

# Environmental pathways to autoimmune diseases: the cases of primary biliary cirrhosis and multiple sclerosis

Carlo Selmi<sup>1,2</sup>, Anna Maria Papini<sup>3,4</sup>, Piera Pugliese<sup>1</sup>, Maria Claudia Alcaro<sup>4</sup>, M. Eric Gershwin<sup>2</sup>

<sup>1</sup>Department of Medicine and Autoimmunity and Metabolism Unit, IRCCS Istituto Clinico Humanitas, Rozzano, Milan, Italy

<sup>2</sup>Division of Rheumatology, Allergy, and Clinical Immunology, University of California, Davis, USA

<sup>3</sup>Laboratory of Peptide and Protein Chemistry and Biology, Dipartimento di Chimica "Ugo Schiff", Università degli Studi di Firenze, Italy

<sup>4</sup>Toscana Biomarkers, Siena, Italy

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**Corresponding author:**

M. Eric Gershwin MD  
Division of Rheumatology,  
Allergy and Clinical  
Immunology  
University of California  
at Davis School of Medicine  
451 E. Health Sciences Drive  
Suite 6510, Davis  
CA 95616, USA  
Phone: 530-752-2884  
Fax: 530-752-4669  
E-mail:  
megershwin@ucdavis.edu

## Abstract

The pathways leading to autoimmunity remain enigmatic despite numerous lines of experimental inquiry and epidemiological evidence. The mechanisms leading to the initiation and perpetuation of specific diseases such as primary biliary cirrhosis (PBC) or multiple sclerosis (MS) remain largely enigmatic, although it is established that a combination of genetic predisposition and environmental stimulation is required. The growing number of genome-wide association studies and the largely incomplete concordance for autoimmune diseases in monozygotic twins concur to support the role of the environment (including infectious agents and chemicals) in the breakdown of tolerance leading to autoimmunity through different mechanisms. In the present article we illustrate the current hypotheses related to an environmental impact on the onset of PBC and MS as two representative conditions investigated with complementary approaches. Indeed, while a role of post-translational antigen modifications has been proposed for MS, this field remain unexplored in PBC where, conversely, most evidence is gathered from geoepidemiology and experimental data on xenobiotics or infectious agents.

**Key words:** tolerance breakdown, multiple sclerosis, autoantibodies, antigen modification, glycosylation.

## Introduction

The field of autoimmunity currently recognizes a wide variety of conditions ranging from pure autoimmune to chronic inflammatory diseases and approximately 100 different entities [1] affecting up to 5% of the general population in Westernized countries [2]. These prevalence rates lead to significant economic costs for healthcare systems worldwide also based on the possible degrees of disability resulting from the multi-organ involvement and the high comorbidity rate [3-5].

Several common factors are shared by the pathogenetic mechanisms of different autoimmune disease and manifest as the coined "mosaic of autoimmunity" to support the multifactorial origin and clinical diversity [6-8]. Four groups include the major factors contributing to the mosaic:

i.e. genetics, environment, immune defects, and hormones [9]. An ideal example to represent the former two groups is given by the disease concordance rates in monozygotic compared to dizygotic twins or other family members [10], as illustrated in Table I. Somehow surprisingly, autoimmune disease concordance rates in monozygotic twins are largely incomplete and are often based on small series, as in the case of primary biliary cirrhosis (PBC). Nevertheless, data support the hypothesis that genetics per se are insufficient to determine disease onset and that additional factors, likely related to the environment, may play a significant role [11]. The present article will discuss the current views in terms of gene-environment interplay based on two paradigmatic examples, i.e. PBC and multiple sclerosis (MS).

Primary biliary cirrhosis is a chronic cholestatic liver disease characterized by the immune-mediated destruction of intrahepatic bile ducts resulting in chronic liver injury and ultimately fibrosis, cirrhosis and liver failure [12]. The disease recognizes an autoimmune pathogenesis as well represented by the specific presence of serum antimitochondrial antibodies (AMA) and autoreactive T cells [13]. On the other hand, MS is a chronic inflammatory neurodegenerative autoimmune disease affecting the central nervous system [14, 15]. Both conditions manifest a largely incomplete concordance among monozygotic twins, 63% in PBC [16] and 30% in MS [17] despite a significant genetic component [18-20]. One interesting feature characterizing both diseases, similar to autoimmunity per se [8], is related to the geographical pattern in prevalence and incidence which also support the environmental influence [4, 7, 21, 22]. These observations are now unified into the novel field of clinical geoepidemiology [6, 23].

## Epidemiology and geoepidemiology

Thanks to the globalization and the consequently easy comparison of data between different geographical areas the discipline of geoepidemiology was developed in recent years [6] to represent the approach by which epidemiological data across different geographical regions and populations are compared [4, 8, 24]. It is then possible to identify genetic, environmental, and socioeconomical factors involved in the autoimmune disease development. On one side it is possible to determine the contribution of ethnogenetic factors in development of autoimmune diseases, analyzing prevalence among different racial groups sharing the same geographical locations. On the other hand, it allows to study the impact of nutrition, light exposure, infections, xenobiotics and other environmental factors. An example is given by the evidence that different diseases share similar geoepidemiological distributions, as in the cases of type 1 diabetes mellitus, multiple sclerosis, and inflammatory bowel disease. These three chronic inflammatory diseases share the highest prevalence rates in European Caucasian populations, particularly in Northern Europe and North America, originally settled by northern-European people [8] and this observation suggests common determinants and offers a cue for future studies, although we cannot overlook the possible role of disease awareness in different areas. Of note, a latitudinal gradient was observed for most autoimmune conditions [4], with highest prevalence in industrialized countries, especially Northern Europe and North America, suggesting a central role played by Westernized lifestyle and focusing the attention also on other factors such as sunlight (UV) exposure affecting vitamin D levels, nutritional habits, and xenobiotics (i.e. chemicals).

**Table I.** Pairwise concordance rates of autoimmune disease in monozygotic and dizygotic twins (n of concordant sets/n of studied sets)

Disease	MZ concordance rate	DZ concordance rate
Primary biliary cirrhosis	0.63	0.00
Primary sclerosing cholangitis	Concordant pair reported	–
Systemic lupus erithematosus	0.24	0.02
Sjögren's syndrome	Concordant pair reported	–
Type I diabetes mellitus	0.21-0.70*	0.00-0.13
Rheumatoid arthritis	12.3-15.4	3.50-3.60
Graves' disease	0.17-0.29	0.00-0.02
Multiple sclerosis	0.25-0.31*	0.03-4.7
Celiac disease	0.75-0.83	0.11

\*Following a minimum of 7.5 years of observation

## The environmental factors and their mechanisms in autoimmunity

The search for environmental factors triggering autoimmunity has led to numerous candidates via four possible mechanisms [25]. The first mechanism, molecular mimicry, is perhaps the most widely investigated mechanism by which infections may induce autoimmunity. It is characterized by the cross-reactivity between epitopes (i.e. proteins, carbohydrates, or DNA sequences) shared by the pathogen and the host, as well illustrated by rheumatic fever, a disease often associated with heart involvement and acute polyarthritis in a large proportion of cases following streptococcal infections. In this case streptococcal epitopes (i.e. N-acetyl-glucosamine and M protein) mimic cardiac myosin and  $\alpha$ -helical proteins present in cardiac valves, resulting in cardiac damage [26]. Another classic example is Guillain-Barré syndrome (GBS), an acute polyradiculoneuritis following various microbial infections or vaccinations. A particular attention was given to the association between GBS and *Campylobacter jejuni*. Antibodies cross-reacting with both *C. jejuni* and peripheral nerve gangliosides are detected in the sera of GBS patients [27] and recently a significant homology was found between lipo-oligosaccharides present in *C. jejuni* and in ganglioside GM1 [28]. Furthermore, antibodies directed against the CMV-derived protein UL94 have been found to cross-react with the cell surface tetraspanin transmembrane 4 superfamily member 7 (TM4SF7 or NAG-2) molecule inducing apoptosis of endothelial cells and activation of fibroblasts in patients with systemic sclerosis [29]. Second, a polyclonal activation of B-cell occurs when a lymphotropic virus infects B cells leading to B-cell proliferation causing enhanced antibody production and the accumulation of circulating immune complexes that may cause damage to self tissues [30]. One evocative example is mixed cryoglobulinemia (MC) induced by hepatitis C virus (HCV). Mixed cryoglobulinemia is considered the result of such persistent polyclonal activation [31] eventually resulting in the appearance of a mixture of monoclonal and polyclonal autoantibodies that may result in autoimmunity [31]. The third mechanism is provided by epitope spreading occurring when, in an inflammatory state, there is strong local activation of antigen-presenting cells (APC). This may result in overprocessing and overpresentation of antigens, thus priming large numbers of T cells with broad specificities, possibly against self antigens [32], as recently described in translational settings [33, 34]. Fourth and last, the bystander activation occurs when pro-inflammatory cytokine production is enhanced after an infection. An inflammatory microenvironment is created, with cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ),

lymphotoxin, nitric oxide (NO), and others, leading to bystander killing of uninfected cells in the proximity. Ultimately, this manifests as bystander activation of autoreactive T cells that were already present within the self tissue but, due to their prior low number, were unable to cause an overt autoimmune disease [35, 36].

### Microbial infections

The most widely studied environmental factors are represented by infectious agents through two pathways: (i) directly causing the breakdown of tolerance irrespective of the individual susceptibility; or (ii) triggering autoimmunity in genetically susceptible individuals. The issue is largely enigmatic as most recently supported by solid data on a hygiene hypothesis [37]. The former pattern is exemplified by rheumatic fever, in which antibodies against streptococcal antigens cross-react with a myocardial antigen causing heart injury [38] while the latter appears more likely in autoimmune diseases [39, 40] as exemplified by Epstein-Barr virus (EBV) infection in systemic lupus erythematosus (SLE) [41] but also in rheumatoid arthritis [42], multiple sclerosis [43], Sjögren's syndrome [44], autoimmune thyroiditis [45], autoimmune hepatitis [46], and Kawasaki disease [47]. Besides EBV, also hepatitis B (HBV) and cytomegalovirus (CMV) infections have been associated with autoimmune diseases, as represented by the prevalence of serum autoantibodies in patients with chronic or acute infections. In particular, relevant titers of antimitochondrial antibody (AMA), liver kidney microsome (LKM), antinuclear antibodies (ANA), and smooth muscle antibodies (SMA) were observed in chronic HBV infected subjects [48-50]. A potential role for CMV in the development of SLE has been suggested [51], similar to inflammatory bowel disease [52], antiphospholipid syndrome [53] and diabetes mellitus [54, 55]. Whether these are stochastic associations or significant pathogenetic links remains to be elucidated.

### Vaccines

One of the hottest debates in modern medicine stemmed from the reports of an association between vaccination and autoimmune diseases, even though vaccinations protect from infections and thus may have an indirectly protective role towards autoimmune diseases. This is well illustrated by the proposed association between autism and vaccines [56]. The most studied associations are GBS after 1976 swine influenza vaccine, immune thrombocytopenic purpura after measles, mumps and rubella vaccine, and myopericarditis after smallpox vaccination [57].

It has been reported an increased relative risk of developing autoimmune manifestations in adults receiving HBV vaccine. The autoimmune manifestations include SLE (OR = 9.1,  $p < 0.0001$ , 95% CI = 2.3-76), rheumatoid arthritis (OR = 18,  $p < 0.0001$ , 95% CI = 3.1-740), multiple sclerosis (OR = 5.2,  $p < 0.0003$ , 95% CI = 1.9-20), and thrombocytopenia (OR = 2.3,  $p < 0.04$ , 95% CI = 1.02-6.2) [58]. The mechanism underlying the proposed relationship between vaccination and autoimmune diseases is not clearly understood, although it is thought to be caused by both vaccine immunogenic content and adjuvants, which are used to increase the immune reaction [59]. Nevertheless, we submit that the data supporting a causative link remain circumstantial [60] and do not justify a blind refusal of vaccination.

### Tobacco

Tobacco smoking has been demonstrated to alter cytokines release and to exacerbate autoimmunity modulating pro-inflammatory cytokines levels (TNF- $\alpha$ , IL-1, IL-6, IL-8, and GM-CSF) and to decrease anti-inflammatory cytokines levels (IL-10) [61-63]. The autoimmune diseases associated with smoking include rheumatoid arthritis, SLE, Graves' hyperthyroidism, Crohn's disease, Goodpasture syndrome, thromboangiitis obliterans, PBC, systemic sclerosis, MS, and fibromyalgia. Indeed, higher risk and worse prognosis have been reported in past and current smokers for these conditions while we should also note that in some cases tobacco was associated with a reduced risk of disease, i.e. ulcerative colitis and Behçet's disease [9, 64, 65]. One significant link has been proposed for rheumatoid arthritis. Citrullination, also termed deimination, is a post-translational modification of arginine side chains catalyzed by peptidyl arginine deiminase enzymes with the potential to alter the structure, antigenicity, and function of proteins. In rheumatoid arthritis, antibodies to cyclic citrullinated peptides are used for diagnosis, though we argue that the identification of specific citrullinated antigens, as whole proteins, is awaited. Four citrullinated antigens, fibrinogen, vimentin, collagen type II, and  $\alpha$ -enolase, have been identified among others. All four proteins are expressed in the joint connective tissue and there is evidence that antibodies to citrullinated fibrinogen and collagen type II mediate inflammation by the formation of immune complexes, both in humans and animal models. Antibodies to citrullinated proteins are associated with HLA 'shared epitope' alleles, and autoimmunity to at least one antigenic sequence, the CEP-1 peptide from citrullinated  $\alpha$ -enolase (KIHACitEIFDSCitGNPTVE), demonstrates a specific association with HLA-DRB1\*0401, HLA-DRB1\*0404, 620W PTPN22, and smoking [66]. In

a complementary fashion, a higher expression of peptidyl arginine deiminase and higher levels of citrullinated proteins have been observed in the bronchoalveolar lavage cells of smokers [67].

### Nutrition

Numerous systematic studies have confirmed that nutritional deficiencies can alter the immune response [68]. In recent years we have witnessed important advances in "nutritional immunology" [69], as well represented by the relationship of vitamins and minerals with autoimmune conditions. Among these, vitamin D has been demonstrated to be a modulator of the innate and adaptive immunity [70] and low levels of vitamin D are associated with numerous autoimmune diseases such as multiple sclerosis, SLE, and psoriasis [71, 72]. Accordingly, there is evidence of the beneficial effects of vitamin D supplementation [73-75]. Other examples include the lower risk of developing rheumatoid arthritis with a higher weekly consumption of fish oil, rich in omega-3 polyunsaturated fatty acids [76], the increased iodine consumption as a trigger for autoimmune thyroiditis in genetically susceptible individuals [21], and effects of low doses of probiotics on disease activity in inflammatory bowel disease [77]. Most recently, an association between anti-gluten or anti-gliadin serum antibodies and autoimmune disease different from celiac disease, such as MS, was reported [78]. We foresee that clinical and epidemiological studies focused on the impact of dietary factors on the geographical gradient of autoimmunity may help focus on this promising field of research with potential improvement in patient management [68].

### Ultraviolet light

Ultraviolet (UV) light represents an additional proposed environmental factor capable of producing direct negative effects in predisposed individuals. The skin is a barrier against external agents while hosting both innate immune and adaptive immune cells and specialized antigen presenting cells, i.e. Langerhans cell [79]. Of note, photosensitivity is the most common skin manifestation in SLE [80]. Cutaneous SLE lesions often arise in sun-exposed areas and it is well recognized that sun exposure may also exacerbate or induce systemic manifestations of this disease [81] via proposed mechanisms [79]. It has been demonstrated that human keratinocytes can bind autoantibodies on their surface following UV exposure and antibodies against La, Ro, Sm, RNP, lupus-specific antigens, are specifically associated to the membrane blebs of cells undergoing apoptosis after UV damage. The ability of UV light to induce keratinocyte apoptosis and autoantigen



exposure suggests the role of UV in the lupus pathogenesis [82]. Conversely, low-dose UV light can also have immunosuppressive effects, as well represented by the beneficial effects in psoriasis. Finally, pre-vitamin D<sub>3</sub> is produced from 7-dehydro-cholesterol by effect of UV light [79].

### Xenobiotics

Xenobiotics represent additional etiologic factors proposed for autoimmune diseases. Xenobiotics include substances of synthetic, natural, or biologic origin that may exert an immunosuppressive effect due to direct toxicity on the immune system cells, or an immunostimulant effect resulting in autoimmune manifestations, as well illustrated by the pseudo-autoimmune conditions triggered by exposure to chemicals. In 1981 the ingestion of contaminated rape oil seed resulted in the toxic oil syndrome with cases of SLE-like disease, scleroderma-like disease and a number of general clinical manifestations that persisted in 10% of the subjects [83]. In the late 1980s in the United States, the eosinophilic myalgia syndrome was reported following exposure to L-tryptophan [84]. Since these earlier reports our knowledge of the links between chemicals and autoimmune conditions has considerably grown. As of now silica exposure is considered a trigger for SLE [85] and scleroderma [86] while organic solvents such as vinyl chloride and epoxy resins may also be associated with scleroderma-like manifestations [87]. Several other associations have been proposed through three mechanisms [88-90]. First, a potential direct toxic effect of xenobiotics may cause cell death by apoptosis or oncosis, inducing the generation of immunogenic autoepitopes. Second, chemical modifications of native cellular proteins by removal and/or exchange of a hapten has been shown to change processing in antigen-presenting cells and may lead to the presentation of cryptic, potentially immunogenic peptides. Third, xenobiotics may have the potential to modify host proteins to form neoantigens. Neoantigen specific T cells and B cells, once primed, may cross-react with the formerly inert native autoantigens.

### The case of primary biliary cirrhosis

As previously mentioned PBC is a slowly progressive autoimmune disease in which the loss of bile ducts leads to decreased bile secretion and the retention of toxic substances within the liver, resulting in further hepatic damage, fibrosis, cirrhosis, and eventually, liver failure. It primarily affects women. Commonly it develops mainly in the fifth decade of life and it is uncommon in persons under 25 years of age [91].

### Geoepidemiology

Although PBC is considered a rare disease, recent data suggest an increasing trend in its incidence and prevalence, but several factors that make difficult to study PBC epidemiology have to be considered. First of all, thus far there are no solid population-based studies on PBC, due to the rarity of this condition, and most of the data we have are descriptive data. Then the accuracy of diagnosis is difficult to obtain, since it does not exist a perfect diagnostic marker. Finally, disease awareness in healthcare providers is raising and may reflect the increase in prevalence and incidence of PBC cases, reported in several studies [92]. Based on the epidemiological data on PBC, it was observed that there are areas presenting a higher prevalence of PBC cases. Reported prevalence and incidence rates of PBC cases are highest in the United Kingdom [93, 94], the scandinavian region [95], and the northern United States (Table II) [96]. In this case it is difficult to determine whether this data are due to a true higher prevalence of cases, possibly influenced by environmental factors, or to a higher number of epidemiological studies lead in these areas. Instead, a lower prevalence and incidence have been reported in other countries, such as Canada, Australia, and the Mediterranean countries [97-104]. An interesting population-based study lead in Australia showed a significant higher PBC prevalence in Italian, British, and Greek immigrants, compared to native population [105], an evidence that may underline the genetic component on the etiology of PBC or specific environmental priming occurring early in life. Finally data on PBC epidemiology are not available in large parts of the world such as Africa or Asia.

### Environmental factors

Based on earlier data on PBC autoantibody reactivity with xenobiotic-modified autoantigens [106], Amano *et al.* [107] studied a large number of xenobiotics with a structure similar to the lipoic acid residue on the epitope of PDC-E2, the E2 subunit of the mitochondrial pyruvate dehydrogenase complex, that is the main autoreactive antigen identified so far in PBC [108]. It was observed that replacement of lipoic acid by certain xenobiotics enhanced PBC sera reactivity against the PDC-E2 epitope. Particularly, one of the xenobiotics, 2-nonynoic acid, induced reactivity of PBC sera stronger than that of the native lipoic acid residue. Interestingly, the methyl ester of 2-nonynoic acid has a viol-/peach-like scent and is used as an ingredient in perfumes. But this is only one of the several example, the number of exogenous compounds potentially leading to modifications is enormous, from food preservatives to home

**Table II.** Distribution of prevalence, incidence and sex ratio of primary biliary cirrhosis in different countries [7]

Geographical area	Year	Number of cases	Incidence (per million)	Prevalence (per million)	Gender (M : F)
Europe	1984	569	4	23	1 : 10
Sweden	1985	111	13,3	151	1 : 6
Newcastle, UK	1989	347	19	154	1 : 9
Canada	1990	225	3.26	22	1 : 13
Victoria, Australia	1995	84	–	19	1 : 11
Estonia	1995	69	2.27	26.9	1 : 22
Norway	1998	21	16	146	1 : 9
USA	2000	46	27	402	1 : 8
Newcastle, UK	2001	770	31	251	1 : 10
Victoria, Australia	2004	249	–	51	1 : 10
Japan	2005	9761	–	78	1 : 9
Canada	2009	227	30	227	1 : 9

detergents to water pollutants [106] and new mechanisms for haptization have been recently described [109]. In addition one such halogenated compound has been shown to induce AMA production in rabbits without requiring the peptide backbone of PDC-E2 [110] but failed to produce liver lesions. It could reflect the evidence that in humans AMA titer is detectable several years before the appearance of liver injury. Finally, xenobiotics are able to induce epigenetic modifications, as DNA methylation and histone modifications, that can lead to gene expression alteration and then phenotypic changes. These processes may play a fundamental role in the onset of PBC [111].

Several lines of evidence support the hypothesis that also some infectious agents are able to be a trigger in PBC development. In particular it was suggested by the observation of a significantly higher rate of urinary tract infections sustained by *Escherichia coli* among PBC patients and the presence of bacterial products in mononuclear cells surrounding damaged bile ducts [112]. In addition it has been demonstrated that a history of urinary tract infections increases the risk of having PBC [113]. The proposed mechanism to explain this hypothesis is molecular mimicry, discussed above. This theory is mostly based on experiments that demonstrated a cross reactivity between AMA and/or autoreactive T cells with prokaryotic antigens of several microbes, including *E. coli* [114] which share an ExDK amino acid motif within their mimicry epitopes.

More recently, *Novosphingobium aromaticivorans*, an aerobic, free-living Gram-negative bacterium has been investigated in PBC [115] as supported by several observations. First, the

bacterium is known to be ubiquitous in the environment (soil, water), non-pathogenic to humans and demonstrates the ability to modulate activation of estrogens [116], which could be linked to the well-documented female predominance of PBC. Then it was observed in 100% of anti-PDC-E2 positive and in a fraction of AMA-negative sera a serum reactivity against two lipoylated proteins of 47 and 50 KDa from *N. aromaticivorans* [117]. Titers of antibodies detected against lipoylated bacterial proteins of *N. aromaticivorans* were 1000-fold higher compared to those against *E. coli* in PBC patients [117]. *Lactobacilli* and *Chlamydia*, which show some structural homology with the autoantigen (although reactivity against them is considerably less than that against either *E. coli* or *N. aromaticivorans*), have also been implicated as putative pathogens, as well as *Helicobacter pylori* and *Mycobacterium gordonae* [118, 119]. However, no compelling data have been provided to show that one individual infectious agent can reproducibly be detected in patients with PBC. In conclusion our current knowledge of the environmental triggers of PBC includes numerous suspected agents involved in PBC development, but no specific agents have been identified as able to be causative of disease.

### The case of multiple sclerosis

Multiple sclerosis is an inflammatory, demyelinating disease of the central nervous system (CNS). The disease is highly heterogeneous particularly in its clinical subforms, with an extremely variable evolution over time. Temporal profile and combination of neurological findings, appearing as relapses and

remissions, or gradually progressive dysfunctions, led to classifying MS in clinical subforms coined relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS.

### Geoepidemiology

The total number of people affected by MS worldwide is estimated to be 2-2.5 million, but the distribution of the disease changes significantly in different part of the world. Its prevalence varies between less than 5 cases per 100,000 people in tropical areas or Asia and more than 100-200 cases per 100,000 in temperate areas such as the United States, Canada, New Zealand and parts of Australia [120, 121]. Furthermore it exists a well-accepted latitudinal gradient for MS [122]. Highest rates are recorded in Northern Europe, the British Isles and Scandinavia, and the areas originally settled from these locations, which are North America, Australia and New Zealand [8, 120]. Interestingly it was observed a lower risk to develop MS in adult migrants from tropical countries to the UK than local population, although these differences were less evident in following generations [123, 124] thus confirming also the familial risk [19].

There are also important differences in MS prevalence in different races. Caucasians seems to be more affected by MS than blacks and Asian populations, whereas Australian Aboriginals, New Zealand Maori and North and South-American Indians have a very low prevalence [121, 125]. After a large study conducted by WHO it was recently proposed a socioeconomic gradient, since it has been observed a higher MS prevalence in Western Countries, even though it may reflect the availability of more precise but expensive diagnostic instruments [126].

### Disease pathogenesis and the role of post-translational modifications in creating neo-antigens

Multiple sclerosis pathogenesis remains unclear, but growing evidence suggests that the disease could be due to a consequence of multiple genetic, immunological, and environmental factors [18, 20, 127].

Different hypotheses have been formulated to explain MS pathology putting into evidence the heterogeneous and homogeneous features of MS [128-130]. However, data support the hypothesis proposed by Lucchinetti *et al.* [128] of a heterogeneous mechanism of demyelination in which the following four MS pathological patterns can be identified: (I) T-cell/macrophage-mediated demyelination, (II) antibody/complement-associated demyelination, (III) distal oligodendroglipathy, and

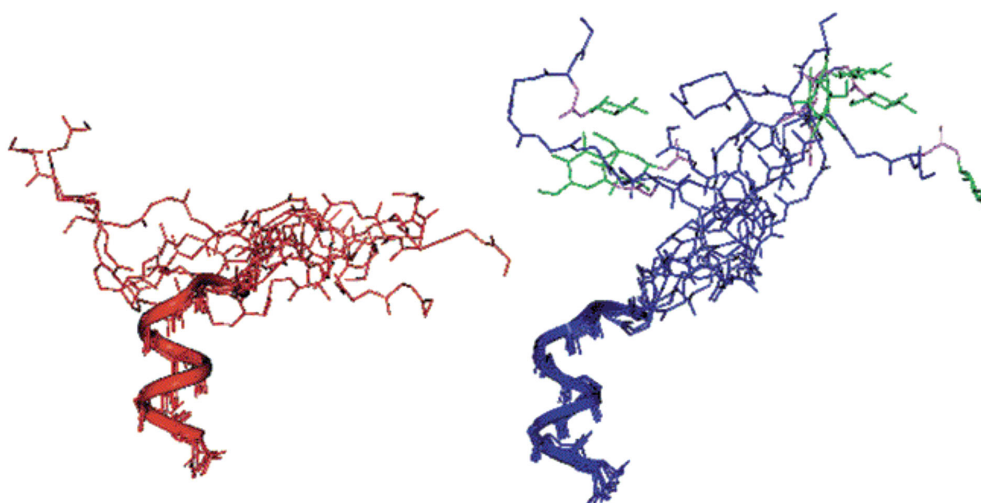
(IV) oligodendrocyte degeneration in the periplaque white matter. Nevertheless, the difficulty in the comprehension of MS pathology can be connected to the presence of post-translational modifications (PTM) that increase the complexity of myelin proteins. It is of note that if there are 22 proteinogenic amino acids (20 of which directly encoded by the universal genetic code), once considering PTMs the possible combinations will become over 400. Moreover, growing evidence indicates that PTMs (i.e., acetylation, lipidation, citrullination, glycosylation, etc.), either native or aberrant, may play a fundamental role for specific autoantibody recognition in autoimmune diseases [131]. The role of PTMs in MS has not been fully understood; in fact, it is currently a matter of debate whether PTMs can have an effective role in MS pathology and if they can be related to an autoimmune response [131-133] or a neurodegenerative process [134, 135]. These observations account, at least in part, for the limited results in the discovery of biomarkers using proteomic analysis and/or protein microarrays. A number of autoimmune diseases have been associated with PTMs, demonstrating that some modifications can generate new self-antigens or even mask antigens normally recognized by the immune system in physiological conditions [131, 136]. It is well accepted that a number of these chemical modifications introduced in proteins, during or after their synthesis, could influence the central and peripheral tolerance, and subsequent inducing autoimmune responses to otherwise ignored self-protein [131]. On the other hand, the neurodegenerative hypothesis is based on metabolic changes in myelin constituents that altering the PTMs destabilize the membrane structure leading to myelin degradation [135]. Once myelin is modified by PTMs some antigenic fragments could be exposed to the autoimmune response. Numerous studies indicate that PTMs, either native or aberrant, may play a fundamental role in generating neo-epitopes and thus triggering an autoimmune response, particularly in MS. For example, rheumatoid arthritis recognizes anti-citrullinated peptide/protein autoantibodies in sera of patients because of an aberrant deimination [137]. In MS, the role of different PTMs in triggering an autoimmune response have been investigated considering the possible mechanisms that could pathologically alter the PTM profile (e.g. environmental factors and oxidative stress, pathogens and molecular mimicry). It was previously reported that Pb metal can enhance proteins immunogenicity [138] while lead exposure (PbOAc<sub>2</sub>) can induce autoantibodies to nervous system proteins, including MBP [139, 140]. MBP represents 20-30% of total proteins of myelin sheath and is important for myelin structure [141].

The putative antigen MBP is widely studied in MS because it acts as an inter-membrane adhesion protein enabling myelin structure stability thanks also to its dynamic pattern of PTMs. MBP binds to the negatively charged lipids in myelin sheath and is characterized by different PTMs that differ in their extent in physiological vs. pathological conditions [142]. Mice immunized with Pb-altered MBP developed elevated anti-MBP antibody titer [143] but a possible correlation with MS disease activity is far from convincing. Nitric oxide (NO) is involved in axon and oligodendrocyte degeneration in MS lesions [144, 145] and increased levels of nitrite have been detected in cerebrospinal fluid of MS patients and correlate with clinical activity [146, 147]. Boullerne *et al.* reported an antibody response against the uncommon nitrosylated epitope: SNO-cysteine-BSA, in which BSA was used as a carrier to expose the modified cysteine [148]. Anti-SNO-cysteine-BSA IgM antibodies when compared with healthy controls were detected in: 8/8 (100%) of RRMS patients in relapse, 6/18 (33%) of clinically stable RRMS patients, and 16/23 (70%) SPSM patients. Further, the anti-SNO-cysteine-BSA antibody titer correlates with disease activity and increases during clinical activity in MS and was

detected also in sera from Lewis rats with actively induced MS-like murine disease and it was shown that anti-SNO-cysteine-BSA antibodies precede anti-MBP antibodies. The specificity of IgM binding to SNO-cysteine-BSA was demonstrated by inhibition experiments at different concentrations. Another PTM widely studied in MS is glycosylation as it can affect the immune response and immune tolerance [131]. Recently, IgM autoantibodies to recombinant MOG, a putative self-antigen component of normal myelin of the CNS, have been reported to be predictors of clinically definite MS after a first demyelinating event [149, 150]. However, these results were not confirmed by other studies probably because differently folded and modified forms of MOG have been used (Table III) [151, 152]. In fact a recently report demonstrated for the first time that a solid-phase ELISA based on a conformationally controlled rMOG<sub>ED</sub>(His)<sub>6</sub> on MS sera and controls failed to detect IgM or IgG antibodies. Interestingly the rMOG<sub>ED</sub>(His)<sub>6</sub> used was expressed in *E. coli* after subcloning the cDNA of the extracellular domain of rat MOG, performing a refolding procedure on column and affinity purification. The far-UV Circular Dichroism (CD) spectra of rMOG<sub>ED</sub>(His)<sub>6</sub> manifested a  $\beta$ -sheet,

**Table III.** Different types of MOG used as antigens for the detection of autoantibodies in multiple sclerosis

Authors	MOG type
Berger <i>et al.</i> [149]	hMOG <sub>ED</sub> – extracellular, unfolded, and unglycosylated hMOG(28-130)
Gaertner <i>et al.</i> [150]	mMOG – full length native, folded, and randomly glycosylated mMOG(1-247)
Mantegazza <i>et al.</i> [151]	hMOG <sub>ED</sub> – extracellular, unfolded, and unglycosylated hMOG(29-149)
Lampasona <i>et al.</i> [152]	hMOG – full length and unglycosylated hMOG(1-241)
Gori <i>et al.</i> [153]	rMOG <sub>ED</sub> – extracellular, folded, and unglycosylated rMOG(1-125)



**Figure 1.** Superposition of the 10 optimized structures of hMOG(30-50) (left) and [Asn<sup>31</sup>(N- $\beta$ -Glc)]hMOG(30-50) (right) with the C-terminal part at the bottom showing that the two peptides adopt similar conformations in water/HFA solution. Autoantibodies detected in a relevant number of MS sera by the MOG glycopeptide and not by the unglycosylated sequence are directed against the N-linked glucose [155]. Copyright © 2001, American Chemical Society



a characteristic feature of the Ig-fold [153]. MOG is a myelin protein presenting a unique N-glycosylation site at position 31 (Asn<sup>31</sup>) and it was previously reported that N-glycosylation of a MOG peptide epitope improved the detection of specific antibodies in MS sera [154]. Moreover, structure-activity relationship studies demonstrated that the two MOG derived peptides hMOG(30-50) and the glycosylated analog [Asn<sup>31</sup>(N-β-Glc)]hMOG(30-50) adopt similar conformations in the environment used for NMR investigations (water/HFA solution). Thus, the specific antibody-recognition of [Asn<sup>31</sup>(N-β-Glc)]hMOG(30-50) is driven by direct interactions of the antibody binding site with the N-linked glucose (Figure 1) [155]. Therefore, specifically glycosylated synthetic peptides, as MS antigenic probes, have been demonstrated to be powerful tools for increasing autoantibodies recognition in MS patients sera, with higher affinity and specificity compared to recombinant MOG.

### Final thoughts on the cases of primary biliary cirrhosis and multiple sclerosis

The etiology of autoimmune diseases remains a complex issue that encompasses epidemiology, clinics, genetics, and experimental data. The genomic predisposition remains preponderant but insufficient to explain the different geographic distribution of these diseases or the incomplete concordance in MZ twins. Environmental factors are thus ideal candidates to fill this gap but the mechanisms remain to be elucidated. Our current knowledge of the environmental triggers of PBC is limited to numerous suspected agents involved in its development, such as xenobiotics or infectious agents (e.g. *E. coli*, *N. aromaticivorans*), but no specific agents have been identified as able to be causative of disease and data obtained in animal models await confirmation. The case of MS, on the other hand, illustrates a different approach, mostly based on the PTMs in creating neo-antigens, but due to the heterogeneity of MS, a clear and rational association of PTMs with the activity of MS has not been yet fully elucidated. Geoepidemiology may provide an important contribution in researching (looking for) potential environmental factors involved in autoimmune diseases. Nevertheless, it remains uncertain whether the latitudinal gradient observed for most autoimmune conditions reflects a real different distribution of the disease, possibly influenced by environmental factors, or it is due to a greater number of epidemiological studies lead in certain regions or to the availability of more precise but expensive diagnostic instruments.

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