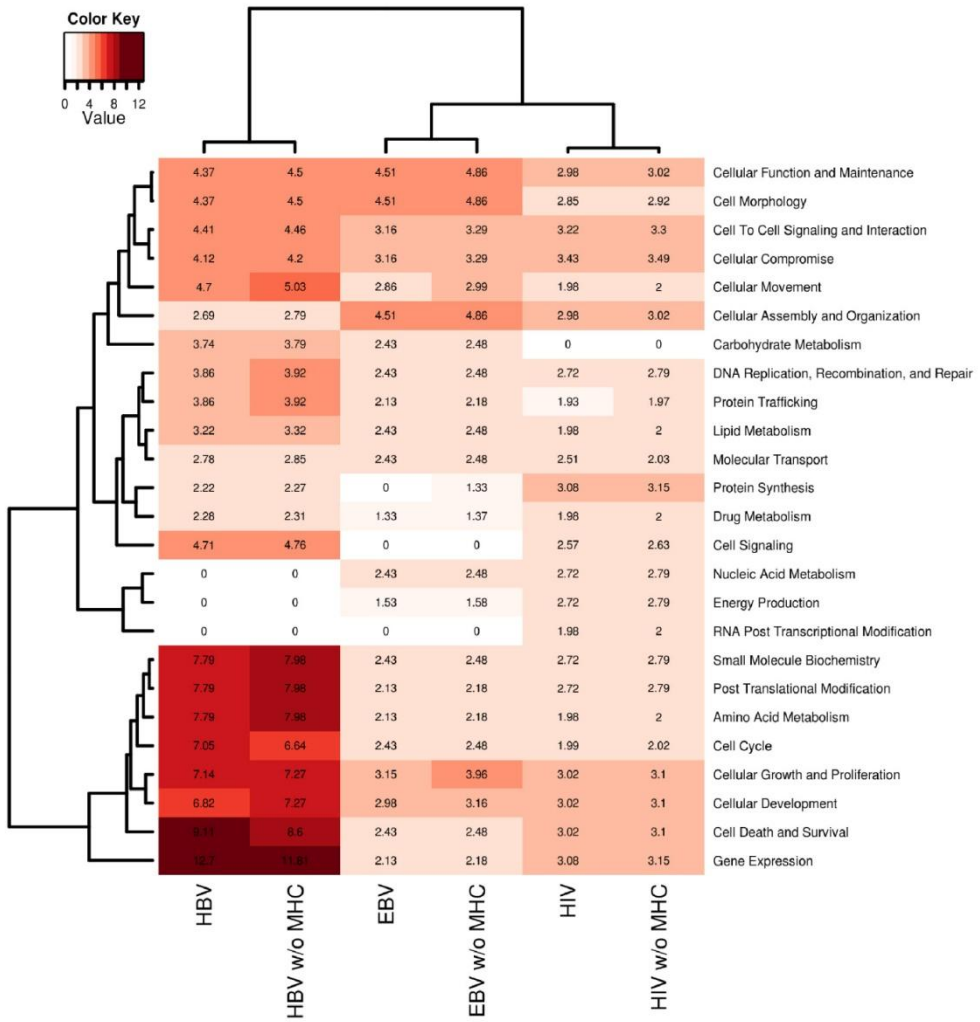

Figure 2. Heatmap from Ingenuity Pathway Analysis of MS-associated interactomes. Statistical significance (in –log[p-value] notation, where $p<0.05$ corresponds to a –log[p]>1.3) of the functional components in each one of the three MS-associated interactomes (Table S3) computed by ALIGATOR (Association LIst Go AnnoTatOR) first flow process. These p-values were obtained through a Comparative Core-Analysis in IPA (Ingenuity Pathway Analysis). The functional components identified at the molecular and cellular level are presented row-wise; the interactome sub-sets are presented column-wise. Each cell in position (*i,j*) contains a number that represents in $-\log$ notation the strength of the association between the functional class *i* and the interactome; this information is also color-matched with a color gradient that moves from white ($-\log[p]=0.0$, $p=1$) to crimson ($-\log[p]=14$, $p<10^{-14}$). Two hierarchical cluster analyses were employed to group functional classes that share similar patterns of associations across all interactome sub-sets (left-side clustering), and to group interactome sub-sets that share similar functional compositions (top-chart clustering).
doi:10.1371/journal.pone.0063300.g002

Hence, the "candidate interactome" approach may become an increasingly meaningful strategy to interpret genetic data in the light of acquisitions from epidemiology and pathophysiology. Notably, this approach appears to be complementary to other studies, which look for statistical enrichment of associations in an unbiased way, and may disclose unexpected pathways in MS susceptibility [40].

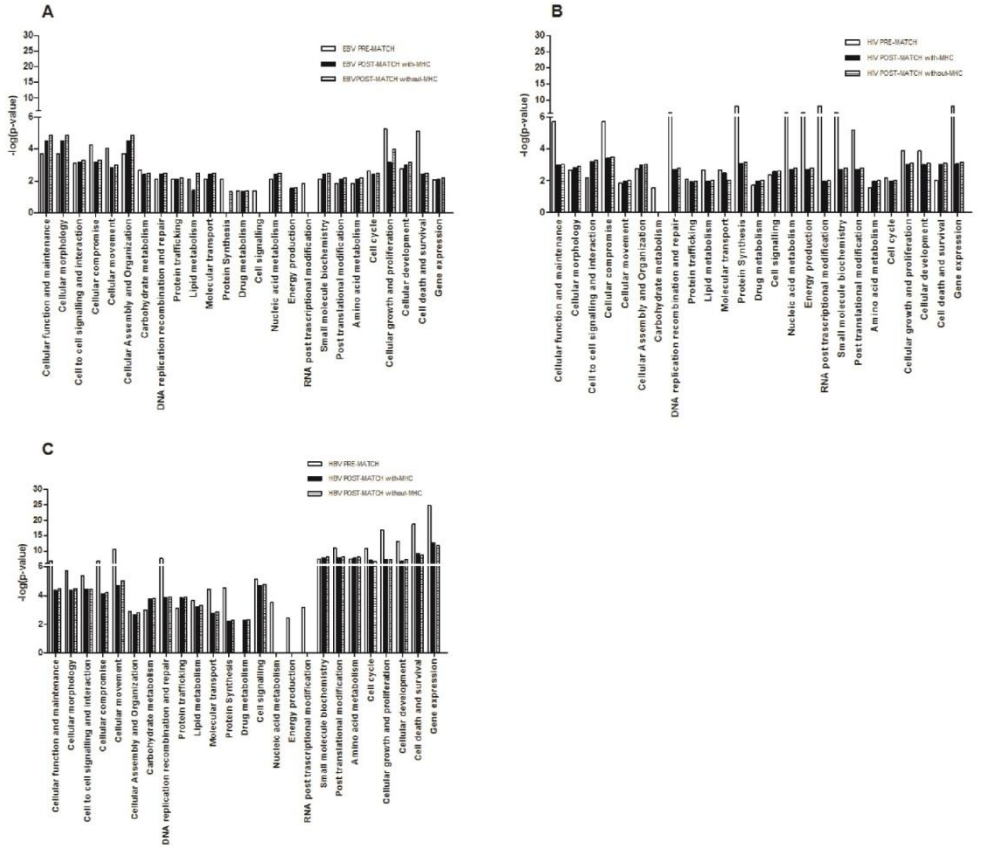

Figure 3. Histograms of functional class distribution of MS-associated interactomes. The histograms show the strength of the association between each IPA functional class and the 3 MS-associated interactomes (EBV [A], HIV [B] and HBV [C]). For each functional class 3 values were derived according to its distribution before (Figure 1) and after (Figure 2, with and without MHC [Major histocompatibility complex]) the ALIGATOR (Association LIst Go AnnoTatOR) statistical analysis of association.
doi:10.1371/journal.pone.0063300.g003

At present, our results support a causal role of the interaction between EBV and the products of MS-associated gene variants. Other viruses may be involved, through common and unique mechanisms of molecular perturbation.

Supporting Information

Table S1 ALIGATOR settings.
(XLS)

Table S2 Composition of all the interactomes. Lists of genes of each interactome as obtained from the literature. VIRORF = Virus Open Reading Frame; HIV = Human Immunodeficiency virus; HCV = Hepatitis C virus; hu-IFN = human innate immunity interactome for type I interferon; EBV = Epstein Barr virus; H1N1 = Influenza A virus; HBV = Hepatitis B virus; VDR = vitamin D receptor; AIRE = autoimmune regulator; CMV = Cytomegalovirus; HHV8 = Human Herpesvirus 8; JCV = JC virus; AHR = Aryl hydrocarbon receptor.
(DOC)

Table S3 List of genes within molecular and functional classes in the three MS-associated interactomes (p-value cut-off<0.05). MS = multiple sclerosis; HIV = Human Immunodeficiency virus; EBV = Epstein Barr virus; HBV = Hepatitis B virus; MHC = Major histocompatibility complex
(XLS)

Table S4 Statistical enrichment of MS-associated interactomes (p-value cut-off<0.005; 0.03). ALIGATOR-obtained interactome p-values (overall contribution given by SNP p-values to each interactome, with and without SNPs falling in the MHC region). MS = multiple sclerosis; ALIGATOR = Associa-

tion LIst Go AnnoTatOR; SNP = single nucleotide polymorphism; MHC = Major histocompatibility complex; HIV = Human Immunodeficiency virus; EBV = Epstein Barr virus; HBV = Hepatitis B virus.
(DOC)

Acknowledgments

We thank Dr. Eliana Coccia (Istituto Superiore di Sanità, Rome, Italy) for her helpful contribution to obtain the interactomes.

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Screening for neurotropic viruses in cerebrospinal fluid of patients with multiple sclerosis and other neurological diseases

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Mult Scler published online 19 August 2013
DOI: 10.1177/1352458513501233

The online version of this article can be found at:
http://msj.sagepub.com/content/early/2013/08/19/1352458513501233

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MULTIPLE SCLEROSIS JOURNAL | MSJ

Multiple Sclerosis Journal
0(0) 1

DOI: 10.1177/1352458513501233
msj.sagepub.com
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Screening for neurotropic viruses in cerebrospinal fluid of patients with multiple sclerosis and other neurological diseases

The paper by Virtanen et al.[1] strengthens the association of herpes viruses with multiple sclerosis (MS). The correlation between the presence of Epstein Barr virus (EBV)/Herpes Virus 6 (HHV-6) DNA in cerebrospinal fluid (CSF) and number of contrast enhancing lesions (CELs) on magnetic resonance imaging (MRI) is of interest and mirrors our results from a screening for neurotropic viruses in cerebrospinal fluid of patients with MS and other neurological diseases.

We performed PCR for herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), cytomegalovirus (CMV), varicella zoster virus (VZV), HHV6, JC virus (JCV) and EBV on CSF samples from 159 patients who had had a lumbar puncture for diagnostic purposes. All CSF samples were analyzed using a commercial RHA CNS kit (Labo Bio-medical Products) for the detection of cell-free and cell-associated viral nucleic acids. Patients were then grouped according to their final diagnosis: 60 with MS (58 relapsing–remitting [RR] and two primary progressive [PP]; mean age 37±13 years, 40 females, 20 males), 48 with other inflammatory diseases (OIND; mean age 57±17 years, 23 females, 25 males), and 51 with other non-inflammatory diseases (NIND; mean age 56±18 years, 43 females, 8 males). Gadolinium-enhanced MRI was performed in all patients within 10 days of CSF sampling.

In the MS group, 14 patients had a positive PCR for EBV, two for HSV1, three for HSV2 and one for HHV6. In OIND patients, 12 had positive PCR for EBV, two for HSV1, three for HSV2, four for CMV, one for JCV and four for HHV6. In NIND patients, two had positive PCR for EBV, one for HHV6 and one for VZV. The frequency of viral DNA in CSF was comparable among MS and OIND groups. A significant bias in the distribution of EBV was observed in MS and OIND groups with respect to NIND ($p = 0.011$ at Fisher's exact test), while CMV infection was more frequent in OIND patients ($p = 0.036$ at Fisher's exact test). In patients with MS we observed an excess of large T2 lesions at MRI in cases with EBV DNA in CSF: T2 lesions larger than 10 mm were present in six out 14 EBV-positive and five out 46 EBV-negative patients ($p = 0.01$ at Fisher's exact test). This finding was not found in OIND patients; it was also independent of the cell count in CSF, which was higher in OIND than in MS patients ($p = 0.02$ at Mann–Whitney *U* test), but was comparable between EBV-positive and EBV-negative MS patients. Thus, the relationship between lesion size and EBV-positive PCR seems not to reflect a non-specific recruitment of leucocytes in the central nervous system in the context of amplified inflammation. The increased chance of detecting EBV DNA in MS patients with more active lesions is instead in accord with Jacobson's group's finding[1] (at least as far as EBV is concerned) and supports evidence of intrathecal reactivation and virus-driven immunopathogenic response in MS.[2,3]

Conflict of interest

None declared.

Funding

This work was supported by Fondazione Italiana Sclerosi Multipla (grant number 2011/R/31).

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