MULTIPLE SCLEROSIS

Perspectives in Treatment and Pathogenesis

Cover image: Pathogenic mechanisms of multiple sclerosis. See page 141, chapter 9 for details.

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Perspectives in Treatment and Pathogenesis

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Multiple Sclerosis: ***Perspectives in Treatment and Pathogenesis***
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FOREWORD

Since finishing my fellowship in neurology at University Hospitals Case Western Reserve University, I have been involved in both the clinical evaluation and treatment of patients with multiple sclerosis for more than 35 years, as well as clinical and translational research on multiple sclerosis. I have seen various therapies used, beginning with steroids and ACTH. In some situations, agents were later developed and were found to be effective in reducing acute inflammatory activity or were agents directed toward symptom management. These agents for disease control often times fell short of anticipated needs. They also were associated with high-cost and significant side effect profiles, and, as a result, patients often times, were non-compliant in taking the medicines.

Because multiple sclerosis is a chronic progressive disease and rarely acutely life-threatening, yet it shortens life span, the treatment has often primarily focused on the patient's symptom management and reduction of acute flares. Funding has been limited in clinical trials because of the potential high cost of implementing prospective studies. Nonetheless the basic science of multiple sclerosis, as well as clinical research, has continued with incremental advances in understanding multiple sclerosis and in seeking improved ways of analysis and treatment.

Basic science research in the field of immunology and neuro-inflammation provides clues of the mechanisms and the complex pathways of multiple sclerosis. Since the mid-1980s publication of exciting studies into the biological role of endogenous opioids and their identified classical and non-classical receptors within the brain and other organs suggest the potential dysregulation of these pathways during the development of immune and various other diseases. These findings open new research opportunities. With a wide acceptance of low dose naltrexone (LDN) as an adjuvant therapy, and, at times, even a stand-alone therapy, attention needs to be directed on this pathway as a potential etiological role in this complex multifactorial disorder. Stem-cell research has also been shown to be a possible novel therapy and is provocative. However, continued research into the types of stem cell treatments and programs are necessary.

In this book on the pathophysiology of multiple sclerosis, several chapters concentrate on the potential etiology and treatment of multiple sclerosis, and other chapters focus on basic science studies discussing potential mechanisms and pathways involved in the development and progression of the disease. In the first section, there is a comprehensive review of the genetics of multiple sclerosis. Genomics is proving to be extremely important as far as determining what medications may be best for the individual patient. However, this is going to require acceptance by the pharmaceutical companies as to limiting what is now considered an open market for their medicines. As the prevalence of the disease rises, there is an increasing need to have understanding into the etiology of multiple sclerosis. A detailed summary of the prevalence of multiple sclerosis in individual European countries provides interesting information. The need to identify and understand biomarkers that are clinically relevant and may be easily obtainable continues to be researched, as are safer treatments. This book offers insight and opportunities in all these areas.

I applaud the authors and contributors of this book for addressing each valuable topic. I hope that clinicians, scientists, patients, and the general public read and learn at least one piece of information that may stimulate further research and understanding about this disabling disorder.

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PREFACE

Multiple sclerosis (MS) is a chronic neurological disorder with potentially devastating, long-term complications. Although not considered a life-threatening, terminal illness, MS is incurable and most therapies may treat only the symptoms, leaving the patient with a reduced quality of life for extended periods of time. Given that the onset of MS can occur as early as the second or third decade of life, patients can be compromised in their lifestyles for many decades. This book focuses on different biological pathways associated with MS and contains current information on the prevalence of MS, novel treatments that target pathophysiology, and new approaches for management of the disorder, as well as general knowledge about the disease process. Basic science research and clinical research continues to make advances into understanding MS. The book focuses on specific deficits related to this autoimmune disorder. Over the last few years, a number of different therapies have gained momentum, and new perspectives on the pathogenesis of MS have been established.

The book is divided into two sections that are related to the etiology and treatment of MS, and the pathophysiology and mechanistic pathways underlying the disease. Section I of the book provides a comprehensive overview on clinical studies, providing details on the prevalence of the disease and current therapies, both defined and postulated, for both pediatric and adult patients with MS. Section II of the book provides current information on fundamental pathways involved in etiology, development, and progression of disease. The contributing authors represent an international group of scientists and clinicians with expertise in a broad range of disciplines, including molecular and cellular biology, immunology, bioinformatics, genetics, neurology, psychiatry, pharmacology, and internal medicine.

The first chapter in Section I by Didonna and Oksenberg provides a comprehensive review on the genetics of MS, highlighting the use of genome-wide association studies to identify nonmajor histocompatibility complex genes that appear to be prevalent in families with MS. This information will be useful in predicting risk and worldwide incidence. The genetic approach extends into Chapter 2 which provides a detailed summary of the prevalence of MS in Europe, with selected information on individual countries. The chapter by Gitto brings to the forefront the need for improved communication among clinicians and patients related to approved and/or novel therapies and research into autoimmune disorders. In Chapter 3, Jancic and coauthors provide a thorough discourse on challenges that are specifically related to the treatment of pediatric patients with MS. This population of patients is symptomatic very early in life and thus has ample time to experience numerous relapses. The authors review the strengths and weaknesses of immunomodulatory therapies including steroid treatment and even plasmapheresis. The message from this chapter is the need for treatment modalities that approach MS longitudinally to reduce both the severity and frequency of relapses. A major symptom of MS that is often overlooked in lieu of the mobilization issues is that of pain. As discussed in Chapter 4, alleviation of pain is not always the primary target of MS treatment, yet many MS patients will self-report that they suffer from chronic pain. Murphy and colleagues discuss

treatment strategies of pain when it becomes sufficiently severe to reduce the quality of life. Unfortunately, research efforts are limited in this area and current strategies may use ineffective drugs such as antidepressants, narcotics, or cannabinoids. The take-home message from this chapter is the need for understanding the mechanisms of MS-related pain and applicable treatment modalities. Chapters 5 and 6 provide information on two novel therapeutic strategies for the treatment of autoimmune disorders including dietary supplementation and stem-cell therapy. In Chapter 5, Zahoor and Haq present compelling information to approach the etiology of MS by targeting vitamin D deficiency. These authors provide mechanistic pathways that support the relationship of sunlight, vitamin D circulatory levels, and prevalence of MS. In summary, vitamin D supplementation may be a valuable, but often overlooked, adjunctive therapy. The final chapter in this section provides a comprehensive evaluation of stem cell biology and the role of stem-cell therapy in autoimmune disorders. This field is still in its infancy, but is gaining research momentum worldwide. Bojnordi and colleagues provide two extensive treatises on stem-cell therapy as a promising approach for reversing MS progression. These authors divide their work into comprehensive discussions on exogenous stem-cell therapy and endogenous stem-cell niches that when stimulated may serve to reduce neurodegeneration by inducing oligodendrocyte proliferation and activation of resident oligodendroglial precursors and adult neural stem cells. Each chapter in this section is provocative and provides insights into the diagnosis, management, and treatment of MS.

Section II of this book includes chapters on the disease pathobiology, highlighting advancements in immunomodulation, endogenous regulatory pathways, and oxidative stress mechanisms underlying the etiology and pathogenesis of MS and other autoimmune disorders. These chapters are no less important than those on treatment and include preclinical, animal research to demonstrate the basis of new and exciting theories on the pathogenesis of MS. Moreover, each chapter adds basic science or clinical data to an underlying theme of identifying or defining new biomarkers that can effectively be used for the diagnosis and treatment of MS. Data are presented on three novel thematic areas including primary neuroinflammation, oxidative stress pathways, and the role of endogenous opioids and their receptors in MS. Each chapter discusses the possibility of the pathway becoming dysregulated during development of the disease. The final chapter provides some insight into the strengths and weaknesses of animal models when studying a multi-modality disorder such as MS. As detailed in Chapter 7, neuroinflammation is a primary response to antigen presentation as well as a secondary immunological response. Dr. Palumbo presents evidence on arachidonic acid metabolism as an active pathway, leading to further demyelination, glial loss, and axonal pathology in animal models with experimental autoimmune encephalomyelitis and humans with MS. The author presents arguments for the treatment of MS with nonsteroidal anti-inflammatory drugs to control COX-2 mediated inflammation following arachidonic acid stimulation. In Chapter 8, Zagon and McLaughlin introduce an endogenous opioid pathway as a homeostatic regulatory axis that can modulate progression of experimental autoimmune encephalomyelitis (EAE) or MS using a number of different paradigms. These authors summarize preclinical work on chronic progressive and relapse-remitting EAE, as well as clinical data from patients with MS. Treatment with endogenous opioids such as opioid growth factor (OGF), chemically termed [Met^5]-enkephalin, or low doses of naltrexone

(LDN) that upregulate secretion of OGF are effective at stalling the onset of disease, reversing the progression of EAE, and inhibiting neurodegeneration. MS patients on LDN report significantly better quality of life, improved ambulation, and have little or no side effects. Moreover, levels of OGF declined in animal models of EAE following immunization, suggesting that this noninvasive measurement of an endogenous peptide might serve as a specific biomarker for the onset of MS. Chapters 9 and 10 continue the thematic concept of identification of biomarkers. Teniente-Serra and collaborators present evidence to validate biomarkers by monitoring peripheral blood mononuclear cells with a characterization of lymphocytes. Adamczyk-Sowa and coauthors provide a comprehensive report on the role of oxidative stress mechanisms and their role in both pathophysiology and therapy of MS. Oxidative stress may enhance processes of demyelination—the ultimate neurological pathology associated with MS. These authors argue that the balance between reactive nitrogen species and reactive oxygen species, and the production of free radicals, supports the environment for demyelination in MS. Furthermore, these compounds could also serve as biomarkers specific for MS. The last chapter by Palumbo and Pellegrini sheds light on the use of animal models to investigate MS. Currently, three *in vivo* paradigms are predominately used to study autoimmune disorders—antigen-producing autoimmune encephalomyelitis, cuprizone intoxication, and Theiler's murine virus. Each model is discussed with the strengths and weaknesses highlighted.

The book is intended to provide an authoritative source of current knowledge on the field. Given that MS is only one of the many autoimmune disorders that have limited definitive etiology and treatment, we hope that the comprehensive studies detailed in the book may stimulate other researchers to explore their specific diseases of interest, thereby adding to the knowledge on autoimmunity.

When organizing and editing this book, it was our intention to combine broad-based reviews of human and animal studies on MS so that the information would appeal to researchers as well as patients with an interest in knowing more about MS. We thank the authors for their time and concerted efforts in organizing the current literature. The intended audience of this book are students, basic scientists, and clinicians who are interested in the basic and/or clinical aspects of MS. The goal of this book is to provide a cohesive, but comprehensive, view of the state of the art on MS and encourage new investigations that could lead to novel insights into the etiology, pathogenesis, management, and treatment of MS.

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Section I

Etiology and Treatment

1 The Genetics of Multiple Sclerosis

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Abstract: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system, characterized by focal inflammation, demyelination, and axonal injury. The etiology of MS is still uncertain, but the most updated working model for disease pathogenesis proposes the interplay between genetic and environmental factors as necessary for MS manifestation. With the notable exception of the major histocompatibility complex (*MHC*), the identity of MS genetic determinants has been elusive for decades. In recent years, the advent of genome-wide association studies (GWAS) and collaborative efforts among international centers have fueled the characterization of several non-*MHC* loci associated with MS susceptibility. To date, after a number of GWAS screenings, 110 MS risk variants have been discovered outside the *MHC* locus in European populations. In the future, functional studies will be required to define the biological pathways and cellular activities connected to these variants.

Key words: Autoimmunity; Genome-wide association studies; Human leukocyte antigen; Multiple sclerosis; Single-nucleotide polymorphism

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Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), characterized by focal lymphocytic infiltrates, the breakdown of myelin sheaths wrapping axons, astrogliosis, microglia activation, and diffuse neurodegeneration (1). Clinical manifestation is heterogeneous, ranging from relatively mild neurological symptoms to a rapidly evolving and debilitating disease. MS typically begins with a relapsing-remitting clinical phase (RR-MS), dominated by inflammatory events, both in the periphery and CNS, and full or partial recovery. In the majority of affected individuals, this initial relapsing-remitting course evolves years later into a secondary progressive MS (SP-MS), characterized by the irreversible accumulation of neurological disabilities as a result of axonal injury and neuronal loss. However, a proportion of MS patients (up to 15%) enter directly into the progressive phase after clinical onset, without experiencing initial relapses (2). This disease subtype is known as primary progressive MS (PP-MS) and is associated with an irreversible and progressive severe clinical phenotype. Significantly, the mean age of onset of SP-MS and PP-MS is similar, approximately 40 years (3). A total of 14 FDA-approved treatments for RR-MS are now available as disease modifiers to control inflammatory lesions and clinical relapsing activity. However, their long-term effects on disease progression remain largely unknown.

With the age of onset ranging between 20 and 40 years, MS represents the most common cause of acquired neurological disability among young adults, affecting over 2.5 million people worldwide. MS affects women more often than men (3:1 ratio), but its incidence also varies according to ethnicity and geographical location, with northern Europeans and their descendants being more susceptible to develop the disease (4). MS etiology is still elusive but there is a growing body of experimental evidence, suggesting that both genetic determinants and environmental factors converge to determine disease susceptibility and clinical trajectory. This chapter will review key milestones in MS genetic research with an emphasis on the technological and conceptual advances that have fueled the identification of discrete genomic loci associated with MS risk.

Multiple Sclerosis Holds a Genetic Component

The discovery of family aggregation in the second half of the 19th century shed light for the first time on the genetic component of the disease. Compared to a lifetime risk of 0.2% in the general population, siblings of affected individuals have a 10- to 20-fold higher risk of developing the disease (2–4%), with monozygotic twins having an even higher risk (30%) (5, 6). In contrast, spouses and adoptees hold a risk comparable to that of the general population (or their original nuclear families), consistent with genetic sharing being the driver of familial aggregation (7). On the other hand, the fact that the relative risk does not reach 100% even in identical twins suggests that other factors beyond DNA sequence identity must concur to create the conditions that cause or allow the dysregulation of the immune response associated with MS. A broad range of determinants lie in this category; they include environmental exposures (e.g., smoking, viral infections,

vitamin D intake, diet, and microbiome) as well as epigenetic signatures (e.g., DNA methylation patterns, histone modifications, and non-coding RNAs) (8).

Another factor supporting MS heritability consists in the distinctive worldwide prevalence of the disease. People living in northern Europe and North America exhibit a higher disease incidence (1–2 in 1000) when compared with southern Europeans. Moreover, MS is uncommon in some ethnic groups such as Uzbeks, Samis, Turkmen, Kyrgyzis, Kazakhs, native Siberians, North and South Amerindians, Japanese, Chinese, African blacks, and New Zealand Maori (9). Although these differences could be partially explained by differential exposure to specific environmental factors (such as certain nonubiquitous pathogens), the presence of MS-resistant or low-incidence ancestral groups suggests that the history and genetic architecture of a population influence its own risk of developing MS.

Altogether, these epidemiological observations—in particular the nonlinear relationship between genetic distance from a proband and the lifetime risk to develop MS—support a polygenic etiology for MS following the "common variant-common disease" paradigm of genetic influences and inheritance. According to this model, the overall MS risk is the result of the contributions of multiple polymorphic genes with risk alleles common in the population, each one determining a moderate portion of the risk (10, 11). This non-Mendelian pattern of transmission is not exclusive of MS but is shared with other autoimmune diseases and chronic disorders such as type II diabetes and obesity. These conditions are collectively known as complex genetic disorders, which are characterized primarily by polygenic risk and multifaceted gene–environment interactions.

THE HUMAN LEUKOCYTE ANTIGEN LOCUS IN MS

The strongest genetic association signal in MS resides within the major histocompatibility complex (*MHC*) in chromosome 6p21.3. This 4-megabase region contains approximately 160 closely linked genes. About half of these genes have important roles in the regulation of the immune system, and include the six classical transplantation human leukocyte antigen (*HLA*) genes—the class I genes *HLA-A*, *HLA-B*, and *HLA-C*, and the class II genes *HLA-DPB1*, *HLA-DQB1*, and *HLA-DRB1* (12). *HLA* genes are highly polymorphic, with over 15,000 alleles identified to date (http://hla.alleles.org/nomenclature/index.html). The first evidence of association between *HLA* and MS risk dates back to 1972, when the frequencies of surface glycoproteins encoded by the *HLA-A3* and *HLA-B7* class I alleles were found enriched in MS patients using serological reagents (13, 14). In the following years, numerous investigations, regardless of sample size and the resolution, have independently replicated the association of the *HLA* locus with MS risk across all populations studied, in both primary progressive and relapsing-remitting patients. Although the initial association was to class I *HLA-A* and *HLA-B* alleles, better powered studies, including genome-wide association studies (GWAS), have shown that the main MS susceptibility signal genome-wide maps to the *HLA-DRB1* locus in the class II region of the *MHC*. The *HLA-DRB1*15:01* allele has the strongest effect, with an average odds ratio (OR, a frequently used measure of effect size) of 3.08 and a clear dose response to 0, 1, or 2 allele copies the individual carries (15). However, complex allelic hierarchical lineages, cis/trans-epistatic and haplotypic effects, and independent protective signals, specifically in the class I region of the locus, have been documented as well.

Using GWAS single-nucleotide polymorphism (SNP) data (5091 cases/9595 controls), the International Multiple Sclerosis Genetics Consortium (IMSGC) reported in 2013 the isolation of 11 statistically independent effects in the *MHC* region: six *HLA-DRB1* and one *HLA-DPB1* alleles in the centromeric class II region of the locus; one *HLA-A* and two *HLA-B* alleles in the telomeric class I region; and one in the class III region between MHC class I polypeptide-related sequence B (*MICB*) and leukocyte-specific transcript 1 (*LST1*) (16). More recently, the analysis of independent high-density *MHC* region SNP data from multiple cohorts of European ancestry has provided, in addition to novel and previously identified *HLA* class II risk alleles (*DRB1*15:01, DRB1*13:03, DRB1*03:01, DRB1*08:01,* and *DQB1*03:02*) and independent *HLA* class I protective alleles (*A*02:01, B*44:02, B*38:01,* and *B*55:01*), evidence for two interactions involving pairs of class II alleles: *DQA1*01:01–DRB1*15:01* and *DQB1*03:01–DQB1*03:02* (17). Larger ongoing studies hold the potential for discovering additional independent and interactive effects.

THE ADVENT OF GENOME-WIDE ASSOCIATION STUDIES IN MS RESEARCH

In the early 2000s, the introduction of chip-based technologies with the capacity to genotype simultaneously hundreds of thousands of SNPs allowed the development of a new analytical methodology known as genome-wide association studies or GWAS—a hypothesis-free method in which SNPs spaced across the entire genome are screened for association with a particular trait in case–control datasets composed of genetically unrelated individuals (18). Compared to classic linkage studies that rely on extended families, the possibility to test unrelated individuals allows collecting much larger datasets, substantially increasing the statistical power of gene-discovery studies. GWA studies have been a determinant to deconstruct the genetics of many multifactorial disorders, characterized by common genetic variants conferring moderate risk to disease susceptibility.

The first MS GWAS was reported in 2007 by the IMSGC employing 931 family trios (one affected child and both parents). The screening confirmed with genome-wide significance the association of the previously identified locus containing the interleukin-7 receptor α (*IL7Rα*) gene, and detected a novel non-*HLA* disease-risk locus, defined by the presence of the interleukin-2 receptor α (*IL2Rα*) gene (19). In the following years, between 2007 and 2011, seven additional GWA studies of comparable size and one meta-analysis were performed, adding 21 new loci to the roster of MS risk variants. However, theoretical power estimations showed that all the studies conducted at that time were substantially underpowered to capture risk variants with odd ratios less than 1.2, which were the values expected for most of the MS risk variants (20). For that reason, the IMSGC decided in 2011 to embark on the largest MS GWAS with the collaborative effort of the Welcome Trust Case Control Consortium 2 (WTCCC2). This new study employed nearly 10,000 MS cases and 20,000 healthy controls of European ancestry and was able to extend the list of genome-wide significant MS loci to 52, of which 29 were never reported before (21). Remarkably, most of the associated variants were found located in proximity to genes with documented immune functions, corroborating the hypothesis that the dysregulation of physiological immune response most likely represents the driving factor of MS. Two years later,

MS genetic association was further refined through a novel multicenter study based on a custom high-density genotyping array named ImmunoChip. Over 80,000 individuals of European descent were analyzed and 48 new susceptibility variants were identified as genome-wide significant (22).

After a decade of GWAS screenings in European populations, the MS genetic atlas currently includes 110 non-*MHC* risk variants belonging to 103 genetic loci (Figure 1). In aggregate, the proportion of the genetic variance accounting for disease risk explained by these polymorphisms has been estimated as roughly 30%,

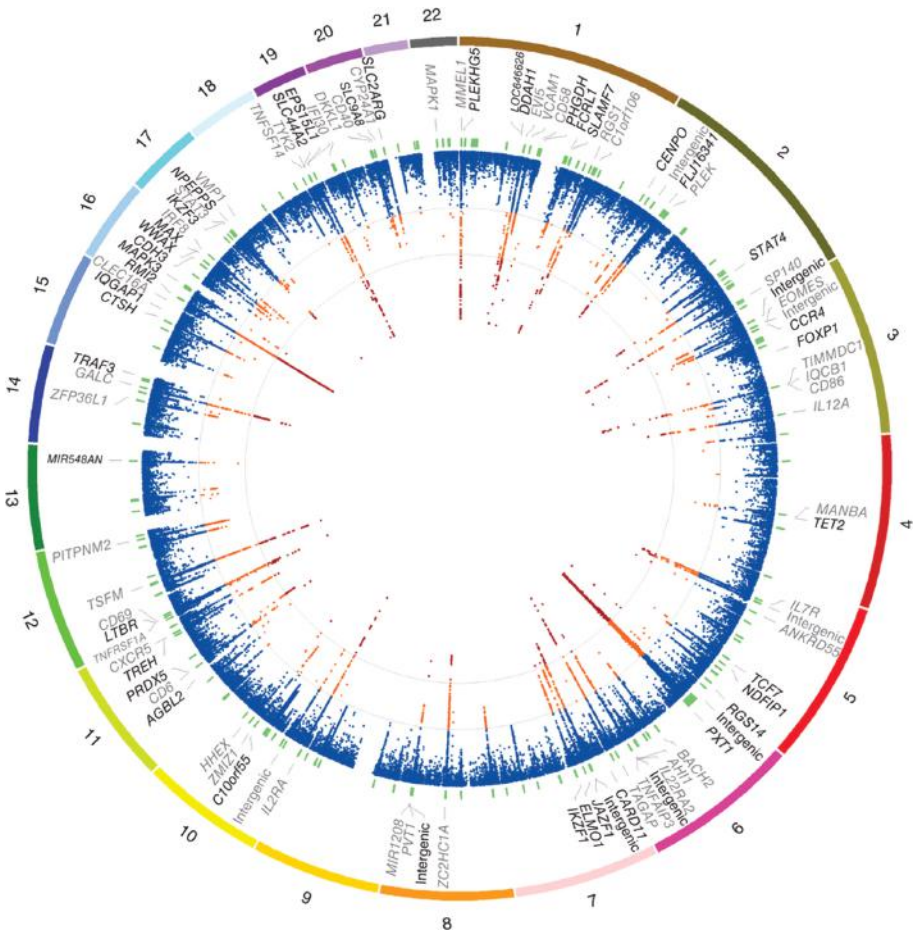

Figure 1 Genetic atlas of multiple sclerosis. The circus plot summarizes all the known MS-associated risk loci. The outer most track indicates the numbered autosomal chromosomes, while the second track shows the closest gene to the top hit within each locus (previously identified associations are in gray). The third track indicates the physical position of the 184 fine-mapped intervals (in green). The inner most track indicates –log(p) for each SNP (scaled from 0 to 12 which truncates the signal in several regions). Also, contour lines are given at the *a priori* discovery (–log(p) = 4) and genome-wide significance (–log(p) = 7.3) thresholds. Orange indicates –log(p) ≥ 4 and <7.3, while red indicates –log(p) ≥ 7.3. (Reproduced from Ref. (22)).

but the mapping of additional risk variants has been proceeding rapidly through ongoing multicenter initiatives utilizing dense, specialized arrays and very large sample collections. In this regard, a recent report anticipated that over 200 risk variants have been identified through the meta-analysis of all previous GWA studies conducted in MS (23). It is not inconceivable, however, that the potential for the discovery of additive risk variance extractable from large genomic screens will be quickly exhausted. The remaining fraction of the risk commonly known as "missing heritability" is likely due to still unknown common variants characterized by much smaller effects, below the detection limits of the GWA studies conducted so far. Some authors have proposed that a substantial portion of the missing heritability lies in genetic interactions between known variants, the so-called phantom heritability (24). Also, likewise gene by environment interactions, cis/trans-regulators of allelic expression, unidentified rare and penetrant semi-private variants, population and/or disease heterogeneity, neglecting the analysis of sex chromosomes, and hidden epigenetic effects may all contribute to the missing heritability.

From Genes to Function: Understanding the Molecular Basis of MS

The translation of GWAS data into biological functions has been challenging. The principal reason for this shortcoming consists in the pervasive linkage disequilibrium (LD) along the human genome, which hinders the identification of true causative variants. LD refers to the tendency of genetic loci in physical proximity to segregate together during meiosis, leading DNA to be inherited in large blocks through generations. This peculiarity of genome architecture substantially impairs GWAS resolution since SNPs in the same LD block are inherited together as well. Thus, statistically significant GWAS risk variants are usually proxy for the real causative variants, which can be located up to several megabases away within the same LD block. In addition, the identification of the causative variants is further complicated by the fact that most of them are not translated but rather map to regulatory elements (promoters, enhancers, silencers, and other transcription factor–binding sites). Nevertheless, substantial effort has been directed in this post-genomic era toward the functional characterization of the huge amount of genetic data generated by GWAS screenings, using either wet lab approaches or *in silico* analyses (or a combination of both).

FUNCTIONAL STUDIES IN MS

A variety of experimental systems have been employed to study the biological functions associated with MS risk variants, ranging from patients-derived primary blood cells to animal models of disease. The first putative causal variant identified in MS was the SNP rs6897932 located within the exon 6 of the *IL7R* gene, coding for the trans-membrane segment of the receptor. This SNP was shown to disrupt an exonic splicing silencer, affecting the relative amounts of soluble and membrane-bound isoforms of the protein (25). Recent evidence has shown that the RNA helicase DEAD box polypeptide 39B (DDX39B) is also a potent activator of

IL7R exon 6, and the SNP rs2523506 located in the *DDX39B* 5'UTR increases MS risk by reducing *DDX39B* mRNA translation (26). A similar effect was described for the intronic SNP rs2104286 in the *IL2RA* gene as well. In fact, this risk variant was also found to alter the soluble/membrane-bound ratio of IL2RA protein by driving the expression of higher levels of its soluble form (27).

Another well-characterized example is the intronic SNP rs1800693 in the *TNFRF1A* gene. In this case, the risk allele promotes the skipping of exon 6 with the production of a novel soluble form of the tumor necrosis factor (TNF) receptor which is able to inhibit TNF signaling inside the cells, mirroring somehow, the exacerbating effects of TNF-blocking drugs on MS course (28). More recently, our group has reported that the nonsynonymous exonic SNP rs11808092 in the ecotropic viral integration site 5 (*EVI5*) gene induces changes in superficial hydrophobicity patterns of the coiled-coil domain of EVI5 protein, which, in turns, affects the EVI5 interactome. In particular, we demonstrated that EVI5 protein bearing the risk allele selectively interacts with sphingosine 1-phosphate lyase (SGPL1), an enzyme important for the creation of the S1P gradient—which is relevant to adaptive immune response and the therapeutic management of MS (29).

Altogether, available functional data pinpoint at a "transcriptional hypothesis" where risk variants increase the propensity to develop MS by affecting primarily the expression of the associated genes. To this extent, recent advances in bioinformatics and computer-based methods of analysis have greatly helped toward the identification of the cellular pathways dysregulated upon disease.

PATHWAY ANALYSIS AND SYSTEMS BIOLOGY APPROACHES

The advent of "big data" in genetic research has been paralleled by the development of computational methods that could handle the size and complexity of this new type of information. In particular, different *in silico* approaches have been optimized to extract biologically meaningful associations from large genomic, transcriptomic, and proteomic datasets. These methods usually rely on the computation of overrepresentation of the input genes in specific gene ontology (GO) categories or biological pathways. More elaborated algorithms instead take advantage of gene interaction networks and search for possible sub-networks (modules) enriched in the input genes. Cell specificity and epigenomic reference datasets add additional layers of complexity to the analysis.

An early application of network-based methods in the context of MS was reported in 2011 by the IMSGC, which analyzed the results of the 2011 large GWAS and a following meta-analysis, comprising together a total of 15,317 cases and 29,529 controls. A large protein network encompassing more than 400,000 interactions among ~25,000 human proteins was created for the analysis. Notably, the intersection network between the two independent studies resulted in 88 genes arranged in 13 sub-networks. Furthermore, GO analysis on the 79 MS risk genes arranged in networks in at least one of the two studies highlighted the categories "leukocyte activation," "apoptosis," and "positive regulation of macromolecule metabolic process" as well as the KEGG pathways "JAK-STAT signaling pathway," "acute myeloid leukemia," and "T cell receptor signaling" (30). Extending pathway analysis to all the 110 non-*MHC* variants identified after the ImmunoChip study also detected the NF-kB cascade to be significantly associated with MS risk genes (22, 31).

In a recent paper, a gene network candidate approach has highlighted the putative role of cellular adhesion molecules (CAMs) in MS pathology (32). By using eight GWAS datasets and considering all the genes interacting in the CAM pathway, five sub-networks were found associated with MS susceptibility, possibly connecting the risk to the regulation of blood–brain barrier (BBB) crossing by T cells.

Genotype-Phenotype Correlations in MS

In addition to genetic factors contributing to MS susceptibility, specific variants also affect the clinical manifestation and the course of disease. Since the *HLA* locus is the first MS risk genetic determinant to be discovered and exerts the strongest influence on MS susceptibility, most of the genotype–phenotype studies are focused on *HLA* alleles. For instance, *HLA-DRB1*15:01* carriage has been found to be consistently associated with lower age at the onset of disease (33). Furthermore, *HLA-DRB1*15:01* seems to modulate the response toward glatiramer acetate, an immunomodulatory drug whose mechanism of action involves its binding to MHC class II molecules as an initial step (34). In addition, this allele was shown to increase the progression of MS brain pathology in terms of decline in brain magnetization transfer and T2 lesion load, as assessed by magnetic resonance imaging (MRI) (35). In contrast, the protective allele *HLA-B*44:02* appears to preserve brain volume and reduce the burden of T2 hyper-intense lesions (36). In a recent work by our group, we carried out an analysis of the global contribution of the *HLA* locus to a number of clinical and MRI outcomes. We calculated the cumulative *HLA* genetic burden (HLAGB) resulting from carrying different alleles in different *HLA* genes in 652 MS patients who had comprehensive phenotypic information and 455 controls of European descent. As suggested by previous studies, we found that higher HLAGB scores are associated with younger age at onset and the atrophy of subcortical gray matter fraction in women with RR-MS. Conversely, *HLA-B*44:02* showed a nominally protective effect for subcortical gray matter atrophy (37).

Genetics of MS Animal Models

Although MS naturally occurs only in humans, different animal models have been developed in which a disease mimicking MS is induced artificially. According to the nature of the inducing agent, the current models can be grouped into three categories: autoimmune, viral, and neurotoxic (38). Among them, the most widely used model is experimental autoimmune encephalomyelitis (EAE), which falls in the first category. EAE is an experimental disease that can be induced in several species (e.g., rodents, primates, cats, dogs, and chickens) via immunization with spinal cord homogenates or, more often, with purified peptides containing specific sequences of myelin proteins such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and myelin proteolipid protein (PLP). EAE recapitulates several features of MS, including the influence of genetic

and environmental factors. This evidence has led to the search for the genetic determinants modulating EAE susceptibility with the intention of getting insights into the human counterpart.

Like MS, the *MHC* locus displays the biggest contribution to EAE susceptibility and manifestation, confirming the important role of T cells and antigen presentation in disease pathogenesis (39). In addition, at least 27 non-*MHC* loci (*Eae1-Eae27*) have been found to be associated with different traits of the disease, including incidence, onset, severity, and histopathology (40–42). Interestingly, a large part of them show sex specificity, possibly mimicking differences between genders in MS susceptibility. Most of these quantitative trait loci (QTL) have been mapped through genetic linkage studies in backcross mice derived from SJL/J and B10.S strains. The choice of these two specific strains is due to the fact that the former is highly susceptible to EAE induction, whereas the latter is characterized by poor encephalitogenic responses. More sophisticated approaches rely on the generation of congenic lines between these two strains, in order to fine-map the loci of interest. A recent study combining phenotype-selected congenic mice and gene interaction network analysis was able to identify candidate genes shared between EAE and MS within several *Eae* loci. Interestingly, most of these genes belong to evolutionary conserved pathways important for $CD4^+$ T helper-cell differentiation (43). Following a similar approach in a panel of consomic lines from the wild-derived PWD strain, the same group has also identified candidate genes associated with sexual dimorphism in CNS autoimmunity, highlighting the possible involvement of the mitogen-activated protein kinase (MAPK) pathway in driving gender-related EAE differences (44).

The EAE model offers an additional advantage through the option to easily engineer the mouse genome and test candidate genes for their putative effects on disease expression. Such an approach encompasses either the knockout of endogenous mouse genes evolutionarily related to the human genes of interest or the introduction of human alleles into the mouse genome. As a paradigmatic example of the first scenario, knockout mice lacking the orthologue of the human *IL7Rα* gene were shown to be refractory to EAE induction, confirming the GWAS statistical association at the experimental level (45). The generation of transgenic mice carrying MS-relevant *HLA* alleles is instead the most common application of the second methodology. For instance, humanized mice expressing *HLA-DRB1*15:01* and *HLA-DRB5*01:01* alone or in combination, along with the human T cell receptor (TCR) specific for the $MBP_{85–99}$ peptide, have been instrumental in demonstrating the functional epistasis between the two alleles. Mice expressing both alleles indeed develop a milder form of a spontaneous MS-like disease as compared to mice expressing *DRB1*15:01* only (46).

Conclusion

GWA studies have undoubtedly energized and changed the field of MS genetics, allowing the discovery of more than a hundred risk loci following decades of unsuccessful attempts. A pressing challenge for the MS research community lies in the organization of the vast amount of genetic data finally available in a coherent

biological frame, which could explain the primary causes of the disease and its pathogenic processes. Considering the heterogeneity of MS and the intrinsic complexity of the human genome, a number of rational approaches can be envisioned to characterize the biological functions connected to MS susceptibility and pathophysiology.

First, fine-mapping projects will be required to refine the association in previously identified genomic loci and prioritize the candidate variants for further studies. This could be done by employing batteries of genetic markers saturating the region of interest as well as by analyzing populations with different LD patterns. In this regard, we recently reported the analysis resulting from genotyping an African American MS dataset with the ImmunoChip platform (47). African American genomes possess shorter LD, reflecting their unique ancestral history, a characteristic that facilitated narrowing down the association to tumor necrosis factor receptor superfamily member 14 (*TNFRSF14*) in a confirmed locus that included tetratricopeptide repeat domain 34 (*TTC34*), *LOC115110*, membrane metalloendopeptidase like 1 (*MMEL1*), *TNFRSF14*, and family with sequence similarity 213 member B (*FAM213B*) as candidate genes. These results support the utility of transancestral studies to better map the relevant variants within MS loci and suggest that common genetic basis underlies susceptibility across different ethnic groups.

Second, the increasing availability in public databases of gene expression datasets with relative genotype annotation can greatly facilitate the assessment of expression quantitative trait locus (eQTL) effects associated with the carriage of genetic variants relevant for MS. In this regard, computational strategies integrating gene expression measurements with summary GWAS data have been recently developed to identify genes whose cis-regulated expression is associated with complex traits, an approach called transcriptome-wide association study (TWAS) (48, 49). In addition, transcriptomic studies in relevant tissue samples from MS patients can also help identifying specific genetic signatures associated with disease susceptibility or progression. For example, following this approach, our group has shown that low levels of transducer of ERBB.2-1 (*TOB1*) transcript in $CD4^+$ T cells are strongly associated with a higher risk of early conversion to clinically defined MS in patients experiencing a first demyelinating event in the CNS (50, 51).

Finally, recent remarkable innovations in genomic editing, such as the CRISPR-Cas9 or the TALEN systems (52), promise to reshape the next generation of functional studies aiming at translating genetic observation into mechanistic insights. These tools afford the modification of the genome at the single nucleotide level in a mono-allelic or bi-allelic fashion. Compared with classical methods of transgenesis, these new methodologies allow assessing the functional impact of genetic variants in physiological conditions via direct modification of the host genome in cell or animal models. These systems will be particularly relevant to efficiently screen regulatory variants mapping outside genes, whose function is less intuitive as compared to variants inducing amino acidic substitutions. Furthermore, the possibility to simultaneously introduce multiple modifications in different genomic regions makes these systems suitable to explore possible epistatic effects between two or more variants (53).

In summary, an integrated approach involving multiple disciplines and technologies is likely to be the most effective way to address the complexity of MS

genetics and identify biologically meaningful correlations between risk variants and specific molecular functions.

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Conflict of interest
The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this chapter.

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2 Living with Multiple Sclerosis in Europe: Pharmacological Treatments, Cost of Illness, and Health-Related Quality of Life Across Countries

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Abstract: More than 700,000 people suffer from multiple sclerosis (MS) in Europe. This implies that more than 1 million people are affected by this disease through their role as caregivers and family members. Given its relevant impact, MS deserves consideration by epidemiologists, clinicians, psychologists, social scientists and other scholars. Such interdisciplinarity is stressed in the present contribution, which focuses on various aspects of socioeconomic burden. Starting from considerations about the epidemiology of the disease in Europe, as outlined by the MS Barometer, a comparative survey based on data collected by the national MS societies and launched in 2008, a brief literature review for each European country mentioned in the report was carried out with the following key terms: "multiple sclerosis," "cost of illness," and "health-related quality of life (HRQoL)." The consideration of the level of assistance provided, the access to rehabilitation centers, and the availability of pharmacological treatments,

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especially innovative therapies, reveal how there are still huge differences across Europe. Literature contributions are mostly oriented toward HRQoL studies and the impact of new pharmacological treatments. There are less studies focusing on compliance: this may be the consequence of a higher awareness of the disease among the patients and a strengthened cooperation with the physicians. Some suggestions about foreseeable and desirable lines of research conclude the contribution.

Key words: Cost of illness; European countries; Health-related quality of life; Multiple sclerosis; Pharmacological treatments

Introduction

More than 700,000 people suffer from multiple sclerosis (MS) in Europe; this implies that more than 1 million people are affected by this condition through their role as caregivers and family members (1). MS is one of the most common causes of neurological disability in young and middle-aged adults (2). It is characterized by various symptoms that can be associated with motor deficits (fatigue, paralysis, and coordination disturbances), sensory problems, speech and vision (blurred or double vision) impairments, and sphincter and bladder malfunctions (3). While MS can be diagnosed at any time in life, it frequently occurs between the ages of 20 and 40; women are more susceptible than men, with a ratio of 3:2. The natural history of MS is highly variable. Initially, about 85% of patients present with relapsing remitting multiple sclerosis (RRMS), which is characterized by unpredictable, self-limited episodes of the central nervous system, and may last from several days to weeks. For the remaining 15% of patients, MS begins as primary progressive (PP) with the gradual worsening of neurological symptoms. Two-thirds of RRMS patients may develop a secondary progressive course (SPMS, secondary progressive multiple sclerosis), which is characterized by neurological deterioration over time (4). Although the disease may manifest and evolve in different ways, it definitely changes people's lives. Due to the consequences of MS, which go beyond the physical symptoms, patients have to limit their daily activities and social relationships, and their self-esteem might be reduced (5). Recent studies recognize how the number of people living with MS around the world is growing: it has increased at least by 10% in the last few years, and in 2013 it reached 2.3 million (6). This is likely to be attributed mainly to diagnostic criteria such as the McDonald criteria, which permit to formulate a diagnosis more often than other criteria such as the Poser's criteria (7). There has been progress in brain imaging too: this leads to a faster diagnosis by employing a special type of scanning which is able to reveal lesions in the brain's white matter (8). The role and importance of information regarding MS as well as other chronic diseases have been stressed in many studies (9). Such information systems enable the identification, collection, and processing of data in order to obtain useful indications. Exchanging data among physicians and health care centers helps to organize better assistance. Hence, an accurate and efficient information system can reduce the expenses and uncertainties associated with the disease and favor an increase in health-related quality of life (HRQoL).

MS in Europe

Currently, information regarding MS in Europe is widespread, thanks to many sources. The MS Barometer is a comparative survey based on data collected by the national MS societies (10). First launched in 2008, the MS Barometer raises awareness about the geographical differences in MS management across Europe. It is a questionnaire with points scored based on the responses: the higher the score, the better the disease management, the level of support, and the HRQoL of people with MS in each country. The questionnaire has been updated in three subsequent editions of the MS Barometer in 2009, 2011, and 2013. It is structured around the priority policy areas defined in the European Multiple Sclerosis Platform's (EMSP) Code of Good Practice, related to access to health care (where health care has to be meant as a comprehensive notion, which includes treatments, new medications accessing the market, therapies, and health workforce involved in MS care); research and data collection system (given that the quality of the information provided is likely to impact expenses determined by the disease); participation in society of people with MS (that aims at strengthening financial support, education for young people affected by MS, and possibility of employment); and empowerment (that should be meant as an objective both for people with MS and for organizations). Twenty-eight countries participated in the MS Barometer 2015, representing more than 500,000 patients. Hence, the MS Barometer 2015 sketched an up-to-date picture of prevalence, incidence, and access to treatment in Europe.

Instead, the EMSP, founded in 1989, group about 40 national MS member societies from 35 European countries and aims at collecting data and evidence on MS with the purpose of being a guide to improve patients' and their families' HRQoL.

Table 1 reports on data about MS prevalence across European countries, collected through the national MS societies joining the EMSP. Further evidence is presented in Table 2, which contains data collected by the EMSP (11), retrieved through the Atlas of MS (www.atlasofms.org), the report Under Pressure, Living with MS in Europe, released by the EMSP (www.underpressureproject.eu) and some recent studies (1, 12). Data are representative of the year 2013 and relate to prevalence and access to disease-modifying drugs (DMDs) and symptomatic treatments, which will be discussed in more detail in the next section. Other information concerns epidemiological data on the course of MS (age of diagnosis, RR form); the impact on working (percentage of reduction in the number of working hours and the percentage of people with MS employed part time and full time); information related to the social impact of the disease such as the awareness of the disease, limitations at work, and the possibility to access rehabilitation centers. This information sheds light on the level of assistance, especially provided to patients experiencing a relapse and the possibility to recover from it. Little information was available for countries such as Cyprus, Latvia, and Slovakia. Overall, there are important consequences for individuals' working activity: on average, half of the people with MS leave their jobs 3 years after the diagnosis (13).

Costs, employment, and quality of life are affected by increasing disease severity in people with MS (14, 15). While, in the early stages of the disease, costs are predominantly driven by pharmacological treatments, when the

TABLE 1 MS in European Countries (in ascending order of prevalence)

Country	Prevalence per 100,000
Slovakia	NA
Romania	30
Bulgaria	39
Portugal	56
Croatia	59
Greece	70
Lithuania	78
Estonia	82
The Netherlands	88
Latvia	90
France	95
Belgium	100
Spain	102
Finland	105
Switzerland	110
Italy	113
Poland	120
Slovenia	120
Austria	140
Ireland	140
Germany	149
Czech Republic	160
Norway	160
United Kingdom	164
Cyprus	175
Hungary	176
Sweden	189
Denmark	227

Source: European Multiple Sclerosis Platform, 2015.
NA = not available.

TABLE 2 Epidemiological and Socioeconomic Information about MS in Europe (in ascending order of prevalence)*

	Prevalence per 100,000	Onset disease	RR form	Reduced working (%)	Working full time	Working part time	Awareness	Incentives at work	DMDs treatments	Symptomatic treatments	Rehabilitation centers
Slovakia	NA	NA	NA	80	NA	NA	0	1	NA	NA	100
Romania	30	22	90	80	NA	NA	1	1	39	60	10
Bulgaria	39	NA	70	80	30	1	0	0	12	35	NA
Portugal	56	29	NA	80	45	NA	0	0	70	85	30
Croatia	59	32	NA	80	21	1	1	1	20	60	100
Greece	70	27	NA	80	NA	NA	0	1	70	NA	12
Lithuania	78	29	77	80	10	40	0	0	70	100	50
Estonia	82	44	90	80	NA	NA	0	0	27	NA	75
The Netherlands	88	30	89	77	25	30	1	1	50	70	100
Latvia	90	28	NA	80	NA	NA	NA	NA	NA	NA	NA
France	95	29	85	82	25	35	0	1	40	80	45
Belgium	100	30	80	76	50	50	1	1	59	70	100
Spain	102	35	85	68	NA	NA	0	NA	50	75	15
Finland	105	32	93	80	35	15	1	1	62	100	85
Switzerland	110	30	90	83	NA	NA	1	0	NA	NA	70
Italy	113	30	85	79	50	10	1	1	47	80	70

Table continued on following page

TABLE 2 **Epidemiological and Socioeconomic Information about MS in Europe (in ascending order of prevalence)*** (Continued)

	Prevalence per 100,000	Onset disease	RR form	Reduced working (%)	Working full time	Working part time	Awareness	Incentives at work	DMDs treatments	Symptomatic treatments	Rehabilitation centers
Poland	120	NA	NA	80	NA	NA	0	1	13	90	NA
Slovenia	120	28	NA	80	30	60	1	1	53	80	90
Austria	140	27	85	75	35	15	1	1	51	85	75
Ireland	140	30	70	80	12	12	0	1	32	70	0
Germany	149	31	87	73	33	13	1	1	69	80	100
Czech Republic	160	32	NA	80	10	20	0	1	39	100	100
Norway	160	31	85	80	18	21	0	1	52	70	100
United Kingdom	164	32	85	77	8	11	1	0	21	NA	70
Cyprus	175	30	NA	80	NA	NA	NA	NA	NA	NA	NA
Hungary	176	27	75	80	5	2	0	1	16	30	15
Sweden	189	33	90	77	NA	NA	0	1	39	NA	60
Denmark	227	34	80	80	8	25	1	1	44	80	100

Source: European Multiple Sclerosis Platform, 2015.

NA = not available.

*In the table, in the column related to "awareness," 1 indicates awareness in assistance programs for MS patients in the workplace and 0 represents the lack of awareness; in the column related to "incentives at work", 1 indicates incentives to recruit people with disabilities, while 0 indicates the absence of such incentives

disease becomes severe, the overall costs increase, and indirect costs (due to the loss of productivity for patients and their caregivers) become more significant. It has been estimated that the average cost per year of all resources relating to MS was €22,800 for those patients with mild disease severity, €37,100 for those with moderate disease severity, and €57,500 for those patients with severe disease (14). The same study outlined how, among people of working age, 18% of patients with mild disease were unemployed; this percentage is about 92% when people with severe disease are considered. Disability is the main driver of reduced productivity and HRQoL; the symptoms due to the disease that impact productivity are fatigue (experienced on average by 95% of patients considered for the study) and cognitive difficulties (experienced by 71% of patients). Data about employment, according to the information provided by EMSP, were not available for Cyprus, Greece, Portugal, Spain, Sweden, Switzerland, and eastern European countries (Estonia, Latvia, Poland, Romania, and Slovakia). With the exception of Belgium and Slovenia, where more than 50% of the people with MS are employed full time, this percentage, overall, is not very high (in Denmark and the United Kingdom, people with MS working full time are, respectively, 8 and 5%). However, data are fragmented and apparently contrasting; for example, Austria, Bulgaria, Croatia, and Hungary present a percentage of people employed part time that is lower compared with people employed full time. Incentives to recruit disabled people are present in the majority of countries, with some exceptions such as Bulgaria, Estonia, Lithuania, Portugal, Switzerland, and the United Kingdom. Such incentives are often coupled with the awareness in programs on MS for the workplace and information directed to employers, coordinated by public or private institutions (according to the evidence reported, this occurs in Austria, Belgium, Croatia, Denmark, Finland, Germany, Italy, the Netherlands, Romania, and Slovenia).

Poland and Hungary have the lowest access to DMDs treatment. In Belgium, Croatia, Czech Republic, Denmark, Germany, the Netherlands, Norway, and Slovakia, 100% of MS patients have access to rehabilitation centers.

The evidence that emerges from the table, which summarizes the information retrieved from several sources, stresses which issues should be investigated in more detail. First of all, information on the labor market and the social consequences for MS patients should be enriched. Loss of productivity due to illness, which, according to data, is 79% (average data), leads to an increase in indirect costs and higher social costs, and this has to be investigated. There are not many studies that have been concerned with this aspect, neither are there detailed analyses on the costs of the disease, including indirect costs and productivity losses (16). Finally, affordability is a key barrier to access MS products. In some countries, patients cannot afford the cost of treatment and the expenses related to the disease. Hence, the organization of an efficient assistance model is crucial.

Treatments for MS

There is no definitive cure for MS as yet, but access to pharmacological preventive and symptomatic treatments may help patients in managing the disease (17, 18).

An early recognition of the inflammatory process allows patients to begin treatment with a DMD even before the technical diagnosis of definite MS; in this way, the degenerative progression of MS can be delayed (16). It has been shown how patients, who had started the treatment at a later stage, had a greater risk of reaching score 4 on the Expanded Disability Status Scale (EDSS). Although this is a moderate disability score (while EDSS scores higher than 4.5 are regarded as more severe, impairing individuals' daily activities), according to clinical evidence, this may increase by 7.4% for every year of delay in treatment start after MS onset (19). Moreover, the early pharmacological treatment is associated with fewer hospitalizations, a reduction of relapses, and a gain of QALYs than delayed treatment (20, 21).

The choices about the most suitable pharmacological treatment and its timing may rely on the patient's and physician's joint decision (2, 22). However, the treatment selected and the type of assistance provided to MS patients depend mostly on the characteristics of the health system in each country. Although many studies have found that a consistent part of costs caused by MS is related to productivity losses (sick leave and early retirement due to MS), nonmedical costs (devices and investments to adapt living conditions) and informal care by family and friends (23), it has been estimated that, on average, more than 50% of the costs associated with the disease come from direct medical costs, which are often due to innovative therapies. The relevance of drug treatment and the weight attributed to pharmaceutical costs have to be considered from the third payer's and societal perspectives. New treatments have been made available in recent years. Innovative drugs are still under development or waiting for approval within a centralized procedure by the European Medicines Agency or through a decentralized procedure, at the national-level reference.

About the type of therapies for MS currently available, disease-modifying therapies (DMTs) include injectable medications (interferon beta 1-a and 1-b, glatiramer acetate, and peginterferon beta 1a), oral medications (fingolimod, teriflunomide, and dimethyl fumarate), and infused medications (natalizumab and alemtuzumab). In addition, there are other treatments with immunosuppressants that can be effective for MS (mitoxantrone, azathioprine, cyclophosphamide, methotrexate, etc.). Other drugs (e.g., corticosteroids or nabiximols) are employed in case of relapse or to alleviate some symptoms of MS. All these agents act by modulating and/or suppressing the immune system at various levels with different mechanisms of action. The efficacy, tolerability, and safety profile vary significantly across treatments, ranging from combinations of modest effect and a good level of safety to those that are highly effective but at increased risk of serious or even fatal adverse events.

First-line treatments are intended as a moderate-efficacy, high-safety drug and include interferon beta 1a and 1b, glatiramer acetate, peginterferon beta 1a, teriflunomide, and dimethyl fumarate. Differences exist in terms of efficacy and tolerability among first-line drugs, although direct comparison data are limited (22). Second-line treatments are used in case of unsatisfactory response to first-line drugs: they are not only more effective but also come with more safety risk, and include, among others, natalizumab, alemtuzumab, and mitoxantrone. Fingolimod is approved as a second-line treatment in the European Union and as a first-line treatment in the United States, Canada, and other countries (22). Azathioprine and cyclophosphamide, which are not registered as treatment for MS, are used as first-line and second-line medications, respectively.

There have been many studies on access to MS treatments in Europe. A well-known study (24) looked at the available evidence on prevalence, the costs to society, and difference in access across European countries, and discusses the determinants of patients' access itself. The authors found that there was a wide variation across European member states: according to 2008 data, in Western Europe around 44% of patients had access to pharmacological treatment, whereas in Central and Eastern Europe, this percentage was between 6 and 42%. Such large variations in the number of patients with access to innovative drugs could be explained by economic differences among European economies that lead to a diverse range of pharmacological treatments guaranteed to patients by each national health system. However, the authors of the study found that price levels did not reflect the affordability levels in different markets. Indeed, they also identified differences in medical practice, the ease of access to care, and the availability of care.

The access to innovative treatments across European countries may depend on health policy issues too: some countries may focus on a particular MS patient sub-population and develop specific treatment guidelines. Hence, depending on where a patient lives, he or she will be, or will not be, entitled to such medication. For example, in Sweden, for the use of immunomodulatory therapy, approximately 75% of patients with RRMS meet the criteria for DMDs therapy. Moreover, Sweden presents a high number of SPMS patients: in this light, a study aimed at comparing first-line and second-line treatments, such as natalizumab and fingolimod, outlined how Scandinavian countries provide better access to innovative second-line treatments, followed by France, Austria, and Belgium. Overall, the access to pharmacological treatment has increased in the past years. The percentage of people treated with DMDs across European countries is shown in Figure 1. Among these patients, the percentage of those who are accessing the most innovative treatments is estimated at around 20% for MS patients in Europe. Instead, in eastern European countries, lower shares can be observed: in 2008, in Poland and Romania, around 3–4% of the patients with MS had access to innovative therapies.

Medical and Socioeconomic Literature Related to MS: Evidence from the Literature in the Countries Joining the MS Barometer

The studies investigating the prevalence of MS across Europe include country-specific studies, cross-country comparisons, and compendia of prevalence statistics. Wilsdon et al. (25) cite, among the international comparisons, Kingwell et al. (26), who carried out a systematic review of incidence and prevalence of MS in Europe between 1985 and 2011. The authors concluded that prevalence and incidence estimates tended to be higher in the more recent studies, especially in the Nordic countries; they also stated that, despite the extent of the literature on the epidemiology of MS in Europe, inter-study comparisons are hindered by the lack of standardization. With the general aim of establishing a Europe-wide platform for systematic analysis and comparison of longitudinally collected MS data in

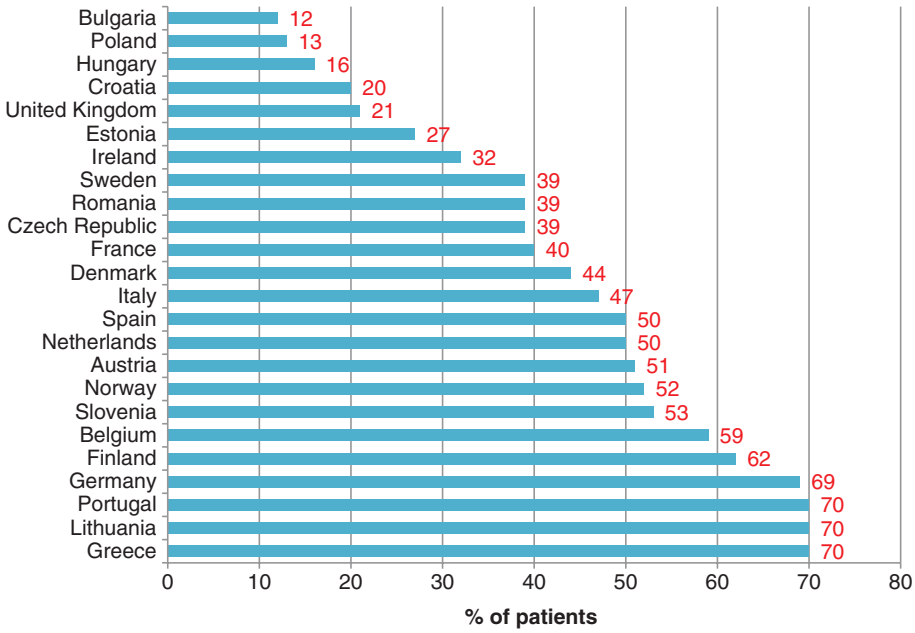

Figure 1 **Percentage of MS patients who have access to DMDs in Europe.**

Source: CRA Analysis, 2014.

Europe, the European Register for Multiple Sclerosis (EUReMS) project was started in 2010 by an international consortium, involving both scientists and patient organizations (27). Detailed information about the number and content of national MS registries in Europe is needed to facilitate the integration of existing data, as well as to carry out comprehensive analyses and comparison across European populations.

In a systematic review of MS registries and databases in Europe, a detailed search identified 17 national MS registries, adding to this list three other registries after contacting European MS societies (28). The registries differ with regard to objectives, structure, data, and the number and type of patients included. In spite of their heterogeneity, all registries had the following common objectives: MS epidemiological and pharmacological surveillance; efficacy, safety, and cost-effectiveness of pharmacological treatments in the long run; provision and quality of health care services; HRQoL and other socioeconomic aspects, such as the burden of disease, both from the patients' perspectives and that of the neurological centers. According to the study findings, registries were available for Austria, Bosnia and Herzegovina, Croatia, Czech Republic, Denmark, France, Germany, Greece, Iceland, Italy, Malta, the Netherlands, Norway, Slovenia, Spain (Catalonia), Sweden, and the United Kingdom.Further information was collected through the national MS societies of Russia, Serbia, and Switzerland.

A literature search for each European country included in the MS Barometer was then carried out in PubMed (period 2012–2017; last accessed, May 20, 2017) using the terms 'multiple sclerosis + country', then 'multiple sclerosis + country + cost of illness' and, finally, 'multiple sclerosis + country + health related quality of life'. Although they are not fully comprehensive, the results gave a picture of the aspects that have received more attention in the 28 European countries considered. Overall, it was noted that MS is often treated in the literature together with other chronic conditions (especially in the studies focusing on HRQoL and carried out at the European level). In some countries, many studies have been carried out within international research projects aimed at assessing the cost-effectiveness and cost–utility ratio for pharmacological treatments, or directed at developing common guidelines and assistance protocols.

The review could be improved by mentioning other aspects in the epidemiology and management of the disease, focusing on cost of illness (COI) and looking at indirect costs that are related to MS patients' reduced productivity and HRQoL. The countries observed through the Barometer, in alphabetical order, are: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, the Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, and the United Kingdom.

In *Austria*, treatment registries, especially for pharmacological "second-line" treatments, such as Fingolimod and Natalizumab, have been instituted. A general search on epidemiology of MS found 314 studies, of which the most recent are focused on the impact of emerging drugs such as ocrelizumab (29). Other economic evaluation analyses concern socioeconomic aspects of some treatments (30). Studies related to QoL have considered some specific rehabilitation programs aimed at improvements in the specific domains of attention and mental fatigue (31).

In *Belgium*, most of the studies retrieved were clinical and were carried out within European research projects. There is a national registry for MS, the *Beltrims*, started in 2012. Organizational issues have been discussed in studies assessing the costs and potential financial benefits of integrated care models for patients with chronic diseases (32). The total burden of the disease relates to the clinical, humanistic, and economic dimension. Crucial information is still missing about MS pathophysiology and other clinical issues. This is a hindrance in reaching the objective of an equal access to care and treatment for MS.

Bulgaria does not have a tradition of studies on MS. Only in 2017, the Bulgarian MS Society announced the realization of a registry of patients (http://www.emsp.org/news-messages/ms-registry-and-national-representation/). The literature search found only 16 studies, of which the last epidemiology study was in 1997 (33), and reported a considerably lower prevalence of MS in Bulgaria comparing with the neighboring countries.

Cyprus neither has any information on epidemiology of MS nor any record based on scientific evidence. The official data of prevalence and/or incidence refers to the information reported by the Atlas of MS 2013; the studies that have been identified through the research were mainly related to the clinical impact of MS or they were meta-analyses (34, 35).

In *Croatia*, the studies carried out in the last 5 years focused mainly on pharmacological treatments and diagnostic tools such as magnetic resonance (36). Croatia has a national registry for MS, started in 2007.

The *Czech* ReMuS started in 2013. The output of the ReMuS is published regularly (http://www.multiplesclerosis.cz/docs/160929_remus_aj_zaverecna-zprava_2016_06_souhrnna.pdf). One COI study used Czech data and extrapolated to Polish patients to estimate costs of MS (37). The mean annual costs from societal and payers' perspective were calculated for patients according to EDSS. Indirect costs (production loss due to early retirement, sick leave, and informal care) cover up to 70% of total costs.

In *Denmark,* all cases of MS have been registered since 1948. In 1996, the Danish MS Treatment Registry was established. Most of the studies adopted a multidisciplinary perspective of MS, with focus on the organization of a multidisciplinary care team and the possibility to support the patient, so that the latter is empowered to manage his or her disease and to implement a physically active lifestyle. Furthermore, some studies have emphasized how dedicated programs for patients and health care professionals, including nonmedical treatment strategies, should be developed at the European level (38).

In *Estonia*, statistical and updated data about MS is not yet available (see http://www.smk.ee/tooandjatele/statistika/). One clinical study, carried out at West-Tallinn Central Hospital, was retrieved (39).

In *Finland*, the focus has recently been on the new therapies (40), the estimation of patients' costs and HRQoL, and cognitive deficits. Although the incidence and prevalence of MS in Finland are high and the structure of the Finnish health care is ideal for taking care of MS, Finland was the only Scandinavian country without a national MS register until 2011. The Finnish Neurological Association assigned a steering board to develop an MS national registry. By 2016, five university hospitals and six central hospitals have joined the register. The burden of illness and HRQoL have constituted the topic of some recent analyses (41, 42).

In *France*, the MS registry is sponsored by the Hospices Civils de Lyon. At the end of 2015, it observed 54,000 patients. One of the latest studies provided estimates of the prevalence and mortality rate of MS and used reimbursement data for disease-modifying treatment, long-term disease status, disability pension, and hospitalization (43). Another study analyzed the social participation in patients with MS, correlating economic costs related to the treatment with social participation, utility, and MS-specific quality of life in a sample of 42 patients receiving natalizumab (44).

In *Germany*, the national MS registry was established in 2001. In the last 5 years, a large number of studies have come out of Germany (about 2063 studies). Despite this, health care utilization data and analyses for MS are still scarce (45). Some studies (46) were related to the effects of new treatments such as alemtuzumab on safety, effectiveness, and HRQoL.

The largest number of researches carried out in *Greece*, where there is a national MS registry, concern clinical issues. There are no recent prevalence studies; the last one dates back to 2008 (47). Some interesting insights came from studies aimed at defining a sort of "stigma" for MS patients, especially neurological disorders, that determines the exclusion from full social acceptance. Although stigma is considered to be present in MS, the factors that influence its levels are ambiguous (48). About the COI analyses carried out for Greece,

the search outlined how there is a North-South gradient for health expenditure for costs and prevalence of the disease (49). The authors of the study stress how health and welfare systems of some countries are not prepared to manage these occurrences. HRQoL is treated in a study that outlines how HRQoL is influenced by self-confidence, which is a direct result of self-ability and mobility, the stage of disease, the social relations, and the risk of sudden substantial of health deterioration (50).

There is no national registry for MS in *Hungary*, but some data are provided by the Hungarian MS Society, established in 1988 (http://www.smtarsasag.hu/). Prevalence studies are related to single centers or to counties. The first epidemiological study on MS was based on the McDonald diagnostic criteria in central Europe (51). There is only one COI study (52) that is aimed at exploring the quality of life, resource utilization, and costs of 68 MS patients in Hungary. About 16 studies focused on the effects of the disease symptoms on HRQoL; a recent study (53) examined the correlations between HRQoL and the level of disability, fatigue, and depression in glatiramer acetate-treated patients with MS and provided suggestions for the management of the disease, recommending immunomodulatory therapy together with improvements of the diagnostics and treatment of the accompanying depression.

Ireland has a high prevalence of MS, which has been increasing in the last 20 years. There is no national registry of people with MS. There are, however, patients' associations which provide an insight into the number of people with MS. Among the first studies aimed at prospectively assessing the incidence rate of MS in Ireland, one epidemiological study ascertained all new cases of MS in the years 2014 and 2015 (54). Another research (55) shows how MS can be associated with significant disability, resulting in considerable socioeconomic burden for both patients and the society. The study found that even low-intensity episodes can have a significant financial impact for the patient. In a prospective study, it has been outlined how there is the potential to significantly reduce the economic burden of the disease through interventions that prevent progression from mild or moderate MS to severe MS, and keep people in the work force (56). A HRQoL study, using EQ-5D-5L correlation with the EDSS score, showed a linear decline in utility with changes in EDSS from 0 to 6, after which point the relationship exhibited greater variability (57).

In *Italy*, the studies on MS are related to various topics, such as clinical outcome, cost-effectiveness analyses, and rehabilitation. Some Italian regions (such as Sicily, in the South) have recently initiated their MS registries. The *Associazione Italiana Sclerosi Multipla* (AISM) provides data about the prevalence and incidence of MS in Italy. A crucial aspect, during the last few years, has been that of adherence and compliance to pharmaceutical treatments as well as communication (58, 59). COI studies are often carried out together with cost–utility analyses and Quality of Life Surveys (60, 61). The focus of the literature is on new therapeutic options as well as the progressive forms of the disease; some research projects concerning palliative approaches to severe MS or communication in SP MS are being carried out (62).

Latvia is often included in international studies on MS among other countries. The national association was instituted in 1995 (http://mslapa.lv/site/30146).

In *Lithuania*, a multicenter MS registry was created in 2013 and the data collection was started in three MS centers and university hospitals. Most of

the studies are related to the experience of single centers and the effectiveness of therapies and adherence (63); other studies relate to specific MS disturbances (64).

The studies carried out in the last 5 years in the *Netherlands* are mainly clinical, evaluating symptoms and the effects of pharmacological treatments. The NEDBase, the national Dutch registry, started in 2007 involves six neurological centers. Some comprehensive studies have measured the burden imposed by MS on the Dutch society, which is higher compared to the results of previous studies (65). Recent studies examine both adherence and persistence and outline how the latter could be predicted by HRQoL (66).

Most recent studies carried out in *Norway* focus on risk factors for MS, mortality data, and life expectancy (67–69). In Norway, there is a national MS registry.

In *Poland*, the National Registry of MS patients was created in 2013 (70). The literature has focused both on COI and HRQoL studies. A study based on real-life data from the Social Insurance Institution in Poland has assessed the indirect costs of six major autoimmune diseases, concluding that MS is associated with great indirect costs (71). Studies on HRQoL employ data from the Polish registry and examine the role of cognitive appraisals, adjusted for clinical, socioeconomic, and demographic variables, as correlates of HRQoL in MS (72, 73).

In *Portugal*, the National Society for MS was established in 1984. Although the literature search retrieved 216 studies, the last epidemiological study was in 2010 (74). There are no studies focused on COI; however, Portugal is often analyzed within international studies (48). Other studies looked at several problems associated with the disease, such as sleep disturbances (75).

In *Romania*, there is a national association of MS patients, which was founded in 1995. Epidemiological studies were carried out in 1989 and 1994 (76, 77). Another study, related to the Multiple Sclerosis Information Dividend (MS-ID) project, aimed at identifying and addressing major inequalities of MS treatment and care, was carried out in 2010 (78): it considered the feasibility of an EU MS register among five countries (Germany, Iceland, Poland, Romania, and Spain).

The *Slovakian* Association for MS was founded in 1990. The studies are mainly clinical or aimed at assessing cognitive impairment determined by MS (79). COI has been investigated in few studies. An MS study in 2015 in Slovakia was the first Slovak study to provide information about health care, social expenditure, and the cost of productivity loss; direct and indirect costs of MS were retrospectively analyzed by prevalence, based on a bottom-up approach (80). The societal and health insurance perspective was used to assess the economic burden caused by MS in Slovakia, using the human capital method for the calculation of indirect costs. HRQoL has been the object of another study that evaluated functional disability measured by patients and neurologists (81).

In *Slovenia*, the national MS association was established in 1973. Most of the studies related to MS focused on the effects of pharmacological treatments. One international multicenter study concerned physiotherapy and rehabilitation (82). HRQoL together with coping was investigated as well (83).

Spain is often mentioned in international studies carried out for Europe and related to treatment experience and MS burden of disease. There is a MS registry for Catalonia. Other registries follow patients in treatments with given drugs,

for example, Fingolimod (84). The most recent studies regard prevalence of MS and suggest an increasing prevalence (85). Several works estimate the COI of MS (86), measure its socioeconomic effects (87), or carry out budget impact analyses (88).

In *Sweden*, there has been a National Registry of MS patients since 1997; many studies are based on real-life data. Prevalence of MS has been analyzed in different areas of the country (89). There are several recent studies on COI that have been carried out for working-aged individuals, reporting that indirect costs contributed to approximately 75% of the estimated costs of MS patients (90). Costs and utility are highly correlated with disease severity, and resource consumption may be influenced by health care systems' organization and availability of services (12). The studies on HRQoL are aimed at assessing several aspects of the pathology, in particular, relapses associated with increased fatigue and reduced HRQoL (91).

The *Swiss* society for MS instituted a register in 2016 (https://www.multiplesklerose.ch/it/attualita/dettaglio/registro-svizzero-sm-partecipanti-colpiti-di-ogni-eta/). The perspectives and expectations of MS patients have been analyzed in a study that outlined how there is no data available about the needs of people living with MS in Switzerland (92). Other studies, related to HRQoL, carried out by Swiss researchers, however, do not employ Swiss data (93).

In the *United Kingdom*, the MS registry was started in 2009. Through the literature research, it was possible to retrieve about 1000 studies. Together with incidence and prevalence (94), studies related to cost-effectiveness, cost utility analyses, and prognostic factors have been carried out (95).

Conclusion

The studies carried out on MS in Europe are mostly oriented toward HRQoL and the impact of new pharmacological treatments. There are less studies focusing on compliance: this may be a consequence of the higher awareness of the disease among the patients and a strengthened cooperation with the physicians. The consideration of the level of assistance provided, the access to rehabilitation centers, and the availability of pharmacological treatments, especially innovative therapies, reveal how there are still huge differences across Europe. The scholars' effort should be directed toward the estimation of the burden of disease and the strategies to implement for the achievement of a higher HRQoL. In spite of many studies on the epidemiological course of the disease, these aspects have not been fully exploited yet, and they need more attention. Costs, employment status, and quality of life are closely linked to disease severity across European countries. In this perspective, the development of a common strategy is essential to ensure consistency in the quality of care over time, to address the variations in service provision for people with MS, and to provide a framework to get access to innovative therapies more rapidly. National registries, linked to an EU comprehensive registry (EUReMS), need to be developed in order to measure the prevalence of MS across countries and to assess the status of people with MS. It is also important that clinical guidelines are kept up to date and, more importantly, that they are actually used in practice.

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3 Multiple Sclerosis Therapies in Pediatric Patients: Challenges and Opportunities

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Abstract: Multiple sclerosis (MS) is an autoimmune, chronic, inflammatory, and demyelinating disease of the central nervous system (CNS). The etiology of MS is most likely multifactorial; it is dependent on genetic, autoimmune, and environmental factors, with a variable course among patients. The two main clinical events that characterize MS are relapses and progression. In recent years, diagnosis and treatment of pediatric MS has drawn attention of the scientific community. Management of pediatric MS focuses on reducing relapses and symptoms via administration of disease-modifying drugs (DMDs) and specific symptomatic treatment. A multidisciplinary approach to pediatric MS treatment is preferred, which aims at alleviating and preventing the accumulation of neurological deficits. MS therapy should be based on DMDs, that is, immunomodulatory drugs. These drugs, which sequester immune system activity, are further subdivided into two categories: first-line and second-line immunomodulatory therapy. First-line immunomodulatory therapy (interferon beta-1a, interferon beta-1b,

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and glatiramer acetate) is ineffective (either no response or partial response) in roughly 30% of patients. Patients with a poor response to first-line therapy require second-line immunomodulatory therapy (natalizumab, mitoxantrone, fingolimod, teriflunomide, azathioprine, rituximab, dimethyl fumarate, daclizumab, alemtuzumab, and ocrelizumab). In addition to immunomodulatory drugs, treatment of relapses also involves the use of high intravenous doses of corticosteroids, administration of intravenous immunoglobulins, and plasmapheresis.

Key words: Etiology; Immunomodulatory therapy; Multiple sclerosis; Pediatrics; Therapy

Introduction

Multiple sclerosis (MS) is an autoimmune, chronic, inflammatory, and demyelinating disease of the central nervous system (CNS) (1). The onset of MS occurs predominantly between the second and the fourth decade of life, but diagnosis in those older than 50, as well as in children, albeit less frequent, has also been observed. In the 19th century, Prof. Jean-Martin Charcot provided the first pathological and clinical description of MS, labeling it *sclerose en plaques* (2). The subsequent decades witnessed extensive etiological, pathophysiological, and pharmacological studies regarding MS, from the discovery of its genetic basis to the implementation of immunomodulatory therapy (3–5). In recent years, diagnosis and treatment of pediatric MS has drawn attention of the scientific community (6). The clinical characteristics, laboratory analyses, and neuroimaging techniques may significantly differ in children versus adults (7), whereas an individual approach remains crucial for the early diagnosis, as well as for the treatment of pediatric MS.

Although the exact etiology is still unknown, MS is most likely a multifactorial disease; it is dependent on genetic, autoimmune, and environmental factors (8). More than 200 genes may play a role in the occurrence of MS, with changes in the human leukocyte antigen (HLA) DRB 1 gene most likely playing the most significant role in initiation (9, 10). Besides genetic factors, the etiopathogenesis of MS may be also associated with an altered immunological response during the Epstein–Barr virus infection, decreased vitamin D levels, and smoking (11–13). Although, some authors reported a link between childhood obesity and MS, this correlation has not been fully clarified; however, the authors believe that this is due to the low levels of vitamin D, since most of the vitamin D is deposited in the adipose tissue (14). Childhood obesity can also increase the risk of MS, independently of vitamin D levels. Low levels of serum vitamin D in mothers, during early stages of pregnancy, can also lead to an increased risk of MS in progeny (14). The consequential production of proinflammatory cytokines during the altered immunological response damages oligodendrocytes and myelin, causing plaques of inflammatory demyelination (15). Moreover, some studies have shown that pediatric patients with MS have 50% higher extent of acute axonal damage compared with adult patients (16). Epidemiological studies show that almost 50% of patients with pediatric and adult MS are from Europe (17). Studies have shown that there are areas with

higher prevalence of MS in the world, such as North America and certain countries in northern Europe (17, 18). The Orkney Islands represent an area that has the highest prevalence of MS, with 300 patients per 100,000 citizens (19), but some studies have also pointed out that Sardinia has the highest prevalence of pediatric MS (20). If we look at the American continent, the rise of African Americans with pediatric MS is noticeable, but still MS is most commonly seen in non-Hispanic white individuals (21).

Clinical Characteristics: Children versus Adults

Although with a variable course among patients, there are two main clinical features that characterize all forms of MS: progression and relapse (22). Progression is characterized by a 6-month period of continuous deterioration in neurological status, while relapse is defined as the occurrence or aggravation of neurological symptoms lasting for more than 24 h (23, 24). These attacks should be separated by at least 30 days in order to be considered a relapse. Normal neurological status is often present during the days between attacks, with some sequelae possible. Pediatric MS is usually diagnosed around 15 years of age (25). The sex ratio varies depending on age (male to female ratio 4:5 at early onset; up to 1:2 after the age of 10), which could indicate the role of sex hormones in its pathogenesis (7, 26). Finally, 6–20% of pediatric patients possess a positive family history for MS (3).

The first attack of neurological symptoms, known as clinically isolated syndrome (CIS), lasts longer than 24 h and is characterized as inflammatory demyelination without encephalopathy (27). According to literature, there is a 30–75% chance of a CIS progressing to MS (28, 29). For the pediatric population, acquired demyelinating syndromes were first classified in 2007 (30), and later updated in 2013 (23). Similar to CIS, radiological isolated syndrome (RIS) has been described in recent years. RIS represents the MRI findings associated with demyelinating diseases. However, a strong correlation between RIS and the development of MS lacks, with approximately 20% of patients with RIS developing MS within the next 5 years (31). Over time, MS eventually leads to significant brain atrophy and thereby loss of brain volume. Global and regional brain atrophy develops gradually in the adult population (32). This is in contrast to pediatric MS, where regional brain atrophy is dominant (33), causing significant cognitive and physical disabilities (34).

The relapsing–remittent (RR) form is most common among children (more than 85% of all patients) (6, 35). Patients with RR MS have no increased risk of advancement to the secondary progressive form despite the growth of the degree of disability (36). Recurrence rates in the pediatric population are higher in the first 3 years than in adults (6). However, the recovery period following a relapse is much shorter in children (1). Long-term disability is slower in pediatric population, but these patients will be more disabled compared to adult-onset MS at a younger age, because of the earlier onset of the disease (37). Furthermore, up-to-date diagnostic techniques have allowed for a much earlier detection of the disease (38). Differential diagnosis should be performed in order to rule out other possible causes with similar clinical signs and symptoms (1, 39, 40).

The revised McDonald's diagnostic criteria are a universally approved scheme for MS diagnosis. Consensus regarding diagnostic criteria for pediatric MS and

related disorders was published in 2007 (30) and most recently updated in 2013 (23). According to Krupp et al., the following criteria should be met prior to the diagnosis of pediatric MS (23, 41). Finally, MRI represents a highly sensitive method for judging disease activity in both adults and children. Children tend to show multiple lesions surrounding the cerebellum and brainstem, in comparison with adults (42). MRI findings with more pronounced lesions are often correlated with increased severity of disability (26).

Treatment of Pediatric MS

Similar to adult therapy, pediatric MS focuses on reducing relapses and symptoms via disease-modifying and symptomatic treatment. Children, however, differ from adults in many physiological and developmental issues, resulting in significant discrepancy for drug efficacy and safety, as well as treatment response. The altered immunomodulatory treatment response in MS may be significantly affected by higher level of CNS inflammation and the differences in neurological damage intensity, restorative capacity, and plasticity (43), as well as by the different immunopathobiological mechanism in children versus adults (6).

IMMUNOMODULATORY THERAPY

MS therapy should be based on disease-modifying drugs (DMDs), that is, immunomodulatory drugs. These drugs are further subdivided into two categories: first-line and second-line immunomodulatory therapy (Figure 1). Current guidelines suggest DMD therapy be also given to pediatric patients, as close to the onset of disease as possible (44). No evident disease activity (NEDA) is the main goal of immunomodulatory therapy, that is, to reduce the number of relapses and disease activity on MRI. At this moment, it is difficult to achieve this in the pediatric population with MS because of the current availability of therapy in the pediatric population (37).

FIRST-LINE IMMUNOMODULATORY THERAPY

Immunomodulatory drugs significantly reduce the frequency and severity of clinical relapses and disease activity, as well as the degree of disability. These drugs, which have been approved by the European Medicines Agency (EMA), are given either intramuscularly (i.m.) or subcutaneously (s.c.) and are generally well tolerated. However, due to their parenteral route of administration, difficulties may arise in pediatric patients (6, 45, 46). Immunomodulatory therapy is a preferred therapy for adults and children older than 12 years of age. Common drugs in this class include interferon beta-1a (Rebif®, Avonex®), interferon beta-1b (Betaferon®), and glatiramer acetate (Copaxone®). Rebif® is given s.c. three times a week in a dose between 22 and 44 μg, whereas Avonex® is given i.m. once a week in a dose of 30 μg. Interferon beta-1b and glatiramer acetate are both given s.c. every other day, at doses of 250 μg and 20 mg, respectively (45). This class of medication reduces relapses in adults by as much as 30% (6, 40). These drugs, with anti-inflammatory and immunomodulatory effects, significantly reduce the

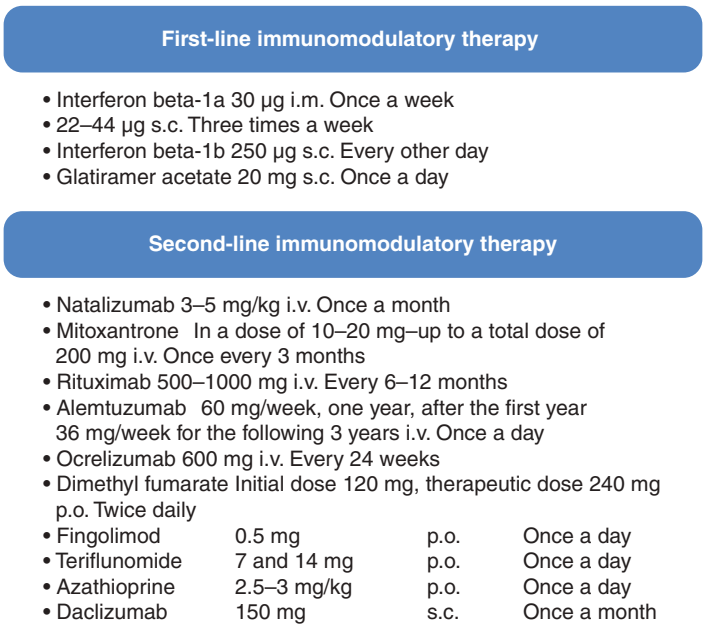

Figure 1 **First-line and second-line immunomodulatory treatment.**

frequency and severity of clinical relapses and disease activity, as shown by MRI of the brain, as well as reduce the degree of disability (39). Results for interferon beta-1a application in young children (aged 2–11 years) versus adolescents (aged 12–17 years) have shown that the safety profile is similar. Younger patients only had increased levels of liver enzymes (47).

Interferons are cytokines crucial for immunoregulation signaling cascades. Their effects range from reduction of lymphocyte cytokines, inhibition of autoreactive T-cells, and induction of anti-inflammatory mediators (6). Interferon beta-1a and beta-1b are DMDs used in MS therapy. Side effects of interferon class medication, based on published findings, include skin reaction at site of injection (more common in s.c. administration than in i.m.), headache, flu-like symptoms, nausea, fatigue, myalgia, anemia, lymphopenia, neutropenia, thyroid dysfunction, allergic reactions (drug eruption, rash, urticaria, and anaphylaxis), epilepsy and convulsive disorder, autoimmune diseases, cartilage and bone disorders, serious infections, and elevated liver enzymes (44, 45). Ibuprofen or paracetamol (acetaminophen) is the therapy of choice for those patients with flu-like symptoms. Monthly liver function tests are necessary during the first 6 months of interferon therapy, followed by once every 3 months until the course is complete. Thyroid function should also be assessed —one to two times per year while on interferon therapy (48).

Glatiramer acetate inhibits effector T-cells and regulates antigen-presenting cells (APCs) and suppressor T-lymphocytes (6). It is a generally well-tolerated immunomodulatory drug and a good option for long-term use (45). In terms of adverse effects of glatiramer acetate use, up-to-date pharmacovigilance studies

are scarce. Available studies suggest that glatiramer acetate may cause a transient flushing-like reaction accompanied by tachycardia (48). Pediatric patients on DMD therapy need to be followed to assess the efficacy and safety of therapy. Their assessment should be performed on MRI every 6–12 months followed by laboratory analyses (blood cell count, kidney function, and liver function) (47).

SECOND-LINE IMMUNOMODULATORY THERAPY

Around 30% of patients are partially responsive or nonresponsive to first-line therapy, requiring second-line immunomodulatory therapy (49). The current recommendation involves switching of these patients to natalizumab or other treatments although these drugs have not been evaluated in children.

Natalizumab (Tysabri®) is a monoclonal antibody that targets α4β1-integrin, a protein located on most leukocytes, and renders the blood–brain barrier (BBB) impermeable to T-lymphocytes and B-lymphocytes (2). It is given as an intravenous (i.v.) infusion once a month in a dose of 300 mg (50) or 3–5 mg/kg (6). Natalizumab has been shown to reduce the activity of MS and its progression in adult patients. Although currently contraindicated for pediatric use, clinical trials have shown that natalizumab decreases disease activity with fewer side effects in pediatric cases as well (51). Natalizumab reduces relapses by 68% (50) and reduces the number of new T2 lesions on MRI compared to placebo by up to 83% (37). However, it has a high risk of serious side effects, such as progressive multifocal leukoencephalopathy (PML), which can lead to serious disability, hypersensitivity, and infections (6, 49, 51, 52). Prior to beginning natalizumab therapy, it is important to perform JC virus serological testing, as well as secondary testing 3–6 months after in seronegative patients (51). If the patient shows any signs of PML, therapy should be stopped immediately.

Mitoxantrone (Novantrone®) reduces the proliferation of lymphocytes (both T and B). It is administered as a single dose of 10–20 mg (maximal dose of 200 mg) through intravenous infusion once every 3 months (50). Mitoxantrone is generally reserved for patients with severe cases of relapse remitting MS or secondary progressive course of disease (53). This drug should be used with caution as it has high rates of adverse reaction (53). The most common adverse effects of mitoxantrone are cardiotoxicity, risk of cardiomyopathy, leukopenia, nausea, infections, alopecia, fatigue, and amenorrhea (37, 50, 53). There have also been reports of increased risk of colon cancer associated with mitoxantrone (54). Due to the increased risk of cardiotoxicity, it is imperative for patients to undergo frequent echocardiograms, as well as subsequent cardiological tests.

Fingolimod (Gylenia®) tablets (0.5 mg) are taken once daily orally, making it a much easier therapeutic option for patients. The Federal Drug Administration (FDA) approved fingolimid as a first-line therapy for MS, while the EMA has it currently as second line. This drug targets the sphingosine-1-phosphate receptor, preventing the migration of lymphocytes from lymph glands, subsequently reducing the number of lymphocytes in the CNS (6). The efficacy of fingolimod is not only considered to be higher than the other first-line drugs but it is also associated with serious adverse effects, such as abnormal heart rhythm (especially bradycardia) after the first dose of the drug, macular edema, lymphopenia and a rise in hepatic enzymes, malignant tumor proliferation and infections (varicella infections, herpetic infections), and PML (37, 55).

Teriflunomide (Aubagio®) tablets (7 and 14 mg) are also administered orally once a day for the treatment of RR forms of MS. Its mechanism of action is the reversible inhibition of dihydroorotate dehydrogenase, thus affecting T-cell and B-cell proliferation (37). This drug is fairly safe, with common side effects such as hepatotoxicity and alopecia (6).

Azathioprine, as an immunosuppressive drug used in adults, antagonizes purine metabolism. Azathioprine is given orally in a dose of 2.5–3 mg/kg/day, and the most common adverse effects include gastrointestinal disturbances, skin rashes, liver toxicity, and cytopenia (50). Cyclophosphamide also represents an immunosuppressive drug with potent cytotoxic effects. In aggressive forms of MS, cyclophosphamide significantly reduced relapse of disease and MRI activity (37, 56). The most common adverse effects include vomiting, transient alopecia, amenorrhea, and osteoporosis, necessitating regular patient follow-ups in order to prevent the development of amenorrhea, sterility, and malignancies, such as bladder cancer and leukemia (6, 37, 50).

Rituximab (Rituxan®) represents a chimeric monoclonal immunoglobulin G1 (IgG1)—kappa antibody that targets the CD20 receptor on activated B-lymphocytes. Rituximab may reduce relapses and MRI activity in MS and Neuromyelitis optica (NMO) in adolescents (37); however, there are only few studies on the use of rituximab in pediatric patients with MS so far (57).

Dimethyl fumarate (Tecfidera®) is administered orally using a dose of 120 mg/240 mg in patients with relapsing forms of MS (58). Although not fully understood, dimethyl fumarate may reduce cytokine production and lymphocyte count, resulting in a decrease in immune cells migratory activity through the BBB (59). Its active metabolite is monomethyl fumarate and the most common adverse effects include itching and redness, nausea and vomiting, abdominal pain and diarrhea, lymphopenia, PML, vision problems, and hypersensitivity reactions (60).

Daclizumab (Zinbryta®) is given s.c. once a month in a dose of 150 mg. It represents a monoclonal humanized antibody that selectively binds to the IL-2 receptor alpha-chain. Daclizumab decreases relapse rate and the incidence of new lesions on MRI (61, 62). The most common adverse effects include serious infections, gastrointestinal disturbances, depression, liver toxicity with an elevation of liver enzymes, and serious cutaneous events. There is only one clinical trial, consisting of seven patients, on daclizumab in children with MS so far (61). It reduced the clinical and MRI disease activity in pediatric patients, while the side effects were mild (61, 62).

Alemtuzumab (Lemtrada®) is administered i.v. with a specific dosage regime. First-time treatment consists of 12 mg/day for the first 5 days (60 mg/week), which is continued for 1 year. After the first year, the patient should receive 12 mg/day for 3 days (36 mg/week) for the following 3 years. Alemtuzumab is a human monoclonal antibody against CD52, which binds to the surface of CD4+ and CD8+ cells, B cells, and monocytes. Its highest efficacy is seen during the active inflammation stage of MS. Alemtuzumab has similar efficacy to natalizumab in patients with RR MS. It is also more efficient in lowering the number of relapses in patients receiving fingolimod and interferon beta (63). For now, a higher risk of infection has been associated with alemtuzumab therapy compared with those receiving interferon beta. The most common adverse effects are infusion reactions (headache, swelling, fever, nausea, urticaria, and fatigue), which are most likely

due to cytokine release after cellular lysis (64). Due to the risk of infusion reactions, it is imperative to monitor patients receiving alemtuzumab infusion therapy very closely, especially 2–3 h post-infusion (64, 65). Furthermore, the same studies have shown idiopathic thrombocytopenia purpura and autoimmune nephropathy as possible adverse effects (64). Thus far, no studies regarding alemtuzumab's efficacy in pediatric MS patients have been published.

Ocrelizumab (Ocrevus®) is a monoclonal antibody with selective affinity for CD20+ B cells. It is given at a dose of 600 mg i.v. every 24 weeks. It is approved by the FDA for use in RR and primary progressive MS patients. This is the first medication that is approved for adults with primary progressive MS. Studies (OPERA I and OPERA II) show that ocrelizumab lowers relapses by an additional 46–47% in comparison with interferon beta-1a therapy (66). Therapy has also shown lowering progressive disability up to 40% as measured by the Expanded Disability Status Scale (EDSS). Furthermore, ocrelizumab also lowers brain atrophy visible via MRI (66). The most common side effects of therapy are infections, infusion reactions, and increased risk of tumor (66, 67).

TREATMENT OF RELAPSES AND SPECIFIC SYMPTOMS

The aim of MS therapy is to alleviate and prevent the accumulation of neurological deficits (68). During a relapse, it is crucial to quickly and efficiently assess the clinical status and begin appropriate therapy (69). High doses (20–30 mg/kg, max 1000 mg/day) of i.v. corticosteroids (methylprednisolone) are recommended once a day, preferably in the morning, alongside gastroprotective medication. Short courses of high-dose corticosteroid treatment reduce side effects of systemic corticosteroid use. Side effects in children include mood disorders, insomnia, hypertension, arrhythmias, facial erythema, higher appetite and body mass, acne, hyperglycemia, and gastric ulcerations (necessitates the use of concomitant gastroprotective agents) (7, 68). Before introduction of corticosteroids, it is necessary to educate the parents and patients about all of the side effects. If even after the completion of i.v. corticosteroid therapy full recovery is not attained, oral prednisone at a dose of 1 mg/kg daily (max dose 60 mg/day) can be initiated (69). If corticosteroid therapy results in little or no improvement in clinical picture, or a deterioration in the patient's condition, a 5-day course of i.v. immunoglobulins at 0.4 g/kg/day can be administered. Another option for patients unresponsive to conventional relapse therapy, or for those patients suffering from rapid progressive disease, is plasmapheresis (1, 69). In severe cases, patients may arrive in a life-threatening condition, wherein primary concern should be the establishment of proper airway and circulatory function (69).

Symptomatic therapy should be directed toward eliminating specific symptoms. The most common symptoms that occur in children are pain, depression, anxiety, fatigue, stiffness, interference with urination, and sexual dysfunction. Adequate and effective symptomatic therapy has a positive effect on the quality of life of pediatric patients with MS. Pain associated with MS should be treated according to the algorithm for neuropathic pain therapy, namely, tricyclic antidepressants, gabapentin doses of 600 mg/day, pregabalin, 5% lidocaine, and tramadol (62, 70). Fatigue is a common symptom in MS, occurring in about 76% of cases (62). Patients who complain of fatigue should be advised to have enough rest, as well as adequate physical activity on a weekly basis. Spasticity in pediatric

cases of MS is most often treated with baclofen or diazepam, botulinum toxin-A, or intense physical therapy (62). Baclofen, a GABA-B agonist, is started at 5–10 mg 3 times a day orally (58). The most common side effects of baclofen therapy are fatigue, seizures, constipation, nausea and vomiting, hallucinations, and hyperthermia (52, 62). Botulinum toxin-A is given at 15–22 U/kg i.m. in children less than 45 kg or 800–12,000 U/kg i.m. in children over 45 kg, every 3–6 months (52).

Current Therapeutic Strategies and Future Directions

The standard first-line therapy of pediatric MS uses different forms of interferon-beta or glatiramer acetate; however, around 30% of pediatric patients with MS discontinue therapy due to side effects, toxicity, persisting relapses, and intolerance or nonadherence. This supports the clear need for new therapeutic strategies. According to the International Pediatric Multiple Sclerosis Study Group (IPMSSG, 71) recommendations, the patients should start first-line immunotherapy (interferon-beta or glatiramer acetate) soon after diagnosis. Patients with poor tolerability or adverse events can be offered to switch the first-line therapy to glatiramer acetate if previously treated with interferon-beta or vice versa. However, these therapies are only partially effective and certain patients may fail to respond. Escalation strategies have demonstrated their benefit in other autoimmune disorders and may also prove to be beneficial in MS. Switching to a second-line therapy should be considered for those patients who do not adequately respond to first-line treatment. The current recommendation involves switching patients to natalizumab or other treatments although these drugs have not been evaluated in children. As in other autoimmune disorders, we need to consider induction therapy at onset. Thus, for patients with severe disease activity at onset, induction therapy with a potent immunosuppressant agent followed by maintenance treatment with interferon-beta or glatiramer acetate may be appropriate.

THE PERSPECTIVE OF DRUG DEVELOPMENT FOR PEDIATRIC MS

According to reference data, there have been no formative clinical drug trials specifically targeting therapy for pediatric MS (72). This is quite unfortunate, considering the vast number of new medications that are becoming available for MS treatment and the incentives available for pharmaceutical agencies willing to undertake pediatric trials. Reasons for the lack of clinical research trials for children could be due to the specific regulations regarding pediatric clinical trials, the off-label use of immunomodulatory medication due to lack of safety and pharmacokinetic data in children, and the number of pediatric patients available for clinic research enrollment.

When conducting future pediatric clinical trials, similar measures as those used in adult trials should be implemented (73). These metrics include relapse rate, time to relapse, and clinical disability with supportive MRI markings. However, there are several additional outcome measures specific for the pediatric population which would be important to incorporate into future clinical trials (74). Quality of life scales would be very important secondary measures in pediatric populations. In addition, cognitive tests are essential, as pediatric MS

has been shown to interfere with cognitive maturation in close to one-third of the children (75). New methods for measuring disability would have to be adjusted in pediatric cases, since most children do not present with measurable physical disability within the first 10 years of the disease. Furthermore, several changes to clinical trial design have been suggested in order to make it more accessible for pediatrics. Designing a trial that cuts down on the number of patients is essential, highlighting the importance of developing international multicentric research and clinical networks. Providing the most successful therapy could also be achieved by deferred treatment/partial crossover, unbalanced arms, and incorporating dose–response studies (72).

The latest study conducted on pediatric-onset MS (POMS) patients with CIS demonstrates the importance of early introduction of DMD on the natural course of the disease (76). This study demonstrated significant reduction in the risk of second attacks, as well as a significant reduction in the risk of worsening in the EDSS and disability rates, in patients who were treated with immunomodulatory therapy early, compared with untreated patients. Most pediatric MS patients experience a second attack between 0.3 and 2.2 years after the first event. In pediatric patients receiving early DMD therapy (before the second attack), there was a 25% reduction of worsening EDSS by the next follow-up. This study, for the first time, consistently supports the beneficial effect of an early DMD exposure in preventing the second attack in CIS and medium- to long-term disability accumulation in POMS.

Conclusion

Pediatric MS is still a challenging diagnostic and therapeutic issue. Advanced MRI techniques (e.g., magnestization transfer, diffusion tensor imaging, and functional MRI) will certainly provide crucial information including cortical involvement in POMS. Possibly they can further explain the different pathophysiological mechanisms of pediatric MS, providing predictive parameters and disease-activity monitoring during different therapeutic protocols (72). Until recently, there have been no randomized controlled clinical trials or safety studies in children with MS (78). According to the US and EU legislation, pediatric studies for new drugs are now required, which have resulted in a notable increase in pediatric studies in the last few years. FDA and EMA encourage a coordinated collaborative approach to product development as an important step toward a more effective product development for children. Nevertheless, the clinicians still have to continue to use new MS drugs in children off-label, since the regulatory authorities have so far not prioritized compounds for potential benefit in children with MS.

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Neuropathic Pain in Multiple Sclerosis—Current Therapeutic Intervention and Future Treatment Perspectives

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Abstract: Chronic pain is defined as any consistent pain lasting more than 12 weeks; chronic pain afflicts 25% of the world's population. The most common form of chronic pain is chronic neuropathic pain, which affects around 8% of the general population and is defined as pain that is initiated or caused by a primary lesion or dysfunction of the nervous system. Neuropathic pain is commonly associated with a variety of neurodegenerative, metabolic, and autoimmune diseases. In multiple sclerosis (MS), chronic neuropathic pain is one of the most frequent symptoms that dramatically reduces the quality of life of MS patients. Current treatment strategies include antidepressants, anticonvulsants, and cannabinoid drugs. However, the efficacy of these drugs varies between patients. Besides providing only insufficient relief of pain, these drugs also lead to severe side effects. Therefore, there is an unmet medical need to identify novel drug targets, which may lead to the development of novel therapeutics with enhanced tolerability profiles and efficacy for the management of MS-associated chronic neuropathic pain.

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Introduction

Pain is an unpleasant sensation that is often provoked by a noxious stimulus and can result in tissue damage. However, it also encourages a person to withdraw from damaging situations or to protect an injured body part while it heals and is therefore an essential component of the protective response of the human body. Pain is often a transient sensation that lasts until the noxious pain stimulus is detracted or the underlying damage or pathology has healed, but some forms of pain may become chronic lasting over months or years, even after the initial injury has healed. Different forms of pain can be classified by their underlying mechanism (1). Nociceptive pain is caused by a noxious stimulus, resulting in damage to body tissue and is usually described as a sharp, aching, or throbbing pain. Inflammatory pain occurs in response to the release of inflammatory mediators from injured tissue, for example, during autoimmune diseases such as arthritis or inflammatory bowel disease. The most common form of chronic pain is chronic neuropathic pain, which is defined as a chronic pain condition that is caused by a lesion or disease of the somatosensory nervous system that is not mediated via a noxious stimulus (2). Chronic neuropathic pain is frequently present in a large number of medical conditions and can result from a variety of injuries to the peripheral nervous system (PNS) or the central nervous system (CNS). Furthermore, chronic neuropathic pain may result as a consequence of a variety of conditions such as cancer, metabolic diseases, autoimmune disorders, and neurodegenerative diseases, including multiple sclerosis (MS). Often, patients with chronic neuropathic pain are more susceptible to pain and experience severe pain. These symptoms are termed "hyperalgesia," which is defined as an increased sensitivity to pain, and "allodynia," a condition wherein typically nonpainful stimuli lead to pain-sensation (3). Importantly, neuropathic pain is not only mediated by a sensory component but also comprises perception, cognition, and higher brain center processing, making it a dynamic multidimensional experience (4).

Neuropathic Pain

ETIOLOGY AND EPIDEMIOLOGY

Chronic pain has been defined as a pain lasting more than 12 weeks, and as irregular somatosensory processing in the PNS or CNS that is sustained beyond the normally expected time course relative to the stimulus (4). Due to its high prevalence, chronic pain is currently the most common human health problem, affecting more than 25% of the world's population, and is rising in incidence as the population ages (5). Chronic neuropathic pain affects around 8% of the general population (6) and is caused by many disparate sources such as cancer, autoimmune

TABLE 1 Overview of disease-associated chronic neuropathic pain

Disease	Prevalence of pain	Pain symptoms
Multiple sclerosis	50–86% (8, 9)	Extremity pain, trigeminal neuralgia, back pain, headaches
Parkinson's disease	40–60% (10, 11)	Musculoskeletal pain, dystonia, central neuropathic pain
Alzheimer's disease	~57% (12)	Musculoskeletal pain
Diabetes	64% (13)	Painful neuropathy, mixed pain symptoms
Cancer	~78% (direct tumor involvement) (14) ~90% of chemotherapy patients (15)	Plexopathies, Painful cranial neuralgias, sensory neuropathy

and metabolic diseases, and CNS injuries and neurodegenerative diseases (7), with prevalence ranging from 40 to 90% depending on the disease (8) (Table 1). Chronic neuropathic pain negatively affects a person's level of functioning and quality of life. Its resistance to available pain therapies means there is an unmet medical need for the development of more efficacious therapeutics for chronic neuropathic pain.

DIAGNOSIS

Physicians typically assess a patient's pain through medical history and conduct a physical exam, but beyond that tests are subjective (16). Historically, neuropathic pain has often been disregarded by physicians, and patients have been labeled as hypersensitive. However, recent research has shown that neuropathic pain can be the underlying cause of a variety of secondary symptoms that severely affect the quality of life of patients (4, 8). There is still a need for greater standardization by which physicians can diagnose neuropathic pain, but newly proposed screening questionnaires and diagnostic procedures such as quantitative sensory testing, pain-related evoked potentials, and skin biopsy have advanced the mechanistic approach to pain management, leading to the development of the so-called sensory profiles (17). Physical and neurological examinations are typically done to assess neuropathic pain, but there are no defined diagnostic guidelines that are universally used among physicians. Only recently, updated criteria were developed by which physicians can more effectively and universally diagnose neuropathic pain (18). These criteria are based on a three-level grading system. For the first level of assessing possible neuropathic pain, patients need to show a history of relevant neurological lesion or disease, and the pain distribution reported by the patient needs to be consistent with the suspected lesion or disease. The second level of certainty to diagnose possible neuropathic pain involves a physical examination of sensory function to ensure that pain is associated with sensory signs in the same neuroanatomically plausible distribution. The third level of certainty to establish definite neuropathic pain requires the use of diagnostic tests to confirm the disease or lesion of the somatosensory nervous system that explains the pain (18).

SYMPTOMS

Individuals that suffer from neuropathic pain exhibit stimulus-independent persistent pain that is characterized by abnormal sensations or hypersensitivity in the affected area and often is combined with, or is next to, areas of sensory deficits (19, 20). Patients often describe the pain as a burning and/or stabbing sensation (21). Neuropathic pain symptoms include tactile or thermal hypoesthesia (reduced sensation to nonpainful stimuli), hypoalgesia (reduced sensation to painful stimuli), loss of sensation, paraesthesia (abnormal sensations such as skin crawling or tingling), paroxysmal pain (e.g., shooting, electric shock-like sensations), spontaneous ongoing pain (not induced by stimulus like, for example, burning sensation), and evoked pain (i.e., stimulus-induced pain), the last of which includes hyperalgesia (increased sensitivity to painful stimuli) and allodynia (perception of innocuous/nonpainful stimuli as painful) (19, 20). In addition to sensations of pain, abnormal sensations have also been reported such as crawling, numbness, itching, and tingling (22). Furthermore, pain can be triggered by typically nonpainful stimuli such as being lightly touched and hot or cold temperatures (22). Secondary symptoms that commonly accompany neuropathic pain include depression, sleep disturbance, fatigue, and decreased physical and mental functioning (23, 24).

GENDER DIFFERENCES

Interestingly, women are affected more often by chronic pain than men (25). Certain chronic pain syndromes occur only in women, for example, endometriosis-related pain, vulvodynia, and menstrual pain (5). Furthermore, several chronic pain syndromes such as chronic fatigue syndrome, fibromyalgia, interstitial cystitis, temporomandibular disorder, headache, migraine, lower back pain and knee pain (mostly osteoarthritis) are more common in women (5). Similarly, chronic neuropathic pain is also more prevalent in females (26, 27), indicating that women are at a greater risk of developing neuropathic pain than men (8). The predominance of females with chronic pain might depend on several indications (5). First, women seek health care services more often than men for both painful and nonpainful disorders, and might be more willing to report pain than men, leading to a higher percentage of women represented in epidemiological studies (28). In addition, multiple reports suggest that pain levels within chronic pain conditions are increased in women compared to men (5). Altogether, these data suggest that women might be more susceptible to chronic pain, and/or have a lower pain tolerance, compared to men. Women may have an increased risk of developing conditions that feature pain as a syndrome, ultimately leading to higher percentages of women crossing the threshold at which the pain experienced rises to the level of a diagnosed “pain syndrome” (5).

AFFECTIVE DISORDER—DEPRESSION

Depression, one of the most common psychiatric disorders, is a mood disorder that causes a persistent feeling of sadness and loss of interest, along with at least four of the following symptoms for a duration of no less than 2 weeks: appetite/weight disturbance, sleep disturbance, psychomotor change, loss of energy,

worthlessness/guilt, concentration difficulties/indecisiveness, and/or thoughts of death or suicide (4, 29). Depression is a common comorbid psychiatric diagnosis encountered in patients diagnosed with chronic neuropathic pain and affects the majority (57%) of chronic neuropathic pain patients, thereby intensifying the patient's disability and impairment as well as the challenge of successful treatment (4). In the general population, depression ranges from 4 to 8% (4). In contrast, patients diagnosed with chronic pain have a two to five times increased risk of developing depression compared to the general population (30, 31). However, since pain and depression are often comorbid, the assessment of depression in the presence of pain is complicated due to shared features between the two syndromes, such as fatigue and sleep disturbance (32).

Multiple Sclerosis–Induced Neuropathic Pain

PATHOPHYSIOLOGY OF MS-ASSOCIATED PAIN

MS is a chronic inflammatory demyelinating disease of the CNS that leads to motor, sensory, and cognitive impairment, and is characterized by demyelinated lesions within the CNS (33). Chronic pain is one of the most frequent MS-associated symptoms that dramatically reduces the quality of life of MS patients and treatment options for chronic neuropathic pain are very limited and often not very effective (20, 34, 35). Estimates on the prevalence of pain in MS vary considerably depending on the population of patients sampled, the definition of MS-associated pain used, and the survey methods employed. Pain prevalence in MS ranges from 25–90% (8, 36, 37), depending upon the assessment protocols used and the definition of pain being applied (34). MS-induced chronic neuropathic pain is typically associated with significant MS-related disability and depression (38) and pain syndromes can be divided into primary pain caused directly by demyelination, neuroinflammation, and/or axonal damage in the CNS from disease, or into secondary pain due to an indirect consequence of the CNS lesion (8, 39). Interestingly, recent imaging studies showed that demyelinating lesions are most commonly reported in the brainstem and less commonly in the spinal cord. Further, most studies reported associations between the localization of lesions and pain (40). The clinical presentation of MS-associated pain can be categorized as stimulus-independent or dependent (41, 42). Whereas stimulus-independent pain includes persistent or paroxysmal pain, evoked pain is characterized by hyperalgesia and allodynia (41, 42).

MS patients can suffer from nociceptive pain, such as pain resulting from musculoskeletal problems, neuropathic pain, or a mixed nociceptive/neuropathic pain (e.g., tonic painful spasms or spasticity) (17). Chronic neuropathic pain is more persistent in nature and is one of the most commonly distressing symptoms experienced by patients even in the early stages of the disease (8, 43). MS patients can experience a wide range of neuropathic pain symptoms (Table 2). The most common MS-associated chronic neuropathic pain conditions are ongoing dysaesthetic pain in the lower extremities, paroxysmal pain, which can be divided into L'hermitte's phenomenon and trigeminal neuralgia, as well as thermal and mechanical sensory abnormalities (8, 17, 34). Other forms of neurogenic pain, including

TABLE 2 Overview of chronic neuropathic pain conditions in MS

Type of pain	Description	Prevalence
Dysaesthetic extremity pain	Burning, tingling, or aching predominately in lower extremities	12–28% (life-time prevalence)
Paroxysmal pain	L'hermitte's phenomenon—shock-like sensation traveling from the back toward the lower limbs	L'hermitte's phenomenon: 9–41%
	Trigeminal neuralgia—sudden, severe, brief stabbing reoccurring episodes of pain in one or more branches of the trigeminal nerve	Trigeminal neuralgia: 2–6.3%
Migraine	Long-lasting headaches, possibly due to brain lesions	34%
Spasticity pain	Excessive muscular work and mechanical muscle pain	<50%
Painful tonic spasms	Spasmodic muscle contractions, ischemic muscle pain	6–11%

migraine with or without aura and tension-type headache, seem to be more prevalent in MS patients than in the general population (44). Dysaesthetic extremity pain is often characterized as a continuous burning, tingling, or aching dysaesthesia, predominantly in the legs and feet that is often worse at night and can be exacerbated by physical activity (8, 34, 39). In patients with MS, dysaesthetic extremity pain is the most commonly reported type of neuropathic pain, having a prevalence of 12–28% (45, 46). Interestingly, MS patients with primary progressive or progressive-relapsing MS are more likely to suffer from dysaesthetic pain than patients with the relapsing-remitting disease form (45). L'hermitte's phenomenon is described as a transient, short-lasting paroxysmal electrical sensation that originates in the neck and spreads down to the lower limbs and is usually related to neck movement. Although this phenomenon is not exclusive to MS, it is frequently reported by patients with MS (45), with a prevalence ranging from 9 to 41% depending on the parameters of the study (47, 48). In most patients, the symptoms resolve within 4 to 6 weeks; however, they may recur occasionally, especially during MS exacerbations (48).

Trigeminal neuralgia (TN) is characterized by sudden, usually unilateral paroxysmal attacks of electric shock-like episodes of pain in specific facial or intraoral areas that affect one or more branches of the trigeminal nerve (49). The prevalence of trigeminal neuralgia in patients with MS ranges from 1 to 6.3%, corresponding roughly to 20 times the prevalence in the general population (8, 41, 50). Importantly, the incidence of MS-associated chronic pain is not correlated with disease severity (36). Further, several studies suggest that pain prevalence and severity are not strongly correlated with age,

physical functioning, disease duration, or disease course (36). However, pain prevalence and severity of MS were found to strongly correlate with reduced social functioning and mental health, and pain severity was found to be significantly related to anxiety and depression, predominantly in women (36). Interestingly, the pathophysiology of trigeminal neuralgia (TN) in MS patients differs from TN in the general population and specifically involves CNS demyelination (51). Recent analyses revealed unique, focal diffusivity changes along the fifth cranial nerve in MS TN patients compared to TN patients or healthy controls. These MS patient-specific diffusivity changes are likely due to MS plaques at the regions proximal to the main sensory nucleus (52).

SEX DIFFERENCES IN MULTIPLE SCLEROSIS AND ASSOCIATED PAIN

Females are more often affected with MS than men, a phenomenon shared with several other autoimmune diseases. The prevalence and incidence of MS is two-to-three fold higher in females, compared with males (33). Similar sex differences were found for MS-associated pain. Whereas female MS patients experienced more severe pain than females in the general population, no difference in pain severity was found between male MS patients and men in the general population (36). Another study also suggested a sex difference in pain prevalence among MS patients, showing a higher female-to-male ratio among MS patients with pain compared to MS patients without pain (53). In contrast, some newer studies did not detect sex differences for pain prevalence in MS (54, 55). Altogether, there is evidence for sex differences in MS-associated pain; however, this has not been sufficiently addressed compared to the general sex differences on pain and, therefore, gender-dependent pain prevalence is still controversially discussed.

Pathophysiological Insights from Experimental Autoimmune Encephalomyelitis Models

In contrast to the wealth of research on the pathophysiology of neuropathic pain induced by peripheral nerve injury, only a limited amount of research on the pathophysiology of central or MS-associated neuropathic pain is available. In the field of MS, the majority of research on pain makes use of the rodent models of experimental autoimmune encephalomyelitis (EAE). EAE animals share many features observed in MS patients, such as pattern of the clinical disease course, histopathological CNS lesions characterized by perivascular cuffs with mononuclear cell infiltration, gliosis, demyelination and axonal damage (56). Furthermore, EAE animals mirror a lot of the pain reactions occurring in humans (34) and similar to clinical administration, pain-like behaviors in EAE mice can be ameliorated by anticonvulsant and antidepressant drugs (34, 57).

NEURODEGENERATION AND DEMYELINATION

Neurodegeneration and demyelination are common hallmarks of both MS and EAE (58) and lead to distinct mechanisms that may cause central neuropathic pain.

A recent report shows that genetic ablation of oligodendrocytes rapidly triggers a pattern of sensory changes that lead to a nociceptive hypersensitivity phenotype that closely resembles central neuropathic pain. Interestingly, this occurred at a time point that preceded apparent demyelination and ataxia and coincided with early axonal pathology in the spinothalamic tract (59). This is in line with data showing that pain-like behaviors occur prior to infiltration of immune cells into the CNS and prior to the development of clinical motor signs in EAE rodents and human patients (57). Mechanistically, oligodendrocyte loss–dependent hyperalgesia and allodynia were not causally associated with microglial reaction or T-cell contributions, demonstrating that central neuropathic pain can be caused by oligodendrocyte death and axonal pathology in the absence of an innate or adaptive immune response (59).

INFLAMMATION AND REACTIVE GLIOSIS

Inflammatory cells and immune-like glial cells are important mediators of central sensitization and contribute to neuropathic pain symptoms (60). Interestingly, typical cellular substrates associated with pain processing and peripheral neuropathic pain, such as altered expression of sensory neuropeptides, do not appear to underlie changes in sensory function in EAE mice (57). In contrast, EAE mice showed a significant influx of T-cells and increased astrocyte and microglia/macrophage reactivity in the superficial dorsal horn of the spinal cord, an area associated with pain processing (57), suggesting that inflammation and reactive gliosis may be key mediators of allodynia in EAE animals. Indeed, activated glial cells can release pro-inflammatory cytokines, glutamate, and nitric oxide during reactive gliosis and may amplify neuronal hyperexcitability, leading to the development of neuropathic pain (60). In addition, pro-inflammatory cytokines were shown to play a pathogenic role in the development of neuropathic pain (61). Moreover, reactive gliosis and a significant increase in the expression of the inflammatory cytokines in the dorsal root ganglia of EAE animals correlates with the onset of neuropathic pain behaviors in EAE rodents (57). In line with the important role of inflammation for pain development, gene therapy with anti-inflammatory IL-10 in EAE animals improved motor and sensory function, prevented allodynia, and reduced glial activation in the lumbar spinal cord (62).

Pharmacological Management of Neuropathic Pain

MANAGEMENT OF MULTIPLE SCLEROSIS–RELATED NEUROPATHIC PAIN

Although some pain relief can be afforded by conventional pain medications, no current therapy provides more than 50% pain relief in the clinic and large randomized and controlled clinical trials for MS-associated chronic neuropathic pain are lacking (34). Therefore, management recommendations for neuropathic pain in MS (Figure 1) tend to be generally guided by findings in other diseases, for example, spinal cord injury–induced chronic neuropathic pain or peripheral neuropathic pain syndrome (45). Since the primary affected brain regions and

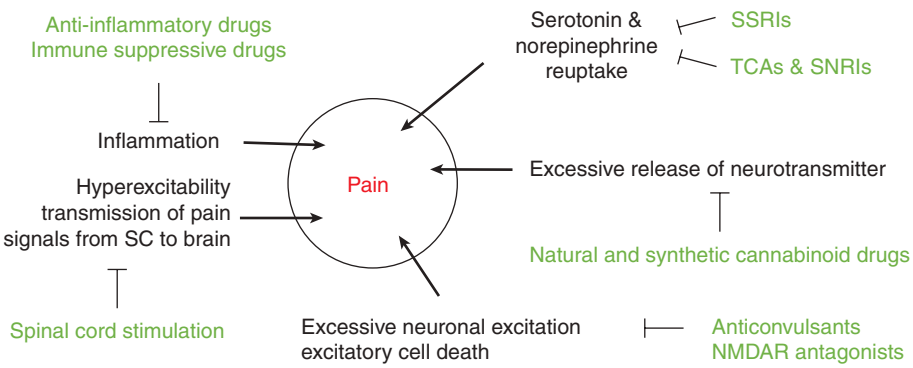

Figure 1 Disruption of MS pain signals by different treatments. Signals in the CNS (brain and spinal cord) and the PNS contribute to the development of neuropathic pain. Different therapies interfere with pain signals (black) and can lead to the alleviation of neuropathic pain. Antidepressants (TCAs, SNRIs, and SSRIs) inhibit the reuptake of serotonin alone or serotonin and norepinephrine, the key neurotransmitters that are hypothesized to be involved in the modulation of neuropathic pain. Natural and synthetic cannabinoid drugs prevent the excessive release of neurotransmitters in the CNS. Anticonvulsants and NMDAR antagonists block excessive neuronal excitation that may lead to excitatory cell death. Spinal cord stimulation blocks hyperexcitability of neurons in the spinal cord and prevents transmission of pain signals from the spinal cord to the brain. Recently, anti-inflammatory drugs were investigated that block inflammatory signaling associated with development of chronic pain.

neuromodulators are shared between chronic pain and depression, the same drugs often are used for both disorders (4). Temporary pain relief is often achieved through antidepressants and anticonvulsants. However, all these therapies have long-term complications and only a short-term efficacy that leaves patients with untreated and constant pain (4). Furthermore, in general chronic pain and in MS-associated chronic neuropathic pain in particular, the conventional analgesics only insufficiently relieve or do not relieve pain at all (8, 46). Adjuvant drugs such as the tricyclic antidepressants (TCAs), serotonin/norepinephrine reuptake inhibitors (SSRIs), and some anticonvulsants, for example, gabapentin or topical lidocaine are utilized as first-line drug therapy for alleviation of MS-associated neuropathic pain (34, 37, 46). Opioid analgesics (e.g., morphine, oxycodone, methadone, and fentanyl) and tramadol (alone or in combination with a first-line agent) are generally regarded as second-line treatments (34, 63, 64). Third-line agents that may be used as second-line treatments in some circumstances include other antiepileptic drugs (e.g., carbamazepine, lamotrigine, oxcarbazepine, topiramate, and valproic acid), mexiletine (orally active lignocaine analogue), and topical capsaicin (34, 63, 64).

ANTIDEPRESSANTS

Often antidepressants are used to treat pain; however, they differ in their efficacy. TCAs are the most studied and clinically used antidepressants for the treatment of neuropathic pain (65). They can be divided into two major groups: tertiary amines, for example, doxepin, imipramine, and amitriptyline, and secondary amines, for example, nortriptyline and desipramine (66). TCAs inhibit the reuptake of serotonin

and norepinephrine, the key neurotransmitters that are hypothesized to be involved in the modulation of neuropathic pain at the synapse, and block alpha adrenergic, serotonergic, histaminic, and muscarinic receptors at the synapse (32, 66). Their activity differs according to their chemical structure, whereas the tertiary amines raise serotonin levels to a greater degree than norepinephrine, the secondary amines have more pronounced effects on norepinephrine (32, 66). Interestingly, the therapeutic effects on pain seem to be independent of the antidepressant effects of these drugs and may be achieved at lower doses compared to clinically effective doses used to treat depression (32, 66). Despite the efficacy of TCAs in pain treatment, their use is limited due to pronounced side effects (e.g., weight gain, anticholinergic effects, orthostatic hypotension, and cardiovascular effects) and a high risk of overdosing, potentially leading to the death of patients (32, 66).

Next-generation drugs include SSRIs that exert their therapeutic efficacy mainly by the inhibition of the reuptake of serotonin (32). However, the use of SSRIs for the treatment of neuropathic pain seems to be less effective than other antidepressants and the number of clinical studies is limited (67). Paroxetine and citalopram, for example, showed just a modest activity for pain management, whereas fluoxetine had no therapeutic activity on pain at all (65). This leads to the assumption that noradrenaline reuptake inhibition is the major underlying mechanism of the analgesic efficacy of TCAs. Positively, the side effects of SSRIs are generally mild, for example, increased risk of weight gain or sexual dysfunction (32).

A therapeutic that inhibits both serotonin and norepinephrine reuptake (SNRI) is venlafaxine. Interestingly, low doses mainly impact serotonin and high doses mainly affect norepinephrine (32). Case reports and empirical studies indicate that venlafaxine can be clinically used to treat neuropathic pain, and its efficacy is comparable to TCAs (65). In general, venlafaxine use may lead to increased blood pressure and has a discontinuation syndrome with abrupt cessation. However, in general, it leads to less severe side effects, and its use is safer compared with TCAs (32). Duloxetine, the only antidepressant approved by the US Food and Drug Administration for the treatment of neuropathic pain, inhibits both SNRI and may cause side effects such as nausea, somnolence, dizziness, and fatigue (32). Interestingly, a randomized double-blind, placebo-controlled clinical trial of duloxetine, in patients with spinal cord injury–induced chronic neuropathic pain, showed that although duloxetine significantly improved allodynia relative to placebo, pain intensity was not significantly reduced compared with placebo (68). Therefore, the efficacy for the relief of MS-associated chronic neuropathic pain is still unclear.

ANTICONVULSANTS

Anticonvulsants or antiepileptic drugs normally suppress the rapid and excessive excitation of neurons during seizures. The efficacy of anticonvulsants, for example, lamotrigine, levetiracetam, topiramate, and gabapentin for MS-associated chronic neuropathic pain relief has been investigated in small clinical trials (69, 70). However, each of these studies showed that the anticonvulsants either led to an incomplete pain relief or that the drug had a limited tolerance and had to be discontinued due to intolerable adverse effects (34).

Carbamazepine is the most effective first-line treatment for MS-associated trigeminal neuralgia. However, due to its poor tolerance, with side effects including leg muscle weakness and micturition problems that can mimic MS relapses, treatment often has to be discontinued (34, 37, 71). Oxcarbazepine, the keto derivative of carbamazepine, has a similar therapeutic efficacy like carbamazepine for treatment of trigeminal neuralgia but shows an improved tolerance compared to carbamazepine (72). In addition, anticonvulsants are often recommended to treat relentless pain due to L'hermitte's phenomenon (46). However, neither drug effectively relieved persistent painful symptoms associated with MS (73).

CANNABINOID DRUGS

Natural or synthetic cannabinoid drugs, which inhibit the function of the endocannabinoid system involved in pain sensation, and alter neurotransmitter release in the CNS (74), demonstrated therapeutic efficacy in relieving MS-associated chronic neuropathic pain. However, several treatment-related side effects were observed, such as dizziness, dry mouth, headache, tiredness or muscle weakness (75). In addition, probable cannabis misuse and the risks of developing acute psychosis have sidelined these drugs to second-line or third-line medications to treat MS-associated chronic neuropathic pain (63).

NEUROSTIMULATION

A notable number of patients do not achieve sufficient pain relief with classical pharmacological medication alone. However, neurostimulation techniques, such as transcutaneous electrical nerve stimulation (TENS), peripheral nerve stimulation, nerve root stimulation (NRS), spinal cord stimulation (SCS), deep brain stimulation (DBS), epidural motor cortex stimulation (MCS), and repetitive transcranial magnetic stimulation (rTMS) show promise in treating chronic neuropathic pain (76). In particular, a recent case report and literature search showed that SCS, a stimulation method that directs mild electrical pulses to the spinal cord, and thereby inhibits pain transition from the spinal cord to the brain, was successfully used to alleviate MS-associated neuropathic pain (77). The exact mechanisms of SCS are not completely understood yet, but attenuated neuronal hyperexcitability was shown to contribute to its therapeutic effect (78).

CURRENT AND FUTURE DEVELOPMENTS

Next to conventional pain therapies using antidepressants and anticonvulsants, novel therapeutic approaches are currently being developed. Since MS is an inflammatory disease, most drugs used to treat MS-related motor symptoms target the inflammatory process. Interestingly, current research also identified the peripheral immune system as a relevant target for therapeutic intervention for pain. An important protein of peripheral inflammation is the mammalian target of rapamycin (mTOR), which has been implicated in behavioral hypersensitivity associated with neuropathy and pain (79). Administration of rapamycin, an

inhibitor of mTOR, not only reversed clinical signs of EAE motor disease but also ameliorated pain in EAE animals (80). Most likely, the therapeutic effect of rapamycin in EAE is dependent on its immunosuppressive activity involving inhibition of effector T-cells, expansion of regulatory T-cells, and inhibition of glial cell activation (80, 81) — all processes shown to contribute to the pathology of MS-associated chronic neuropathic pain. In line with this, anti-inflammatory cytokine gene therapy reduced EAE disease course and prevented mechanical allodynia (62). In addition, fingolimod, an immune suppressive drug that reduces MS relapse rates and lesion frequency (82), has been shown to promote pain alleviation in animals with peripheral nerve injury–mediated pain conditions (83).

Next to immunosuppressive therapies, glutamate receptors are promising targets for MS pain therapy. The N-methyl-D-Aspartate (NMDA) receptor has been proposed as a primary target for the treatment of neuropathic pain, and several clinical trials show beneficial effects of NMDA receptor antagonists on pain relief (84). Glutamate homeostasis is altered in MS patients, with higher levels of glutamate or altered glutamate uptake in the CNS of MS patients (85, 86). These excessive glutamate concentrations can allow prolongation of calcium-permeable ionotropic glutamate receptor activation on neural and glial cells, ultimately leading to excitotoxic CNS tissue damage (87). Similarly, dysregulation of the glutamatergic system, caused by reduced glutamate transporter expression in spinal cords, has been implicated in abnormal pain sensitivity in EAE mice (88). Furthermore, administration of drugs that promote glutamate transporter activity has not only been shown to limit and improve clinical motor symptoms but also to significantly alleviate pain and normalize performance in cognitive assays in EAE rodents (88).

Conclusion

Patients with MS develop, among other ailments, chronic neuropathic pain. Unfortunately, there is a lack of adequate controlled trials in MS patients to assess the efficacy of established pain-relieving agents. Hence, treatment recommendations for MS-related pain largely rely on experience from other diseases with associated neuropathic pain. Currently, the number of medications for the treatment of MS-mediated chronic neuropathic pain is limited, and their use is often associated with severe adverse evets. Therefore, there is an urgent medical need to identify novel drug targets which may lead to the development of therapeutics with improved tolerability, low toxicity, and enhanced efficacy for the management of MS-associated chronic neuropathic pain. Some promising targets are mTOR, glutamate receptors and NMDAR (N-methyl-D-Aspartate receptor).

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Conflict of interest: John R. Bethea and Roman Fischer are named inventors on patent applications covering novel techniques for the treatment of neuropathic pain.

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5 Vitamin D and Multiple Sclerosis: An Update

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Abstract: Multiple sclerosis (MS) is a long-lasting inflammatory neurodegenerative disease of the central nervous system caused by an inappropriate attack of the body's immune system on its own cells. To date, its etiology remains highly enigmatic, with insufficient evidence on the exact cause triggering the disease. Many studies have highlighted the role of different environmental and genetic factors in its etiopathogenesis, each adding a new wedge to MS conundrum and therefore making it a multifactorial and polygenic disease. One of the entrants in the risk factor category for MS is vitamin D, and there is sufficient evidence to suggest its role in increasing the risk of MS development. MS patients have lower levels of vitamin D, and in conjunction with other factors like low sunlight intensity and genetic variations in vitamin D metabolic pathway genes, vitamin D has been adjudged as a potent risk factor for MS. The biological effects of vitamin D in the body are mediated by the vitamin D receptor that acts as a transcription factor after activation by vitamin D and subsequent heterodimerization with the retinoid-X receptor. This allows regulation of protein expression of target genes involved in diverse cellular processes including immune response and vitamin D metabolism. It clearly suggests use of vitamin D supplementation as an unconventional option for MS treatment; however, much work needs to be done to precisely determine the level and/or dosage of vitamin D required for achieving optimum therapeutic response in patients without causing adverse effects.

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Key words: Deficiency; Exposure; Multiple sclerosis; Sunlight; Vitamin D

Introduction

Multiple sclerosis (MS) is a chronic multifactorial and polygenic autoimmune disease of the central nervous system (CNS), affecting predominantly young to middle-aged adults, especially females (1). It was Jean-Martin Charcot who described MS for the first time in 1868 (2). Escalating evidence has shown that it is the outcome of inappropriate immune response, characterized by auto-inflammation, making it a highly unpredictable disease (3). It is accompanied by a wide continuum of signs and symptoms which vary from person to person depending on the area of CNS damage (1, 3). Its epidemiology is variable across the globe, which indicates that MS etiology is governed by numerous geographic and environmental factors (4, 5). Presently, it is estimated that there are over 2.3 million people in the world living with MS, clearly indicating an increase in the number when compared to the 2008 estimate (6). A large body of epidemiological evidence supports the consensus view that it is a heterogeneous disease which results from complex interactions between susceptibility genes and one or more environmental factors during the course of growth and development of a person (1, 7–11). However, no single gene or environmental factor has been unambiguously identified as the causative agent, and it is likely that the cumulative effects of several genes and environmental factors lead to disease onset (12). To date, the exact cause of this debilitating neurological disease remains convoluted; however, significant attempts have been made to discover environmental agents associated with it (8).

Epidemiological and experimental data suggest low vitamin D levels to be associated with disease predisposition in cancer, schizophrenia, cardiovascular ailments, rheumatoid arthritis, and autoimmune diseases such as systemic lupus erythematosus, type 1 diabetes, and MS (13–17). The association between vitamin D and MS has become a burning issue across the globe and in the recent years there has been a tremendous increase in studies on the same (18, 19). The aim of this chapter is to explore the association between vitamin D deficiency and MS risk, and to present the latest knowledge and developments on the role of vitamin D as a risk factor for MS

Vitamin D and Its Biological Role

Vitamin D is a pro-hormone belonging to the category of fat-soluble group of vitamins. It is a secosteroid and is primarily responsible for maintaining calcium homeostasis by facilitating absorption and utilization of minerals; as a result, it acts as a major contributor toward bone formation and homeostasis (15, 20–24). The naturally occurring form of vitamin D is biologically inactive and requires hydroxylation in the liver and kidney for activation (25). It exists in two main forms in humans: D_2–ergocalciferol (plant derived) and D_3–cholecalciferol (animal derived) (25). Small quantities of vitamin D can be obtained from food; however, its primary source is generated by exposure

to sunlight (15, 25–27). Vitamin D in skin is present in the form of pro-vitamin D3 or 7-dehydrocholesterol and is converted to pre-vitamin D3 photochemically by ultraviolet B (UV-B) rays from the sun and later on converted to vitamin D3 by isomerization (23). This vitamin D3 from skin, food, or supplements is transported to liver by vitamin D–binding proteins (GC group-specific component), where it is converted to 25-hydroxyvitamin D3 (25(OH)D3) or calcidiol through the process of hydroxylation by one or more cytochrome P450 vitamin D 25-hydroxylases like vitamin D-25-hydroxylase (CYP2R1 cytochrome P450, family 2, subfamily R, member 1) (28, 29). In kidneys, 25(OH)D3 is further hydroxylated to 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) or calcitriol by 25-hydroxyvitamin D-1-alpha-hydroxylase (CYP27B1 cytochrome P450, family 27, subfamily B, member 1) (15, 29). The schematic pathway for vitamin D synthesis is given in Figure 1. The breakdown product of vitamin D is calcitroic acid which is generated through hydroxylation of 1,25(OH)2D3 by 1,25-dihydroxyvitamin D 24-hydroxylase (CYP24A1 cytochrome P450, family 24, subfamily A, member 1) (15, 30).

In humans, the most biologically active form of vitamin D is 1,25(OH)2D3; however, the vitamin D levels in the body are represented by 25(OH)D3 concentrations due to its longer half-life than 1,25(OH)2D3 (31). The optimal concentration of vitamin D in the body remains a perplexing issue and as a result there exist several definitions for defining vitamin D status of a person. Generally, vitamin D deficiency and insufficiency has been defined as a serum level of 25(OH) D3 <50 nmol/L or 52.5–72.5 nmol/L, respectively (32, 33). Vitamin D deficiency

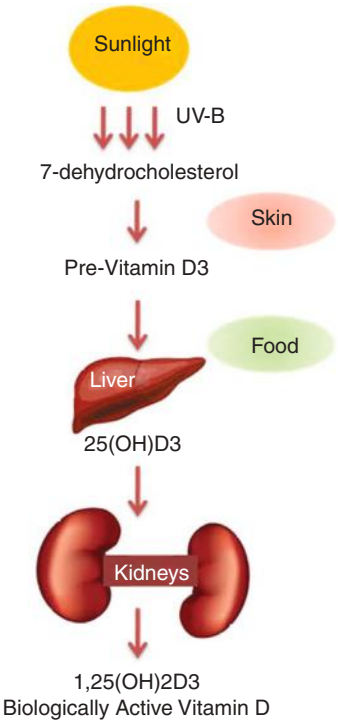

Figure 1 Biosynthetic pathway of vitamin D in humans. Vitamin D is synthesized in a series of events involving sunlight exposure and hydroxylation by liver and kidney enzymes.

is highly prevalent across the globe, affecting almost every population irrespective of age and gender (15, 17).

Vitamin D plays an essential role in innate and acquired immunity by acting as an immunomodulator regulating the production of type 1 and type 2 helper T-cell cytokines (Th1, Th2) (34), suggesting its key role in governing immune and inflammatory responses within the body (35). It plays a key role in several other processes like cellular growth, proliferation, differentiation, and apoptosis; DNA repair and oxidative stress; and membrane transport and adhesion (15, 22–24). Recent studies have proposed that its supportive role in immune response reflects its involvement in the prevention of various diseases including brain disorders and cancer (15, 33, 36, 37). The graphical representation of diverse roles played by vitamin D at the cellular level is shown in Figure 2.

The various biological responses of vitamin D are mediated through the vitamin D receptor (VDR) signaling due to its ubiquitous expression in immune cells as well as within CNS (38, 39). The binding of vitamin D (1, 25 (OH)2D3, calcitriol) to VDR and its subsequent activation leads to its heterodimerization with the retinoid-X receptor (RXR), resulting in modulation of vitamin D responsive gene expression by translocation of heterodimer complex (1, 25 (OH)2D3-VDR/RXR) to nucleus, and its recruitment on vitamin D response elements (VDRE) of target genes (24, 40). The schematic pathway for vitamin D–based signaling is given in Figure 3. Depending on the site of recruitment of VDR complex, it may result in induction of transcription at the promoter site or regulate expression at enhancer sites (41, 42). This allows for the regulation of protein expression of target vitamin D–sensitive genes involved in diverse cellular processes including immune response and vitamin D metabolism and therefore the outcome of this mechanism could be changed from pro-inflammatory to anti-inflammatory, thereby modulating the disease risk (38, 43). The direct manifestations of immunomodulatory effects of vitamin D are inhibition of Th1 cytokine production and Th17 cell differentiation, and stimulation of Th2 cytokines and T-regulatory cells, resulting in a shift in immune response (34).

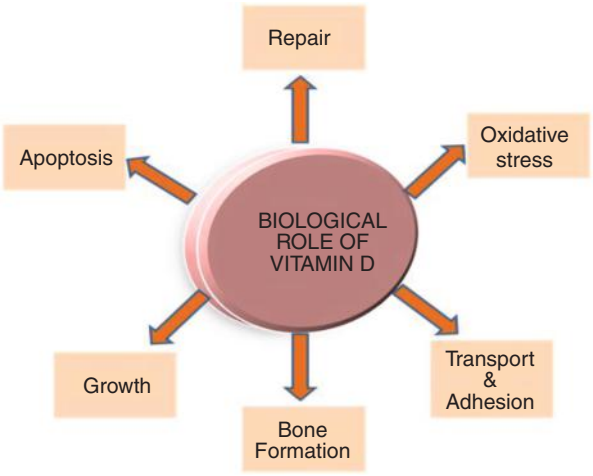

Figure 2 Pictorial representation of diverse roles played by vitamin D.

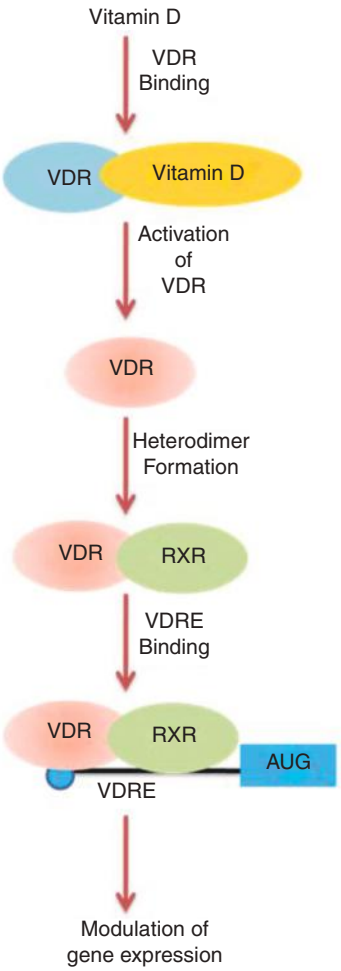

Figure 3 Vitamin D signaling pathway. The expression of vitamin D responsive genes is regulated via vitamin D receptor (VDR) through its activation by binding of vitamin D and then heterodimerization of activated VDR with retinoid-X receptor (RXR) followed by binding of heterodimer complex to vitamin D response elements (VDRE) present in genes.

Status of Vitamin D in MS

MS RISK AND VITAMIN D

The geographical distribution of MS is highly variable (4) and the causal factors known to play a role in its development are fusion of genetic and environmental components, thereby reflecting the role of epigenetics in its development (7, 44, 45). The pattern of its distribution across the globe is believed to be irregular with several exceptions; however, it shows higher prevalence in regions away from the equator (higher altitudes) where there is lower sunlight exposure (46, 47). A recent study has provided substantial evidence in support of latitude gradient shown by MS prevalence (48). Globally, vitamin D is low in general population and also in certain diseases including MS (17). The first report to suggest connection between

MS and sunlight was the one by Goldberg et al. (49). Several studies have suggested that reduced levels of vitamin D are associated with a higher risk of MS as serum levels of vitamin D have been found to be lower in patients than controls (50–56). Many studies have observed a correlation between season of birth and MS risk as is evident from the fact that there is lower sunlight intensity in winter when compared to summer, reflecting the possibility of an association between mother's exposure to sunlight during pregnancy, vitamin D levels or its dietary intake, and MS susceptibility (57).

Since the major source of vitamin D is sunlight-induced synthesis, it is evident that decreased sunlight exposure leads to reduced levels of vitamin D and thus higher MS risk (53–55). The decreased MS susceptibility has been linked to early sunlight exposure in life, especially during childhood and adolescence (50–52). The graphical representation of the link between sunlight, vitamin D, and MS risk is shown in Figure 4. Interestingly, migration studies have shown that MS risk changes with migration from one place to another; however, age at migration plays a key role in determining the disease risk of the migrant (58, 59). Recent studies have suggested an association between vitamin D levels and MS relapse rate as well as the degree of disability, and it was seen that patients with higher serum levels of vitamin D showed a lower relapse rate while lower levels of vitamin D appeared to be associated with higher levels of disability in patients measured in terms of expanded disability status scale (EDSS) score (60–63).

Although there has been a lot of research on vitamin D status and MS risk in adult-onset cases, there is lack of data on association with pediatric-onset MS (64–66). A recent meta-analysis based on Mendelian randomization has used instrumental variable analysis to provide evidence for causal and independent association between low vitamin D levels and increased body mass index (BMI) with the risk of developing pediatric MS (64). In addition, there is evidence suggesting vitamin D–based regulation of klotho and nuclear factor-erythroid-2-related factor 2 (Nrf2) signaling pathways to be responsible for MS development as they are believed to maintain calcium and redox homeostasis within the body (67) and as a result klotho and Nrf2 in conjunction with vitamin D (vitamin D-klotho-Nrf2) act as keepers of several cell signaling pathways including myelin synthesis pathway (68). Even though there is evidence suggesting the role of vitamin D as a potent environmental risk factor for MS, further studies are required to evaluate

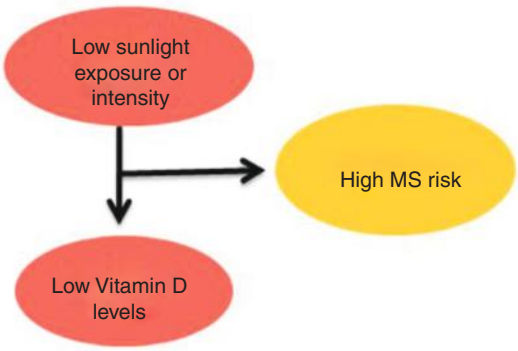

Figure 4 Pictorial representation of association between sun exposure, vitamin D, and MS risk.

precisely whether vitamin D status governs MS susceptibility independently or in combination with sun exposure. Furthermore, research to elucidate the duration and time of exposure and the role of other related epidemiological factors on MS susceptibility are warranted.

GENETICS OF VITAMIN D AND MS

Genetic link of vitamin D status in MS has long been hypothesized and several small-scale studies have been carried out to explore the association of polymorphisms in vitamin D–related genes with MS risk. The most consistent genetic regions found to be associated with the status of vitamin D in MS are vitamin D metabolism genes—CYP24A1, CYP27B1, and DBP/GC (encoding vitamin D–binding protein) (69, 70). It is anticipated that these genes may increase MS risk by modulating vitamin D metabolic pathway, thereby affecting vitamin D levels (70). The other crucial gene has been the vitamin D–based signaling gene VDR, particularly FokI, ApaI, TaqI, and BsmI variants, although a recent study has reported conflicting results (71, 72). A meta-analysis by Huang et al. provided evidence against their association (73). A recent investigation provided strong evidence for the role of VDR in the regulation of gene expression in immune cells of myeloid lineage which clearly indicates the importance of these genes in maintaining cellular tolerance (74). At the same time, it was observed that MS susceptibility loci including CYP27B1 and CYP24A1 showed high expression in myeloid cells, clearly reflecting the role of this interconnected regulatory pathway in therapeutic intervention of MS (74). In addition, it has been demonstrated that the main MS susceptibility governing genetic variant-major histocompatibility complex, class II region, DR beta 1 (HLA-DRB1) contains VDRE in its promoter region, which strongly suggests that their expression is governed by vitamin D (75). In fact, strong correlation has been observed between the increase in expression level of HLA-DRB1 and vitamin D, providing solid evidence for functional implication of vitamin D in MS (75). Several other genes implicated in predicting serum concentrations of vitamin D and subsequent risk of developing MS include NADSYN1 (nicotinamide adenine dinucleotide synthetase) and DHCR7 (7-dehydrocholesterol) (76). Moreover, several genes involved in MS predisposition are also regulated by vitamin D as predicted by *in silico* analysis, clearly signifying the role of vitamin D as a modulator of MS risk (77) (Figure 5).

Furthermore, a recent cross-sectional study by Laursen et al. showed the association between age at onset of MS and vitamin D–related genetic and environmental factors including GC, CYP2R1, CYP27B1, CYP24A1, and HLA-DRB1*1501 (78). Significant association was observed between younger age of MS onset and low sunlight exposure, higher BMI at the age of 20, and HLA-DRB1*1501, reflecting their independent effect on age at disease onset. Also, no association was found between age at onset and rest of the vitamin D–related genetic and environmental factors (78). Accordingly, vitamin D appears to be a potent environmental risk factor in MS, exerting its effect at the genetic level by interacting with genetic elements associated with MS. The concordance observed within genetic and epidemiological data clearly signifies the application of vitamin D supplementation as a promising treatment option for MS.

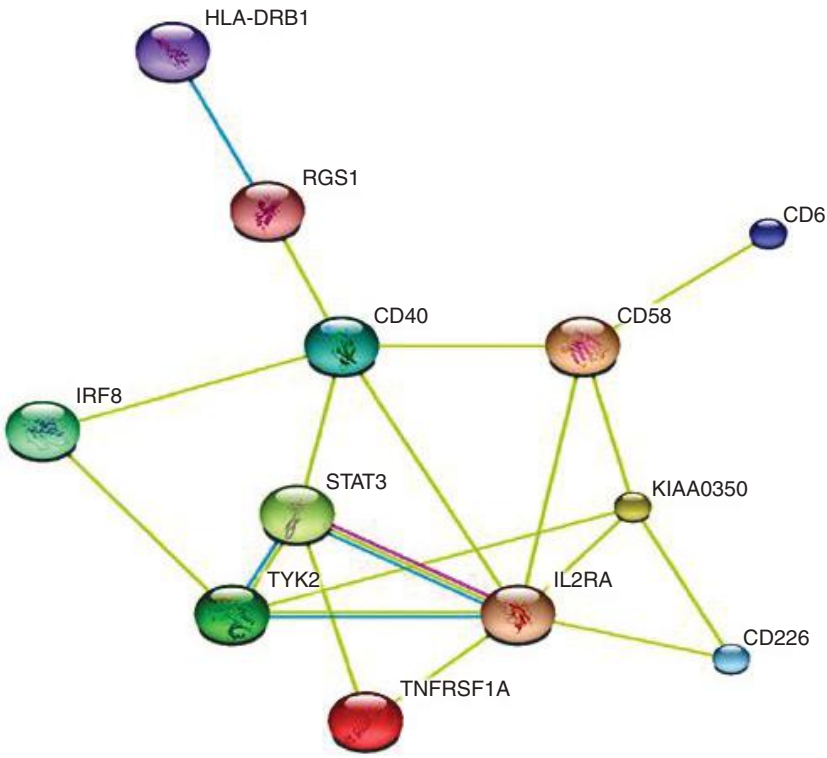

Figure 5 ***In silico*** **analysis depicting interactions between several vitamin D responsive MS candidate genes, confirming the effect of vitamin D on MS (http://www.stringdb.org).**

VITAMIN D AS A FUTURE TREATMENT OPTION FOR MS

There is compelling evidence to suggest that reduced risk of MS is associated with higher sunlight exposure and increased levels of vitamin D, thus suggesting a protective effect of vitamin D supplementation on MS (79). The current research based on large datasets is being targeted on using vitamin D supplementation as an alternative approach for MS treatment; however, there is still lack of convincing evidence for its effect on disease progression (80). The exact mechanism governing vitamin D–mediated regulation of immune response has to be completely elucidated for exploiting it as a future treatment option for MS. The experimental studies hitherto have suggested that the immune effects of vitamin D are not exerted at physiologic concentrations which results in hypercalcemia, reflecting an increase in calcium levels within the body (34). The previous studies based on low sample numbers have not been able to reveal convincing clinical effects of vitamin D in mitigating MS symptoms (81). Hence, there is lack of concrete evidence providing substantial support in using vitamin D intervention for MS management. At the same time, the outcome of

numerous genetic studies reiterate the fact that the studies based on vitamin D and MS should be conducted by considering the independent effect of different vitamin D–linked genetic and environmental factors on vitamin D levels within the body (82).

Keeping in mind the role of vitamin D as an immunomodulator and a risk factor for MS, its supplementation could be the most promising cost-effective treatment for MS in comparison with conventional disease-modifying therapy; thus, it could eventually prove beneficial for lowering MS burden across the globe. However, the major concerns that remain undetermined regarding its application are precise dosage, timing, response, and efficacy. Since MS is highly prevalent in women than men, it will be interesting to study the effect of gender on immunomodulatory response of vitamin D intervention. Also, keeping in view the role of genetic background of a person in determining treatment response, it becomes mandatory to conduct vitamin D–based randomized controlled trials to study the ultimate effects in different individuals with a particular genotype.

Conclusion

MS remains a mysterious disease posing several challenges for investigation; however, considerable progress has been made in unscrambling its etiology. Although there has been a remarkable progress in the research focusing on the role of vitamin D as a risk factor for MS, studies are warranted to explore the exact mechanism behind the impact of vitamin D levels on disease course, severity, and relapse. The precise effect of vitamin D on MS progression is yet to be determined. There is an urgent requirement for understanding the molecular mechanisms behind this association and exploring vitamin D supplementation as a future therapeutic option for MS. At the same time, increased attention should be given to establish the optimum levels of vitamin D that can be used for achieving desired clinical and immunomodulatory effects in MS patients with lesser adverse reactions of hypercalcemia.

Since vitamin D exerts its immunomodulatory effects through binding of VDR, cellular expression of VDR can be a crucial determinant for MS pathogenesis.Vitamin D, being the ligand of VDR, is highly dependent on environmental influences; thus VDR analysis provides an excellent possibility to investigate gene–environment interaction. Understanding how polymorphisms in vitamin D metabolic pathway genes can affect expression at mRNA as well as at protein level may help in delineating the role of vitamin D–based pathway behind MS risk, enabling therapies targeting vitamin D–based signaling pathway. Furthermore, it will help in defining the critical targets involved in vitamin D metabolism and its regulation. This will aid in revealing the clinical immunomodulatory application of vitamin D for MS patients, and provide the basis for using vitamin D supplementation as a future therapeutic alternative for MS management. In addition, this approach can also provide evidence as to whether vitamin D can serve as a reliable clinical marker for MS progression, degree of disability or severity, and for predicting the outcome of disease for better management.

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6 Stem Cell Therapy: A Promising Therapeutic Approach for Multiple Sclerosis

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Abstract: Multiple sclerosis (MS) is an inflammatory disease of the central nervous system which is accompanied by demyelination of the nerves, axonal loss, and disability. Currently, no definitive treatment is recognized for MS. Stem-cell therapy for MS has shown promising results and has attracted attention as an alternative therapeutic option. Various stem cell sources such as mesenchymal, embryonic, and neural have been identified. This chapter gives an overview of the advances made in our understanding of these stem cells under two broad categories: exogenous and endogenous. Stem-cell therapy in MS and the substantial literature regarding their

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therapeutic potential for MS are discussed. Much of the promising data are still in experimental stage, and further clinical trials are needed to rigorously evaluate the safety, validity, and feasibility of these stem cells for the treatment of MS.

Key words: Endogenous stem cells; Mesenchymal stem cells; Multiple sclerosis; Pluripotent stem cells; Stem-cell therapy.

Introduction

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system (CNS) and leads to demyelination of neural fibers, severe neurological symptoms, and progressive disability (1, 2). None of the currently available drugs are effective in supporting regeneration of the demyelinated areas, and preventing disease progression (2). Stem cells, because of their self-renewal and differentiation capacity into various cell types, appear to be suitable candidates for alternative therapeutic strategies for MS (3, 4). A wide variety of stem cells that have therapeutic potential in neurodegenerative diseases have been identified; these include, but are not limited to, mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), and neural stem cells (NSCs) (3–5).This chapter gives an overview of stem cells and their therapeutic potential for MS.

Exogenous Stem Cell Therapy in MS

BONE MARROW MESENCHYMAL STEM CELLS

Bone marrow mesenchymal stem cells (BMSCs) are multipotent stem cells that are derived from the bone marrow and have chondrogenic, osteogenic, and adipogenic differentiation capacities. They can also differentiate into neurons and glial cells (6, 7). The anti-inflammatory, low immunogenicity, and multipotency characteristics of BMSCs render them as a desirable cell source in regenerative medicine (6, 7). Unlike other source of stem cells, ethical concerns or tumorigenic activity is not a concern with BMSCs. They can be cultured and propagated easily *in vitro*, and autologous transplantation can be achieved without rejection (8, 9). BMSCs exhibit migration and homing ability into damaged parts of CNS. Transplantation of this cell population into damaged neural tissues leads to functional improvement via formation of glia and neurons that is identifiable at molecular and cellular levels (10–12). Furthermore, BMSCs have the ability to secrete many autocrine and/or paracrine factors that prevent apoptosis, and mediate neurogenesis and angiogenesis (13, 14). These neurotrophic and neuroprotective factors increase viability and proliferation of neuroglial cells and promote repair and recovery (15, 16). Several studies have confirmed the capacity of BMSCs to improve remyelination following experimental autoimmune encephalomyelitis (EAE) (17, 18). These results suggest that BMSCs are promising cell sources for functional recovery in MS patients. Auto transplantation of BMSCs in patients leads to significant recovery, and limits disability (19, 20).

The transplantation of differentiated BMSCs results in better glial cell engraftment than undifferentiated BMSCs. Transplantation of neuroglial progenitors derived from BMSCs enhances the homing and functional maturation rate of the cells (21, 22). Although the mechanisms that control neuroglial differentiation of BMSCs are not clearly understood, they can be differentiated into neuroglial phenotypes using growth factors, retinoic acid, and cytokines (23, 24). Recovery of the demyelinated areas and promotion of remyelination following transplantation of glial progenitors derived from BMSCs in animal MS models have been documented (25, 26). In experimental animal models, BMSCs have been shown to reduce immune attack to myelin sheets by suppressing T-lymphocyte proliferation (27, 28), diminishing inflammation and demyelination, inducing oligodendrogenesis (12), and improving remyelination (29) and tissue regeneration (10). Clinical trials suggest that BMSCs have the potential to reduce infiltration, decrease demyelinated areas, and improve axonal formation and functional recovery (30).

HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells are isolated from bone marrow and give rise to hematopoietic and lymphopoietic precursor cells, and lymphoid to myeloid lineage cells. Cell-therapy strategies based on engraftment of hematopoietic stem cells have been shown to result in neurological regeneration and repopulation of the immune system (31–35). In animal models, similar positive effects have been reported; however, controversial results also exist (36, 37). Engraftment of hematopoietic stem cells causes clinical improvement in MS patients (38, 39), and auto transplantation of hematopoietic stem cells show positive results in the management of progressive MS (40, 41). Some systematic reviews show that hematopoietic stem-cell therapy in patients with progressive MS leads to recovery of neurological function and prevents mortality of patients (42–45).

UMBILICAL CORD MSCS

Several studies have shown the therapeutic potential of human umbilical cord–derived mesenchymal stem cells (hUC-MSC) in MS patients. hUC-MSCs are promising candidate sources of MSCs that can be collected without pain. They have a faster self-renewal ability compared to other MSCs (46), and they differentiate into a variety of cell types such as bone, cartilage, adipose, muscle, cardiomyocyte, neuron, astrocyte, and oligodendrocyte (47). There is compelling evidence that hUC-MSCs, compared to BM-MSCs, have higher proliferation and differentiation abilities, and stronger immune tolerance because of lower human leukocyte antigen-1 (HLA-1) expression (48, 49). hUC-MSCs can improve clinical manifestations in the animal model of EAE. hUC-MSC-treated EAE mice showed long-term (50 days) recovery of behavioral functions and improvement of histopathological characteristics, including suppression of perivascular immune cell infiltrations and reduction of demyelination in the spinal cord (50). The first report of successful treatment of an MS patient with hUC-MSC was published in 2009 (51). After transplantation of hUC-MSC in a patient with refractory progressive MS, the disease course was stabilized with signs of improved sensory function

and muscle strength, and the patient could even stagger along with the help of family (51). In subsequent clinical experiments, during a 1-year observation period, no significant adverse effects were found in groups treated with hUC-MSC, indicating a better safety profile of these stem cells (52). Administration of hUC-MSC showed lower relapse occurrence and EDSS (Expanded Disability Status Scale) scores in MS patients. Assessment of inflammatory cytokines demonstrated a shift from Th1 to Th2 immunity in treated patients. An increase in HGF was also observed in hUC-MSC-treated group which may have played a role in the improvement of MS. HGF is a multifunctional cytokine which is important for tissue regeneration with its ability to stimulate mitogenesis, cell motility, and matrix invasion (52). According to a case report, a 25-year-old MS patient, throughout the 4-year treatment period (2008–2012) with BM and UC-MSC, was completely free of clinical and radiological disease activity. Also, the patient had good recovery from severe relapse and was able to walk unaided. No new lesions were observed on the MRI performed at the end of the treatment period, and many lesions had resolved (53).

HUMAN WHARTON'S JELLY MSCS

Wharton's jelly is a mucoid connective tissue that surrounds the umbilical vessels. Human Wharton's jelly–derived mesenchymal stem cells (hWJ-MSCs) are a valuable alternative to BM-derived stem cells (54). They can differentiate into many different cell types, including fat, bone, cartilage, and neural cells (29, 55–58). In an experimental model of EAE, transplantation of hWJ-MSCs-derived oligodendrocyte progenitor cells into the brain ventricles of mice reduced the clinical signs of EAE and significantly increased remyelination (59). In another study on rat EAE model, hWJ-MSC suppressed proliferation of activated T-cells with contact-dependent and paracrine mechanisms. Indoleamine 2,3-dioxygenase 1 was shown as the major effector molecule responsible for T-cell suppression (60).

ADIPOSE-DERIVED MSCS

Adipose tissue is an abundant and accessible source of MSCs that can be obtained easily in sufficient quantities with a minimal invasive procedure. These adipose-derived mesenchymal stem cells (AdMSCs) are multipotent and differentiate into chondrocyte, myocyte, neuronal, and osteoblast lineages (61, 62), and are effective in the treatment of immune-related diseases, including GVHD, MS, and rheumatic disease (63).The differentiation and immunomodulatory potencies of AdMSCs are equivalent to that of BMSCs. Whereas hAdMSC derived from elderly and young donors showed similar proliferation, differentiation, and senescence marker patterns, BMSCs from the elderly showed reduced proliferation, decreased differentiation, and increased senescence (64). The therapeutic potential of AdMSCs in a mouse model of peripheral nerve sciatic crush has been demonstrated (65). The therapeutic efficacy of AdMSCs isolated from lean and obese persons indicated that obesity reduces the anti-inflammatory effects of human AdMSCs such that they may not be a suitable cell source for the treatment of autoimmune diseases (66). AdMSCs are a valuable source of adult MSC with neuronal differentiation ability, and are a useful remedy to treat neurodegenerative diseases (67).

Recent studies suggest that AdMSCs have a significant beneficial effect on chronic EAE model, both in the preclinical phase of the disease and after the disease has entered an irreversible clinical course (68). In EAE lesions, the amelioration of clinical scores was accompanied by a strong reduction of spinal cord inflammation as well as demyelination and axonal damage. Administration of AdMSCs in chronic EAE induces a Th2-type cytokine shift in T-cells. The penetration of AdMSCs within demyelinated areas is accompanied by increased number of endogenous oligodendrocyte progenitors (69). Additional studies showed that murine AdMSCs (mASCs) suppress T-cell proliferation via inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) activities. mASCs also prevented lipopolysaccharide (LPS)-induced maturation of dendritic cells (DCs) (70). The efficacy of intravenous AdMSCs transplantation in remyelination, in mouse cuprizone model of MS, can be significantly enhanced by 17β-estradiol (E2) administration (71). AdMSCs can upregulate immunomodulatory cytokines, such as TGF-β, and downregulate inflammatory cytokines, such as IFN-γ, and transcription factors, such as t-bet (72). Brains and lymph nodes of EAE rats treated with AdMSCs show a significant expression of human leukocyte antigen G (HLA-G) gene. The immunomodulatory effects of AdMSCs may be related to their secretion of HLA-G (73). Engineering of AdMSCs as carriers for IFN-β delivery, or secretors of IL-10, has shown beneficial effects in experimental models of MS (74, 75).

NEURAL STEM CELLS

NSCs are unipotent stem cells found in the subventricular zone (SVZ) of the lateral ventricle. This part of the CNS is routinely used for isolation of NSCs (76, 77). The unipotency and migratory properties of NSCs help to repopulate neural cells in the CNS following inflammation (4, 78). The potential of NSCs to differentiate into neuroglial cells and oligodendrocytes suggests their application as a beneficial method for the treatment of MS (79–84). NSCs can also be derived from bone marrow, and these cells also exhibit the capacity for neuroglial differentiation (81, 82).

ENDOMETRIAL STEM CELLS

Human endometrium contains a small number of endometrial stem cells (hEnSCs) that can be considered as a source of MSCs for cell-based tissue engineering applications to repair bone, neural cells, osteoblasts, cartilage, and muscle (85). It is well understood that endometrial stem cells (EnSCs) are responsible for the remarkable regenerative capacity of endometrium (86). hEnSCs can differentiate into high-efficiency cholinergic and dopaminergic neurons with confirmed formation of functional neurons (87). EnScs alleviate neuroinflammation through the impairment of Th17 and Th1 CD4 cells (88). hEnSCs can be differentiated into Schwann cells (SCs) in both 2D and 3D cultures. These differentiated cells in fibrin gel could present new opportunities for tissue engineering approaches and subsequent treatment of neurodegenerative disorders (89). hEnSCs can differentiate into oligodendrocyte progenitors with characteristic oligodendroglial precursor cells (OPCs) morphology, and express markers such as PDGFRα, Sox10, A2B5, Olig2, and O4 (90). hEnSCs reduced perivascular

infiltrate and EAE scores, and improved overall tissue appearance (91) in experimental mice. Intravenous or intrathecal administration of hEnSCs to four patients showed a good safety profile. After 1 year of follow-up, the patients showed no immunological reactions or treatment-associated adverse effects; based on radiological and functional assessment as reported by radiologists, no disease progression was observed (92).

EMBRYONIC STEM CELLS

ESCs are derived from the inner cell mass of blastocyst-stage embryos. ESCs are totipotent cells that can differentiate into all tissues and cell types, including hematopoietic precursors, heart and skeletal muscles, and neural cells. ES cells can be considered as a valuable source of cells for deriving glial precursors that can interact with host neurons and efficiently myelinate axons in brain and spinal cord and also promote improvement of motor function (93, 94). Human embryonic stem cells (hESCs) have proved a promising source for the generation and replacement of mature oligodendrocytes (95). Accordingly, hESC-derived oligodendrocytes can play a supportive role in the repair of CNS injuries (96). Intracerebroventricular transplanted hESC-derived oligodendroglial progenitor (hESC-OPs) cells ameliorated the clinical symptoms and promoted recovery from EA E paralysis. EAE mice that received hESC-OPs induced Foxp3-positive T-regulatory cells and produced a new population of TREM2-positive cells that has anti-inflammatory and tissue regeneration promoting properties (97). Also, transplanted hESC-derived neural precursor cells into the brain ventricles significantly reduced the clinical signs of EAE mice. Transplanted neural precursors migrated into the host white matter; however, differentiation into mature oligodendrocytes and remyelination were insignificant (3). In the EAE model of MS, the therapeutic effect of hES-MSCs, including reduction of clinical symptoms and prevention of neuronal demyelination, was significantly higher than BM-MSCs (98). Transplantation of ESCs in adult rat spinal cord had the ability to survive, migrate, and differentiate into mature myelin-producing cells in areas of demyelination (99). Clinical reports of transplantation of hESC in patients with MS and Lyme disease have shown remarkable improvement in their functional skills, overall stamina, cognitive abilities, and muscle strength (100).

INDUCED PLURIPOTENT STEM CELLS

Induced pluripotent stem cells (iPSCs) are generated via reprogramming of mouse fibroblasts into ESCs that overexpress four genes: *Sox2,Oct3/4*, *Klf4,* and *c-Myc* (101, 102). iPSCs exhibit similar phenotype of ESC, and proliferate and differentiate into all cell types of the body as well as teratomas formation (103, 104). Remyelination activity of iPSCs was assessed in mouse EAE models. The formation of oligoprogenitor cells and myelinating oligodendrocyte confirms the therapeutic effects of cell therapy based on iPSCs. Also, iPSCs have the neuroprotective effects via secretion of growth factors such as LIF that amplify the viability of endogenous oligoprogenitor stem cells and remyelination (105, 106). iPS cells can provide the allogeneic and autologous stem cell therapy and hold promise for specific treatment.

SPERMATOGONIA STEM CELLS

Spermatogonia stem cells (SSCs) are derived from seminiferous tubules in testes, and *in vitro* studies show the pluripotency of these cells (22, 107–109). They differentiate into ES-like cells, with a similar phenotype and differentiation capacity (110–112). They can be considered an alternative cell source to ESCs without the ethical limitation and immunological problems associated with ESCs. Neural and glial differentiation of ES-like cells derived from testes have been reported by several groups. The efficiency of neural differentiation was confirmed using action potentials recorded by Patch-clamp electrophysiological examinations, and the capacity of SSCs to form functional neurons and oligodendrocytes has been reported. Our findings showed functional recovery and significant remyelination, following transplantation of oligoprogenitor cells derived from mouse SSCs, in an animal model of demyelination (22). Further investigations should be done to confirm the recovery outcome of this novel pluripotent cell source in animal models of MS.

Endogenous Stem Cell Niches Reactivation in MS

Apart from the exogenous sources of stem cells described above, the endogenous stem cell population opens up a new perspective for MS treatment (113). Studies on patient brain tissue samples and animal models of MS show that in the adult CNS, endogenous regeneration activities exist; however, repair efficacy is low and tends to diminish during disease progression (114, 115). Mature oligodendrocytes are extremely degenerative due to primary insult, or secondary to oxidative and excitotoxic stress; thus, they do not participate in myelin repair activities (116). However, resident OPCs (117) or adult neural stem cells (aNSCs) (118–120) become activated and are recruited to lesion sites in order to perform remyelination and restore axonal functionality. There is evidence that OPCs produce the vast majority of remyelinating oligodendrocytes (121), which can also originate from the stem and precursor cells of adult SVZ (122). In response to injury or demyelination, OPCs in the surrounding area convert from a quiescent state to a regenerative phenotype (123). Injury to the CNS activates microglia and astrocyte cell types and disturbs tissue homeostasis, resulting in OPC activation (124). These two cell types are the main factors that induce proliferation and migration of OPCs to the site of injury in demyelinating insults (124, 125). During the regeneration phase of demyelination, some factors have been shown to contribute to the regulation of OPC differentiation into myelinating oligodendrocytes (126). Several studies have provided evidence for the inhibitory effects of some factors such as semaphorin 3A (127), Nogo receptor (128), LINGO-1 (129, 130), and wnt signaling pathway (131) on OPCs differentiation during development and remyelination. Remyelination can occur in demyelination conditions but is very limited. Remyelination failure is due to the impact of numerous inhibitory mechanisms (132, 133). To improve functional recovery, therapeutic approaches should be developed by either potentiating endogenous stem cell populations or by providing exogenous source of repair-mediating cells for the injured CNS. In this section, we describe recent studies related to the

endogenous stem cells of the central and peripheral nervous systems, and their potential therapeutic application for the treatment of MS.

CNS Neural Stem Cell pools

Within the adult mammalian brain, NSCs are located in the SVZ of lateral ventricles, hippocampal subgranular zone (SGZ), and the central canal (CC) of the spinal cord where they divide and give rise to new neurons in a process termed adult neurogenesis (4, 134, 135). Other germinal regions have been identified in the third ventricle, hypothalamus, the subpial layer of the cerebellum, and the meninges (136, 137). NSCs located in very specific microenvironments, called niche, and their cellular makeup have been shown to consist of a variety of cells including NSCs and their immature progeny accompanied by endothelial, astroglial, and ependymal cells (138, 139). They receive structural and trophic signals from cell-to-cell and cell-to-extracellular matrix (ECM) contact. This communication provides critical spatial and temporal information, which in turn allows stem cells to act in response to both physiological and pathological stimuli (138, 140).

SVZ OF LATERAL VENTRICLES

SVZ is the largest neurogenic niche in the adult CNS that is capable of sustaining neurogenesis throughout life (141). The adult SVZ displays a high degree of organization with stem cells and other cell types which is an important feature of the neurogenic region of SVZ (142). The SVZ is composed of heterogeneous cell types including nondividing ependymal cells (E1) with a large apical surface and multiple long cilia (143), astrocyte-like type B cells (B1) (slow dividing) that give rise to type C cells (fast dividing), which in turn differentiate into neuroblasts (type A) and migrate to olfactory bulb and provide new interneurons (144, 145). The en face view of the lateral ventricle revealed that the apical cilium of one or more B1 cells was surrounded by E1 cells in striking pinwheel architecture which is specific to neurogenic area (142). B1 cells contact the ventricle via their apical cilium and blood vessels at the basal processes. They are quiescent and slowly proliferate in normal condition but can become activated in different pathologies (146).

Intense research in the last decades on animal models of MS and tissue samples of MS patients has shown that the adult SVZ niche is reactivated in response to various types of proximal insults by producing new progenitors that migrate toward the injury site and differentiate into oligodendrocytes (118, 147–149). In addition, it has been reported that type B (150), type C (147), and type A cells (151) have all been indicated as sources of newly generated oligodendrocytes in physiological and pathological conditions. Furthermore, we recently found that ventricular pinwheel organization and structure are modified and E1 cells are reactivated in response to inflammatory demyelination (152). However, SVZ progenitor's recruitment into the lesion site in the demyelination condition was relatively poor and their differentiation potential to oligodendrocyte is limited because of some inhibitory factors in mature environments during MS.

SGZ OF THE HIPPOCAMPUS

The second major region that sustains neurogenesis in the adult brain throughout life is the SGZ of the hippocampus, which is located at the border of the granule cell layer (GCL) and the hilus of dentate gyrus (DG) (153). Neurogenesis in the adult hippocampus occurs throughout life and mainly contributes to the processes involved in learning and memory; however, the ultimate function of neurogenesis in DG remains to be clarified (154). Radial glia-like cells (RGL) in DG represent a quiescent population which may be provoked to generate the proliferative precursors identified as intermediate progenitors, namely, IPC1 and IPC2 cells (155). These cells produce novel immature granule neurons (type 3 cells), which migrate into the inner GCL and differentiate into granule cells of the DG (153). They extend their dendrites and axons toward the CA3 region and become functionally integrated into host circuitry (119).

Cognitive impairment and memory dysfunction affect more than 60% of MS patients (156). It has been reported that cognitive dysfunction is correlated with hippocampal demyelination (157). Although the molecular mechanisms that control hippocampal NSC proliferation and differentiation in physiology and pathological conditions are unknown, recent findings reveal that acute inflammatory demyelination in animal model of MS could provoke the hippocampal stem cell niche and enhance proliferation of NPCs in SGZ (158). Thus, inflammatory factors such as cytokines and chemokines can affect the proliferative capacity of NSCs and alter neurogenesis in the SGZ (159). Huehnchen et al. (2011) reported that NPC proliferation in the DG increases not only in the acute phase but also in the chronic phase of the disease(160). Furthermore, it has been found that the neurogenic niche of the hippocampus was reactivated in animal models of MS (161).

CENTRAL CANAL OF THE SPINAL CORD

The spinal cord is the caudal part of CNS that consists of 33 nerve segments, from the cervical to coccygeal sections. There is a central canal at the center of the spinal cord which contains the cerebrospinal fluid (CSF) (134). The ependymal layer of the spinal cord has an important role in embryonic development and is well known for its function as a neuroprogenitor niche (162). In the late 1990s, multipotent stem cells were discovered in the adult mammalian spinal cord. Isolated NSC from central canal of rat and mouse can produce neurospheres that are able to self-renew, proliferate, and differentiate into the three major CNS cell types (163). Moreover, it was shown that NSC resides at the central canal and is able to self-renew and generate mature oligodendrocytes during injury (164). The adult central canal stem cells are quiescent under physiological conditions; however, some proliferation has been observed at the dorsal and ventral tip of the CC that contacts the lumen or the subependymal position (135, 164). Dorsal ependymal cells show radial glial morphology and express GFAP, nestin, CD15, and/or brain lipid–binding protein (BLBP) (165). It has currently been shown that ependymal cells at both dorsal and ventral point of the central canal are able to generate progeny of multiple fates under physiological and pathological conditions (166). Further research is needed to fully unravel the neurogenic properties and/or potential of the central canal in MS.

OTHER GERMINAL AREAS OF THE CNS

Beyond the classic NSC niches referenced above, other germinal niches have been identified. These germinal regions include the hypothalamus, the third ventricle, the meninges, and the subpial layer of the cerebellum (167). The parenchyma of the cerebral cortex and spinal cord are mainly comprised of restricted neuroglia precursors and these niche are referred to as nongerminal regions of CNS (168). These neurogenic niches are composed of a heterogeneous population of NSC that is able to self-renew and give rise to most of the neuronal and glial precursors (4). Several studies showed that the third ventricle and hypothalamus neurogenic zone contain multipotent cells that can give rise to neurons, oligodendrocytes, and astrocytes *in vitro* and *in vivo* (169–171). Xu and others reported that the third ventricle ependymal layer cells were able to migrate into hypothalamic parenchymal regions and differentiate into functional neurons in response to injury (172). Our previous study also showed that progenitor cells in the third ventricle surroundings could be reactivated by local demyelination in the optic chiasm (128, 171). Also nestin and DCX-positive cells have been found in the meninges of the brain and spinal cord (138, 173). We concluded that there are widespread sources of stem cells in the CNS that can be activated in different pathological situations, especially in MS.

Peripheral Endogenous Stem Cells and Their Role in MS

SCHWANN CELLS

In the peripheral nervous system (PNS), a different source of cells has been identified that can be used for the treatment of CNS diseases like MS. SCs have been intensely studied in CNS repair and have been shown to support and myelinate regenerating axons (174). Several studies that transplanted neonate or adult SCs in different animal models of CNS demyelination had shown that SCs efficiently remyelinate CNS axons (175). The myelin formed by a grafted SC was stable for up to 5 months post-graft and improved conduction of demyelinated axons (176, 177). Neuroregenerative effect of SCs has also been reported in spinal trauma models which highlighted the ability of these cells to regenerate axons in the injured area (178). However, the important limitation concerning the use of SCs as a therapeutic approach to promote remyelination in MS is their inability to migrate efficiently when grafted in injured CNS (179). Modifying SC-intrinsic properties, like boosting expression of neurotrophins (e.g., BDNF and NT3), promote SC migration and myelinating potentials (180, 181). Also, SC-mediated myelination and axonal regeneration increased when the environment of the SC was modified (182).

OLFACTORY ENSHEATHING CELLS

Olfactory ensheathing cells (OEC) are very similar to SCs and belong to the peripheral olfactory system that ensheathes the axon of the first cranial nerve but does not myelinate it (183). Recently, it was shown that the origin of OEC

during development was from neural crest cells (NCCs) (184). Although OEC does not usually myelinate axons of the first cranial nerve, the vast studies have shown that OECs are capable of extensive functional remyelination when grafted into demyelinated lesions (185, 186). Numerous studies proposed that OEC migrates better than SC when faced with CNS elements (187, 188). From a therapeutic point of view, OEC transplantation appears to be better than SC.

PNS PROGENITORS

PNS progenitors include Schwann cell precursors (SCps), boundary cap cells (BCs), and olfactory epithelial progenitors (OEps) that all originate from NCCs (175). It has been reported that SCp has greater capacity for remyelination after grafting in demyelinated CNS or spinal cord injury (189). BC is the potential stem cell of spinal roots (190) that could migrate freely in the demyelinated CNS and compete with endogenous myelin-forming cells to remyelinate axons of far distant lesions (191). BC can also differentiate into central myelin-forming cells *in vitro* and *in vivo* (192). OEp was extracted from olfactory epithelium with a less invasive method and when pieces of olfactory lamina containing OEp were grafted into injured rat spinal cord, they promoted functional recovery in paraplegic rats (193). OEp provided extensive remyelination upon transplantation into demyelinated lesion (194).

Endogenous Neural Stem Cell Niche Modulation as a Therapeutic Approach

The niche microenvironment regulates NSC survival, proliferation, and differentiation during health and disease (142, 152). Therefore, different molecular strategies have been studied in an effort to enhance the NSC niche potential for facilitating repair and aiding in functional recovery of various neurodegenerative disorders by using new pharmacological targets (138). Administration of exogenous growth factors such as EGF, PEDF, HGF, and CNTF in mice has been reported to enhance NSC proliferation (195, 197). In addition, other factors such as bFGF, EGF, and BDNF have also been shown to enhance neurogenesis and eventually enhance functional recovery in animal models of neurological disease (198–200). Administration of valproic acid has been shown to attenuate symptoms of EAE, and increase endogenous myelin repair by recruiting NSCs and oligodendrocyte progenitors to the lesion sites (201). Moreover, treatment of EAE animals with polymerized nanocurcumin showed promising results in enhancing neuroprotection and myelin repair (202). Certain antidepressants like fluoxetine have been revealed to be capable of increasing neurogenesis (203). Administration of small interfering RNA (siRNA) or specific antibodies against various inhibitory targets such as Nogo, Nogo receptor (NgR), LINGO1, and Sema3A in different animal models of MS and spinal cord injury enhance proliferation, migration, and differentiation potential of endogenous stem cells and facilitate axonal regeneration, myelin repair, and functional recovery (128, 204–207). Khezri and coworkers reported that administration of cyclic AMP inhibits the progression of EAE disease and potentiates recruitment of endogenous NSCs and myelin repair (208).

Conclusion

The existence of NSCs and neurogenic niches in the adult mammalian CNS is clearly recognized. The functional implication of adult neurogenesis and gliogenesis continues to grow as new researches describe their critical roles in both health and disease. In spite of this growing body of evidence and progress in our understanding of NSC and niche functions in physiological and pathologic situations, several critical issues remain to be answered. The main issue is the translational relevance of the basic biology, that has been described in animal models, to human neurogenesis, and clinical trials. Moreover, the ultimate molecular mechanisms that influence endogenous stem cell migration will also be a key in developing appropriate treatments and strategies to prevent, alleviate, and treat MS. Further studies to identify the definitive nature, location, and behavior of NSC are warranted to realize the full therapeutic potential of these stem cells for the treatment of MS.

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Section II

Pathophysiology, Mechanistic Pathways, and Animal Models

7 Pathogenesis and Progression of Multiple Sclerosis: The Role of Arachidonic Acid–Mediated Neuroinflammation

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Abstract: Multiple sclerosis is characterized by inflammatory processes occurring within the central nervous system. In multiple sclerosis, inflammation could be either a physiological response secondary to the immune system activation or a phenomenon triggered by primary cytodegeneration of neurons and/or oligodendrocytes without the involvement of immune cells. The arachidonic acid metabolism is activated via cyclooxygenases (COXs) and lipoxygenases (LOXs) in postmortem brain samples and in the cerebrospinal fluid of multiple sclerosis patients. It has been hypothesized that the arachidonic acid–mediated neuroinflammation could play a role in the pathogenic mechanisms triggering demyelination, oligodendrocyte loss, axonal pathology and, ultimately, motor dysfunctions, which are hallmarks of multiple sclerosis. COX-2 and 5-LOX selective inhibitors efficiently inhibit each of the hallmarks mentioned above in different animal models of multiple sclerosis. Thus, it is suggested that the arachidonic acid pathway represents a potential pharmacological target to ameliorate multiple sclerosis pathology and symptoms.

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Key words: Arachidonic acid; Cyclooxygenases; Inflammation; Lipoxygenases; Multiple sclerosis

Introduction

Multiple sclerosis is a multifactorial degenerative disease of the central nervous system characterized by immune system activation, inflammation, and demyelination. The genesis of the inflammatory process and its role in the onset and progression of the disease is still under debate, although advances have been made over the past decades of scientific research. For instance, it has been hypothesized that the central inflammation observed in multiple sclerosis is a physiological response secondary to the immune system activation. Different subtypes of $CD4^+$ T helper lymphocytes—Th1 and Th17—and cytotoxic $CD8^+$ lymphocytes have been shown to trigger neuroinflammation in multiple sclerosis (1). These activated lymphocytes migrate to the brain, recall peripheral monocytes/macrophages, and ultimately lead to myelin loss and apoptosis and/or necrosis of mature oligodendrocytes. Resident astrocytes and microglia are activated after lymphocytes infiltration. As a consequence, several inflammatory mediators like cytokines (chemokines, IL2, IL3, TNFα, IFNγ, and many others) are released by these cells in the extracellular compartment where they exert cytotoxic activity against oligodendrocytes (2–5).

In some types of multiple sclerosis, the disease seems to develop independently of the autoimmune mechanisms, particularity in those disease types—histological patterns III and IV—that show no evidence of immune activation at demyelinated lesions (6, 7). In these cases, inflammation maybe triggered by primary cytodegeneration of neurons and/or oligodendrocytes without the involvement of immune cells (8). Regardless of the biological process underlying inflammation, it has been consistently shown that inflammation is directly involved in the progression of multiple sclerosis (9). In recent years, there has been a growing interest in understanding the role of inflammatory mediators derived from the activation of arachidonic acid metabolism (e.g., prostaglandins and leukotrienes) in the disease (10). Prostaglandins and leukotrienes are abundantly produced in the central nervous system of multiple sclerosis patients, contributing to the severity of the disease. Therefore, it has been suggested that anti-inflammatory treatments targeting the arachidonic acid pathway, by using nonsteroidal anti-inflammatory drugs (NSAIDs), might be beneficial for treating multiple sclerosis.

Activation of the Arachidonic Acid Cascade in Multiple Sclerosis

Scientific evidences show that arachidonic acid metabolism is excessively activated in the central nervous system of multiple sclerosis patients as well as in the brain of animals from experimental models of multiple sclerosis. It has been hypothesized that arachidonic acid products could play a role in the pathogenic mechanisms underlying demyelination, oligodendrocytes loss, and axonal pathology that represent common hallmarks of multiple sclerosis. Arachidonic acid is a

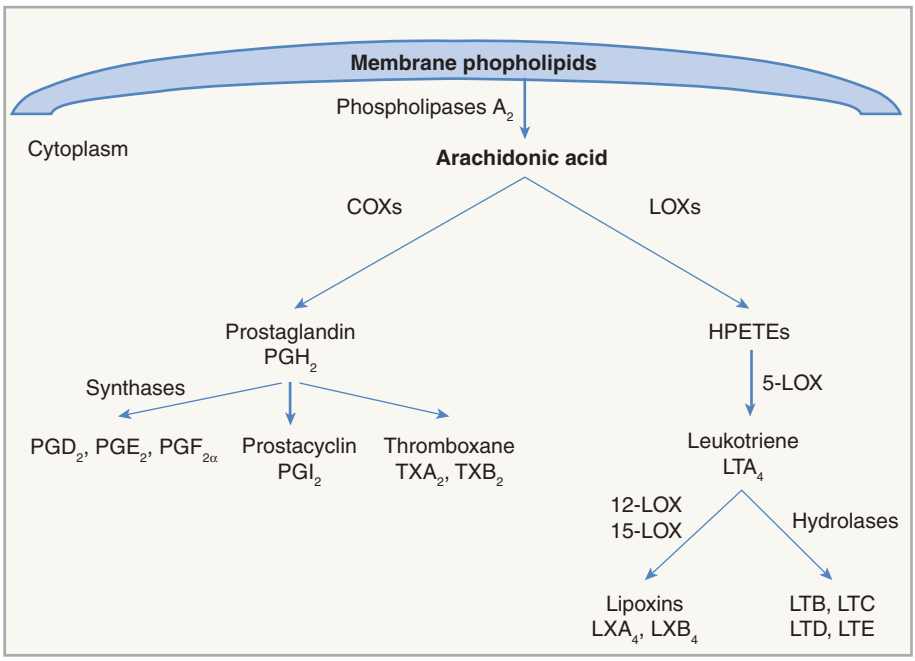

Figure 1 Schematic representation of the arachidonic acid metabolic pathway.
COX= cyclooxygenase, LOX= lipoxygenase, HPETE= hydroperoxyeicosatetraenoic acid.

membrane omega-6 fatty acid molecule released in the cytoplasm by the hydrolytic activity of the cytosolic phospholipase A_2 ($cPLA_2$) (Figure 1). It has been shown that the concentration of several molecules that activate $cPLA_2$, such as reactive oxygen species and cytokines, is increased in multiple sclerosis (11–14). After being released into the cytoplasm, arachidonic acid is metabolized by the activity of cyclooxygenases (COXs) 1 and 2 into prostacyclins, prostaglandins (PGs), and thromboxanes (TXs), and by the lipoxygenases (LOXs), 5-LOX, 12-LOX and/or 15-LOX into leukotrienes (LTs) and lipoxins (LXs). As far as COXs are concerned, both isoforms lead to the production of PGE_2. COX-1 is constitutively expressed, whereas COX-2 is induced during inflammation and seems to be the major source of PGE_2 production. Particularly, COX-2 expression appears to be induced in oligodendrocytes and immune cells during the processes of demyelination (15–17). The proinflammatory PGs and LTs that are upregulated in multiple sclerosis represent promising therapeutic targets as suggested by animal models of multiple sclerosis.

ARACHIDONIC ACID PATHWAY ACTIVATION IN PATIENTS AFFECTED BY MULTIPLE SCLEROSIS

Arachidonic acid activation has been found in the cerebrospinal fluid and in post-mortem brain of multiple sclerosis patients (see Table 1 for details of primary data). It has been shown that COX-2 is expressed in active demyelinating lesions (15), and also in dying oligodendrocytes (16) suggesting a potential role for

TABLE 1 **Primary data concerning arachidonic acid pathway alterations in the CSF, brain tissue, and peripheral blood of multiple sclerosis patients and in the brain of EAE, TMEV, and cuprizone mice**

			Animal Models of Multiple Sclerosis		
		Multiple Sclerosis Patients	EAE model	TMEV model	Cuprizone model
Cyclooxygenase (COX) pathway	COX-1	NT	4-fold increase of mRNA (27); expressed in microglia/ macrophages (29)	NT	Up to 30% increase in protein, 70% increase in mRNA (34); expressed in microglia and/or macrophages and astrocytes
	COX-2	Expressed in brain tissue within apoptotic oligodendrocytes and microglia and/or macrophages (15–17)	Up to 5-fold increase of mRNA (27,31); expressed in microglia and/or macrophages and in endothelial cells (28–29)	Expressed in apoptotic oligodendrocytes and in astrocytes (15–16)	No change in protein, 50% increase in mRNA (34); expressed in apoptotic oligodendrocytes (34, 35)
	PGD_2	Expressed in the CSF of patients only (18)	No change—50% decreased levels (27,31)	NT	Up to 1-fold increased levels (34, 35)
	PGE_2	Increased levels in the CSF (18–20) and in peripheral lymphocytes (21)	1-fold increased levels (27,31)	NT	Up to 5-fold increased levels (34, 35)
	$PGF_2\alpha$	Increased levels in the CSF (18–20)	No change (27)	NT	NT
	PGI_2	Increased levels in the CSF (18)	2-fold increased levels (27)	NT	50% increased levels (34)
	TXA_2	NT	NT	NT	NT
	TXB_2	NT	50% decreased levels (27)	NT	Up to 1-fold increased levels (34, 35)

Table continued on following page

TABLE 1 **Primary data concerning arachidonic acid pathway alterations in the CSF, brain tissue, and peripheral blood of multiple sclerosis patients and in the brain of EAE, TMEV, and cuprizone mice** (Continued)

		Multiple Sclerosis Patients	*Animal Models of Multiple Sclerosis*		
			EAE model	TMEV model	Cuprizone model
Lipoxygenase (LOX) pathway	5-LOX	NT	8-fold increase of mRNA (27)	NT	30% increase of protein, 3.5-fold increase of mRNA (39)
	12-LOX	NT	10-fold increase of mRNA (27)	NT	NT
	15-LOX	NT	10-fold increase of mRNA (27)	NT	NT
	LTB_4	40–100% increase in the CSF (22, 23)	50% decrease (27)	NT	NT
	LTC_4	0–30% increase in the CSF (18, 22, 23)	80% decrease (27)	NT	NT
	LTD_4	No change in the CSF (23)	60% decrease (27)	NT	NT
	LTE_4	No change in the CSF (23)	NT	NT	NT
	LXA_4	NT	NT	NT	NT
	LXB4	NT	NT	NT	NT

NT= not tested, CSF= cerebrospinal fluid, PG= prostaglandin, TX= tromoxane, LT= leukotriene, LX=lipoxin, EAE= experimental autoimmune encephalomyelitis, TMEV= Theiler's murine encephalomyelitis virus.

COX-2 in the biological mechanisms underlying the death of oligodendrocytes. Moreover, COX-2 is also expressed by inflammatory cells like macrophages and microglia that are located at active lesions (17). These data are in line with previous findings showing that COX-derived prostaglandins are excessively produced in the central nervous system of multiple sclerosis patients. The levels of prostaglandins PGD_2, PGE_2, and PGF_2, and prostacyclin PGI_2, were upregulated in the cerebrospinal fluid of patients during relapsing and remitting phases (18–20). PGE_2 levels were also elevated in lymphocytes extracted from the peripheral blood of patients; the highest levels were reached at the onset of the disease or just before symptoms, suggesting that PGE_2 could be involved in disease initiation (21).

As far as the metabolism of arachidonic acid by LOX enzymes is concerned, the levels of LTB_4 and LTC_4 in the cerebrospinal fluid of multiple sclerosis patients were elevated (18, 22). The same authors, in their second publication on the same topic, were able to replicate the results for LTB_4, but not for LTC_4, LTD_4, and LTE_4 levels (23). Overall, these data have suggested that, in multiple sclerosis, the metabolism of arachidonic acid through 5-LOX enzymatic activity was augmented. In 2010, a study, conducted in postmortem white matter specimens of multiple sclerosis patients, identified the *5-LOX* gene as a top risk gene for multiple sclerosis (24).

ARACHIDONIC ACID PATHWAY ACTIVATION IN ANIMAL MODELS OF MULTIPLE SCLEROSIS

The arachidonic acid metabolic pathway is activated in three different animal models of multiple sclerosis: the experimental autoimmune encephalomyelitis (EAE), the Theiler's murine encephalomyelitis virus (TMEV), and the cuprizone model (see Table 1 for details of primary data). In the EAE model, the upstream enzyme $cPLA_2$ has been shown to play a key role in the pathogenesis of the disease as $cPLA_2$ knockout mice and naïve mice treated with a $cPLA_2$ specific inhibitor were both resistant to EAE induction (25, 26). Downstream $cPLA_2$, COX-2, inducible PGE_2 synthase, and PGE_2 levels were all increased in the brain of EAE mice (27). COX-2 was expressed in the resident microglia, infiltrating macrophages, and endothelial cells of the brain of EAE mice (28–29). Concerning the four receptors of PGE_2, EP1, EP2, and EP4 were upregulated by one-, two-, and threefold, respectively (30). EP2 and EP4 have been implicated in the stimulation of lymphocytes CD4+ release and their activation in EAE model (30). Moreover, COX-1 expression and PGI_2 levels were upregulated in the brain of EAE mice, whereas the concentration of PGD_2 was downregulated, and the concentration of $PGF_{2\alpha}$ was unchanged (27). However, one study conducted in a chronic relapsing type of EAE showed conflicting findings. While the increase of COX-1, COX-2, and PGE_2 was confirmed, the PGD_2 levels remained unchanged in all the analyzed brain tissues (cerebral cortex, cerebellum, and spinal cord) (31). Interestingly, the increase of COX-2 expression and PGE_2 levels was observed in early stages of the disease (31), suggesting a pathogenic role.

In the TMEV model, COX-2 expression was observed in the spinal cord (15). Specifically, COX-2 was expressed in oligodendrocytes undergoing apoptosis as indicated by immunohistochemistry experiments that found colocalization of the COX-2 protein and the apoptotic mediator caspase-3. These data were confirmed

in a further study published in 2010 (16). The latter also showed that COX-2 mediates mechanisms of excitotoxicity against cultured oligodendrocytes (16). COX-2 and PGE_2 gene expression were also found in primary cultures of astrocytes from TMEV-infected mice (32). The inhibition of PGE_2 signaling at a downstream level using AH23848, which is a mixed EP1 and EP4 inhibitor, resulted in decreased pathogenesis of demyelinating disease (about 20% decrease) and severity of viral load (about 85% decrease) in the central nervous system (33).

Similar results were obtained in the cuprizone model of demyelination. Cuprizone takes about 5 to 6 weeks to induce a maximum demyelination in the brain, but oligodendrocytes express apoptotic markers earlier, starting from the first week of intoxication (34). In the brain of cuprizone-treated mice, both COX-1 and COX-2 were significantly upregulated, but the change in the expression showed different courses (34). COX-2 gene expression was found to be upregulated in the early phases of the cuprizone treatment when demyelination was not yet detectable, whereas COX-1 was upregulated later on at the peak of astrogliosis and microglia and/or macrophages activation concomitantly with severe demyelination (34). Interestingly, this observation led to the hypothesis that COX-2 precedes oligodendrocytes loss and is involved in the apoptotic processes. COX-2 was expressed in apoptotic caspase-3-expressing oligodendrocytes as early as after 1 week of cuprizone treatment (35). Further investigation in the COX-2 pathway showed that the cortical levels of several prostaglandins (PGE_2, PGD_2, PGI_2, and TXB_2), were upregulated (34, 35). The increase in PGE_2 concentration was more than the other prostaglandins, and the expression of its receptors, EP1, EP2, and EP4, was upregulated at the peak of demyelination (35). Interestingly, only EP2 protein expression was increased in the early stage, after 1 week of cuprizone treatment, and has been implicated in the initiation of demyelination and oligodendrocytes loss (35).

Regarding LOXs, there is an increasing consent supporting the role of 5-LOX and its downstream products in the mechanisms of immune cell recall in the brain, and in the development of axonal damage and of motor disabilities. The *5-LOX* gene was found to be a top risk gene in EAE (24). The brain concentrations of 5-LOX products, LTB_4 and LTD_4, were upregulated (18, 22–23), and favored the migration of inflammatory cells and lymphocytes in the brain of EAE mice (36–38). In the cuprizone model, the brain expression of 5-LOX was highly increased (39). In addition, 5-LOX has been implicated in cuprizone-mediated axonal damage and motor dysfunction development (39). Overall, the data generated from the animal research indicate that the arachidonic acid pathway contributes to the development of multiple sclerosis–like pathology, especially via COX-2 and 5-LOX metabolism.

Anti-inflammatory Therapy in Multiple Sclerosis

Arachidonic acid–mediated inflammation is typically inhibited with nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs have variable specificity against the two isoforms of COX. While some NSAIDs (e.g., ibuprofen, indomethacin, and naproxen), have mixed inhibitory effect on both COX-1 and COX-2 others, like the coxibs (e.g., celecoxib, rofecoxib, and valdecoxib) and nimesulide, specifically inhibit COX-2 (40). NSAIDs have been administered to patients affected by

multiple sclerosis to counteract symptoms related to flu, but no clinical trials have ever evaluated whether NSAIDs could reduce multiple sclerosis pathology as well. Animal models of multiple sclerosis have demonstrated the beneficial effects of NSAIDs. Furthermore, the pharmacological inhibition of LOX-mediated metabolism of arachidonic acid exerts some beneficial effects. The following paragraphs describe the available evidence on the potential of COX and LOX inhibitors as therapeutics for multiple sclerosis.

NSAIDs TREATMENT IN PATIENTS AFFECTED BY MULTIPLE SCLEROSIS

It is not known whether NSAIDs have an inhibitory effect on the pathology of multiple sclerosis. To date, NSAIDs have been administered to patients to treat flu-like symptoms without taking into consideration of their potential role in oligodendrocytes survival and myelin protection (41–46). Nevertheless, some NSAIDs were shown to ameliorate fatigue (approximate percentage of improvement: 10–20% with aspirin, 30% with naproxen, and 20% with ibuprofen) and improve cognitive abilities (approximate fold change of improvement: 1-fold with naproxen, 0.5-fold with ibuprofen, and 2-fold with acetaminophen) (46, 47). It could be hypothesized that these effects may be secondary to the attenuation of brain pathology due to NSAIDs treatment, as suggested by the following data from experimental models of multiple sclerosis.

EFFECT OF NSAIDs IN ANIMAL MODELS OF MULTIPLE SCLEROSIS

Non-selective COX inhibitors and COX-2 selective drugs have shown protective effects in EAE, cuprizone and TMEV murine models of multiple sclerosis. In the EAE model, mixed COX-1/2 inhibitors (indometacin and naproxen) delayed the onset (about 8 days delay with naproxen) and the severity of the disease (about 30% improvement with indometacin and 70% with naproxen) (26, 48, 49). In the cuprizone model, COX-1 knockout mice normally develop demyelination in the same extent as matched wild type mice, indicating that COX-1 is not involved in the demyelination process. Conversely, knocking out the *COX-2* gene inhibited demyelination (about 40% inhibition in the corpus callosum and complete recovery in the cortex) and restored motor functions (35).

Selective targeting of COX-2 has provided a large number of evidence, supporting the prominent role of this isoform in disease initiation and severity. The administration of selective COX-2 inhibitors (LM01, LM08, LM11, and NS398), or coxibs (rofecoxib, celecoxib, and lumiracoxib) interfered with EAE induction by decreasing physical dysfunctions, inflammation, and demyelination; the protective effects of these compounds were mediated through the inhibition of adhesion and chemoattractant molecules, and the reduction of monocyte infiltration (48–51). Specifically, LM01, LM08, LM11, and NS398 inhibited the paralysis period (percentage inhibition: 48, 95, 76, and 43, respectively), inflammation (percentage inhibition: 85, 84, 78, and 81, respectively), and demyelination (percentage inhibition: 74, 67, 53, and 61, respectively) (50). Celecoxib prevented EAE induction, reduced the expression of adhesion and chemoattractant

molecules (histological nonquantitative data), and inhibited the number of infiltrating monocytes (49). Rofecoxib and lumiracoxib reduced inflammation by 90% and 85%, respectively (51).

In the TMEV model, the COX-2 selective inhibitor CAY10542, reduced demyelination by 25%, and prevented the death of oligodendrocytes (16). The efficacy of COX-2 targeting has been confirmed in the cuprizone model as well, as celecoxib greatly reduced demyelination (about 30% reduction in the corpus callosum and complete recovery in the cortex) along with a full recovery of motor abilities (35). In this model, COX-2 expression exerts deleterious effects on the oligodendrocytes through the production of PGE_2, with in turn contributes to loss of oligodendrocytes by interacting with the EP2 receptor: the administration of an EP2 antagonist to cuprizone mice showed similar protective effects as the ones induced by celecoxib (35).EAE mice treated with an inhibitor of $cPLA_2$ showed marked beneficial activity (about 85% inhibition of disease severity) (26). Because of this observation, the question arises whether, the protective effect is mediated merely through the inhibition of the COX pathway or the inhibition of LOX activity is also involed. It has been shown that 5-LOX selective inhibition delayed the onset of EAE by about 5 days (26). Similarly, in the cuprizone model, 5-LOX inhibition resulted in reduced axonal pathology and ameliorated motor disabilities without any improvement in the demyelination severity (39). Overall, these data suggest that COX-2 and 5-LOX inhibition have some nonoverlapping activities (52).

NSAIDs Administration: Future Perspectives

Most of the currently available pharmacological medications for multiple sclerosis counteract the activity of the autoimmune system. Lymphocytes are the leading factors in the autoimmune-mediated mechanisms implicated in the disruption of myelin proteins and the death of oligodendrocytes. First-generation drugs (interferons and glatiramer acetate) and second-generation drugs (fingolimod, mitoxantrone, rituximab, ocrelizumab, ofatumumab, and others) reduce disease severity, progression, and relapses; their main mechanism of action include sequestration of lymphocytes in the lymph node, and reduction of their access to the brain (53–56). However, these drugs do not directly target the arachidonic acid metabolism. Based on the literature, NSAIDs are currently administered to patients if flu-like symptoms occur. However, growing evidence supports the hypothesis that COX-2 and 5-LOX enzymes promote downstream mechanisms that ultimately lead to oligodendrocyte degeneration and axon pathology, respectively, and that both contribute to the development of motor disabilities. The combination of COX-2 and 5-LOX selective inhibitors has the potential to improve multiple sclerosis pathology. Moreover, multiple sclerosis has been associated with platelet activation and augmented cardiovascular risk, which are considered as causal factors in the pathogenesis of the disease (57, 58). Interestingly, it has been recently observed that peripheral blood platelets of patients highly express COX-2 (58). In the light of these evidence, the administration of COX-2 selective NSAIDs could reduce both cardiovascular risk and the progression of multiple sclerosis.

Conclusion

Several pharmacological studies, conducted in experimental animal models of multiple sclerosis, suggest that NSAIDs that selectively inhibit the COX-2 isoform represent promising medications for reducing oligodendrocytes apoptosis, demyelination, and motor dysfunction. In addition, it is suggested that 5-LOX inhibitors could be beneficial to counteract axonal pathology and to inhibit motor disabilities as well. The coadministration of COX-2 and 5-LOX inhibitor is a promising way forward for multiple sclerosis treatment.

Conflict of interest: The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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8 Endogenous Opioids in the Etiology and Treatment of Multiple Sclerosis

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Abstract: Endogenous opioids are enkephalins and endorphins that are primarily produced in the brain and have multiple actions throughout the body. Enkephalins and endorphins act at opioid receptors and their activity can be blocked by opioid antagonists. A small pentapeptide termed opioid growth factor (OGF), and chemically termed [Met^5]-enkephalin, has been shown to have causative and therapeutic roles in experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS). Enkephalin levels are reduced in animals and humans during MS relapses, and may play a role in etiology. Exogenous therapy with OGF or endogenous stimulation of OGF by low dosages of naltrexone (LDN) reverse the course of progressive EAE and limit the number of relapses in relapsing-remitting EAE. Individuals prescribed LDN report less fatigue and a better quality of life while using LDN. This chapter summarizes the information from studies using two different animal models of EAE, as well as two different treatment regimens of two different compounds—OGF or LDN. In all investigations, the presence of enkephalins resulted in beneficial effects.

Key words: β-endorphin; Endogenous opioids; Enkephalins; Receptor mediation; Relapsing-remitting EAE.

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Introduction

Endogenous opioids are a class of molecules that are produced in the brain and circulate widely throughout all organ systems. Endogenous opioids are neuropeptides and are derived from one of the two precursor genes—pre-proenkephalin A or pro-opiomelanocortin (POMC). These opioid peptides have a variety of neural-related functions and are often termed neuromodulators or neuro-immunomodulators. The designation "opioid" is based on their confirmed or presumed binding site of an opioid receptor within the brain tissue. However, this chapter details the role of enkephalins in multiple sclerosis (MS) that is neural-like but not necessarily associated with the brain or spinal cord function. As will be discussed, enkephalins also inhibit cell replication, and blockade of their interaction utilizing low dosages of the general opioid receptor antagonist naltrexone (i.e., low dosages of naltrexone [LDN]) reduce the symptoms of MS and improve the patient's quality of life. The discovery of endogenous opioid peptides in 1975 by Hughes and colleagues (1) followed the identification in 1973 of native opioid receptors in the brain and the gastrointestinal tract (2–4). The first endogenous opioids to be confirmed by radioactive ligand binding were [Met^5]-enkephalin and [Leu^5]-enkephalin (5, 6). These neuropeptides will be the focus in this chapter. [Met^5]-enkephalin is also termed the opioid growth factor (OGF) to distinguish its role in cell replication (7).

Endogenous Opioids—Source, Distribution

Precursors for both enkephalins and endorphins are posttranslationally modified to yield single or multiple copies of the end product endogenous peptide. The primary location for synthesis and regulation is the brain, in particular the pituitary.

STRUCTURE, SOURCE, AND DISTRIBUTION OF β-ENDORPHIN

Endorphins are derived from a single prohormone termed POMC (8–10). The POMC gene consists of three exons and when processed yields two large fragments identified as adrenocorticotrophin hormone (ACTH, 16 kD) and β-lipotrophin hormone (β-LPH). These proteins are further processed to yield the corticotrophin-like intermediate protein (CLIP), various forms of melanocyte-stimulating hormone (α-MSH, β-MSH, and γ-MSH), and β-endorphin. The POMC gene is conserved throughout evolution and is located on chromosome 2p23.3 in humans. Although the first five amino acids of β-endorphin code for [Met^5]-enkephalin, it is not considered a primary source for enkephalins. Most endorphins, of which there may be as many as 20 different derivatives, originate primarily in the pituitary and act as neurotransmitters, pain modulators, and anxiety suppressors. POMC is primarily expressed in the anterior and intermediate lobes of the pituitary, with each lobe being responsible for different peptide products (8). Corticotroph cells in the anterior pituitary secrete POMC peptides that control adrenal function, while melanotrophs of the

pars intermedia secrete α-MSH-associated peptides that influence hair and skin pigmentation. Nonneural tissues expressing POMC products include the adrenal, small intestines, reproductive tract, spleen, lung, liver, heart, and placenta. Given the diffuse presence throughout the body, POMC exerts a number of diverse functions (9, 10).

STRUCTURE, SOURCE, AND DISTRIBUTION OF ENKEPHALINS

The gene for several enkephalin peptides is pre-proenkephalin A (PPE) (11) from which six copies of [Met^5]-enkephalin and one copy of [Leu^5]-enkephalin, as well as a heptapeptide and octapeptide, are produced. The PPE gene is conserved, with prominent expression in the posterior pituitary, as well as axon terminals and cell bodies throughout the body. Comparable to POMC expression patterns, PPE has been detected in a variety of noncentral nervous system tissues including the adrenal medulla; the visual, gastrointestinal, and cardiovascular systems; and the placenta (12). Subcellular distribution of [Met^5]-enkephalin in epithelium was determined by dual-labeled immunoelectron microscopy (13). OGF (i.e., [Met^5]-enkephalin) and its receptor were colocalized on the paranuclear cytoplasm and in the nuclei of keratinocytes in the *stratum basale*. Ultrastructural studies of immunolabeled material using 5 and 10 nm gold particles demonstrated that while OGF was not always bound to the OGF receptor (i.e., OGFr), it was frequently associated with the outer nuclear envelope (13).

Mechanisms of Action and Receptor Mediation

Enkephalins and endorphins are opioid receptor agonists (3, 4, 14, 15), and their activity is very much dependent on receptor mediation. Opioid receptors include the mu, delta, and kappa classical opioid receptors that have a seven-member transmembrane binding site on the cytoplasmic membrane. Another receptor, with little or no gene or protein homology to the classical opioid receptors, was identified and termed OGFr—this receptor is located on the outer nuclear membrane and mediates OGF's inhibitory action on growth (13).

RECEPTOR MEDIATION—AGONIST ACTIVITY

Opioid activity associated with β-endorphin is dependent on its C-terminal residues and loss of these amino acids substantially decreases the analgesic property of the peptide. β-endorphin shares many of the physiological actions of exogenous opiates such as morphine and has been documented in animal studies to have effects on analgesia, respiratory depression, vasopressin release, and cardiovascular homeostasis (8). β-endorphin levels have been shown to increase during pregnancy, with the most elevated levels reported during labor and delivery. Studies over the last few decades have suggested that endorphins can bind to any or all of the classical opioid receptors (mu, delta, and kappa), and some studies have suggested that there is a specific receptor for endorphin termed the epsilon (ε) receptor (16, 17).

RECEPTOR MEDIATION—ANTAGONIST ACTIVITY

Receptor antagonists bind with different affinities to each opioid receptor disrupting the interaction between the enkephalin/endorphin agonist and the receptor (16, 17). Because the interactions can be reversible depending on the longevity of the antagonist–receptor complex, it is often the duration of the opioid receptor blockade that confers the action. Of importance to the therapeutic treatment of MS is the set of data showing that intermittent opioid receptor blockade based on LDN or single dosages of naloxone resulted in biphasic responses (18–20). Dichotomous biological responses following different dosages of naltrexone and thus different durations of opioid receptor blockade were first reported in 1983 (18). Low dosages (0.1 mg/kg) of naltrexone inhibited the growth of the neuroblastoma tumors, but higher dosages (10 mg/kg) of naltrexone were not more inhibitory and, in fact, resulted in enhanced tumor growth. This was the first indication that the action of receptor blockade did not directly correlate with antagonist dosage (18). These observations have been optimized to work in favor of therapeutic treatment of MS. Thus, LDN has become a widespread therapeutic used to safely inhibit inflammatory processes by inhibiting proliferation of T-lymphocytes and B-lymphocytes following a peripheral autoimmune trigger, and to inhibit T-cell infiltration into the CNS (17).

Functions of Endogenous Opioids

In general, β-endorphin binds to multiple opioid receptors and depending on the receptor, functions to diminish pain, equilibrates food metabolism, mediates cardiovascular regulation, as well as drives euphoric responses attributed to higher order emotional and neurological systems (9). It is suggested that since β-endorphin has few central nervous system–mediated effects when administered systemically because of the inherent difficulty for β-endorphin to cross the blood–brain barrier, the effects of mediating analgesia and respiratory depression are not directly attributed to the peptide (9). Classical functions of enkephalins include neurotransmission and pain modulation (1–6, 21, 22). Along with its role as a neurotransmitter, enkephalins alter calcium influx and cause direct hyperpolarization of neurons (22, 23). In regions of the spinal cord (e.g., substantia gelatinosa), pain perception is integrated by enkephalin-enriched fiber tracts. The periaqueductal gray region contains enkephalins that resolve analgesia and inhibit the release of excitatory neurotransmitters (6). High concentrations of enkephalins in the hypothalamus suggest a role for endocrine modulation. Other major enkephalin pathways are associated with motor activity, intestinal tract motility and peristalsis, limbic system regulation of emotional behavioral, and the hypothalamic neuroendocrine axis.

ENKEPHALINS AS GROWTH FACTORS

Although enkephalins were initially considered to function as neurotransmitters, in the early 1980s, it was demonstrated that one specific enkephalin—[Met^5]-enkephalin—regulated the growth of normal and abnormal cells and tissues, and

hence was renamed opioid growth factor (OGF) (7, 12, 24). OGF is a potent, reversible, species-unspecific, and tissue-nonspecific negative growth regulator with action that is opioid receptor mediated (3, 7, 12, 24). The peptide is autocrine and paracrine produced, secreted, and effective at concentrations consistent with physiological behavior. OGF is rapid in biologic action, quickly degraded, and obedient to intrinsic rhythms of the cell (e.g., circadian rhythm). With regard to the role of OGF in disease, OGF was successful at reducing tumor burden, limiting metastatic growth, and had few side effects (18). However, direct application of OGF is difficult to achieve outside of a clinical setting because OGF is rapidly metabolized and requires repeated infusions. Most cancer patients have normal or even elevated OGF serum levels but appear to lack sufficient numbers of intact OGFr.

Another group of diseases—autoimmune disorders—manifests with too little OGF. The hypothesis is that diminished levels of serum enkephalins are unable to control rampant proliferation of immune cells during a trigger event or flare. The etiology of MS remains a black box and most likely, there is no singular cause of MS. Endogenous opioids, or the lack thereof, may be a contributing factor, but the data are insufficient. At best, we are able to work with animal models, but unfortunately, animal models do not imitate MS precisely. The most consistent animal model establishes progressive MS, but most patients present with relapsing-remitting MS (RR-MS), and this form of MS has the least reliable animal model. Nonetheless, hypothesis-driven, controlled studies on the role of endogenous opioids and experimental autoimmune encephalomyelitis (EAE) have generated data, suggesting that enkephalins play an integral role in the disease process.

Preclinical Studies of Enkephalins and EAE

Two different animal models were established to study progressive EAE (25–29) and relapsing-remitting EAE (RR-EAE) (30–32). In the first model, C57Bl6/J black mice were immunized with myelin oligodendrocytic glycoprotein ($MOG_{35–55}$), whereas the SJL/J white mouse along with proteolipid protein ($PLP_{131–165}$) is required to establish the RR-EAE (25–32). Each animal model was established and subgroups treated with either OGF or LDN beginning either at the time of immunization (induction of disease) or after disease symptoms were visible for 2 days (established disease). In addition to clinical behavior, pathology, sensitivity, motor activity, as well as immune system responses were investigated.

CHRONIC EAE WITH OGF TREATMENT BEGINNING AT THE TIME OF INDUCTION OF DISEASE

Initial studies on the onset and progression of EAE examined OGF treatment beginning at the time of disease induction and reported that severity and disease indices were markedly reduced in OGF-treated mice relative to MOG-immunized mice receiving saline (25–27). Significant reductions in activated astrocytes and damaged neurons were observed in CNS tissue of animals treated with OGF;

likewise, no lumbar spinal cord demyelination was detected in the mice receiving OGF or LDN. This was in sharp contrast to mice receiving a high dose of naltrexone which blocked receptors continuously from OGF activity, and again, supported the mechanism that duration of opioid receptor blockade is critical in defining the outcome. Thus, OGF and LDN had no deleterious long-term repercussions and did not exacerbate EAE but halted progression of disease, reversed neurological deficits, and prevented the onset of neurological dysfunction over a considerable period of time.

CHRONIC EAE WITH OGF TREATMENT BEGINNING WITH ESTABLISHED DISEASE

OGF given at the time of induction arrested the progression of disease; however, the effects of OGF on *established* disease are more clinically relevant (28, 29). Studies wherein mice were immunized and then treated with OGF or saline beginning 2 days after showing signs of clinical EAE disease were established. Within 6 days of OGF treatment, animals demonstrated significant reductions (45% reduction) in their behavioral scores relative to mice receiving saline (Figure 1) (28). Behavior was attenuated for at least 40 days. Mice receiving OGF had only limp tails and wobbly gait in comparison with saline-treated EAE mice displaying paralysis of one or more limbs. OGF treatment initiated after the appearance of chronic disease also reduced the number of activated astrocytes and damaged neurons, and decreased demyelination and T-cell proliferation. More specifically, T-lymphocyte infiltration was evaluated by staining lumbar spinal cord sections with a CD3 antibody. After 20 days of drug treatment, CD3+ cell infiltration was reduced by 68% in EAE+OGF mice compared to the EAE+Vehicle group. Spinal cord demyelination was assessed by Luxol fast blue staining, and

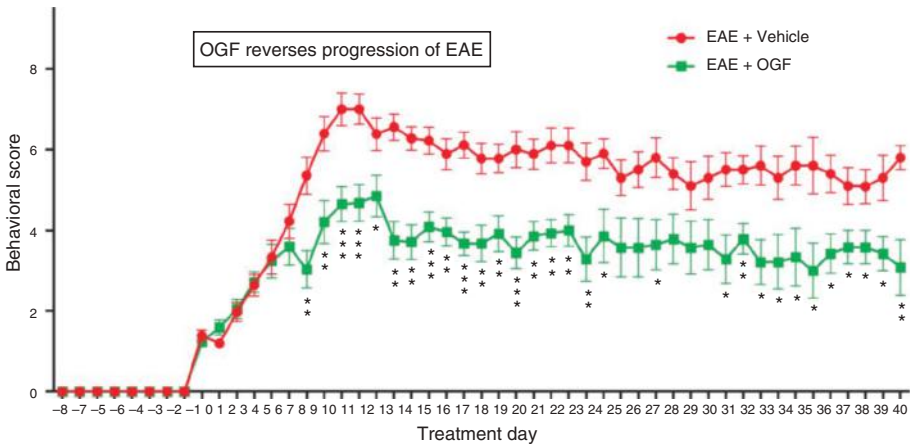

Figure 1 Clinical behavioral scores in C57Bl/6J mice immunized with $MOG_{35–55}$ to induce chronic, progressive EAE and treated daily beginning at the time of established disease with either saline (EAE+Vehicle) or 10 mg/kg OGF (EAE+OGF). Values represent behavioral scores (scale of 0–10) ± S.E.M. for at least 12 mice per group. Significantly different from saline controls at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). (Modified from Ref. (28).)

after ~20 days of EAE disease, EAE+Vehicle mice had approximately 13% demyelinated white matter in spinal cord cross sections, compared to 8% or less in EAE+OGF animals. Neuronal damage as assessed by staining with SMI-32 antibody revealed that after 20 days of treatment, EAE+Vehicle mice had 4-fold elevations in SMI-32-positive neurons compared to normal controls, whereas EAE mice receiving daily OGF had only 2-fold elevations in SMI-32-positive neurons. In summary, the data from studies on exogenous therapy with enkephalins (i.e., OGF) and the progressive model of EAE support the use of OGF as a biotherapy for MS (28, 29).

RR-EAE WITH OGF TREATMENT BEGINNING AT THE TIME OF INDUCTION

Nearly 85% of the 2.5 million patients worldwide have RR-MS. Disease manifestation involves proliferation and activation of T-lymphocytes, microglia, and astrocytes, leading to inflammation, demyelination, and axonal damage. An animal model of RR-MS using proteolipid protein ($PLP_{139–151}$) immunization of SJL/ mice was established to study RR-EAE (30–32). Within 9 days of immunization, behavioral signs of RR-EAE were observed. When OGF was administered at the time of disease induction, OGF-treated RR-EAE animals had less severe clinical disease than mice receiving saline and exhibited 66% reduction in median cumulative disease scores as well as prolonged periods of remission and diminished number and length of relapses (30). Neuropathological examination of lumbar spinal cord revealed reductions in the number of T-lymphocytes, microglia/macrophages, and activated astrocytes, with cell proliferation being targeted by OGF. Areas of myelination and neuronal damage were markedly reduced following OGF treatment during the 55-day observation period. OGF treatment led to the prevention of behavioral relapse for more than 36 days following the initial flare, with 85% of the mice returning to behavioral scores of 0 or 0.5 over the course of 5.5 weeks, and more than 70% of the mice showing remissions for more than 2 days. However, OGF administration at this dosage did not prevent the disease, nor did it "cure" the disease completely in any mouse.

RR-EAE WITH OGF OR LDN TREATMENT BEGINNING AT THE TIME OF ESTABLISHED DISEASE

Given the importance that OGF therapy was effective for relapsing EAE when the drug was given at the time of disease induction (30), a study was conducted on the effects of OGF treatment (31) or LDN (32) on established RR-EAE, with injections beginning 2 days after initial clinical signs of disease. Mice were immunized with subcutaneous injections of 100 mg of myelin proteolipid $protein_{139–151}$. Clinical disease appeared with 9 days of immunization, and either OGF or LDN treatment was initiated. OGF reduced clinical behavioral scores and increased the number and duration of remissions (Figure 2). Over the course of 40 days of treatment, 42% of mice in the RR-EAE+OGF group had at least one remission compared to only 1 of 13 mice in the RR-EAE+saline group. Five OGF-treated mice appeared to remain in a permanent remission. Spinal cord neuropathology was suppressed in OGF-treated mice. In particular, astrogliosis

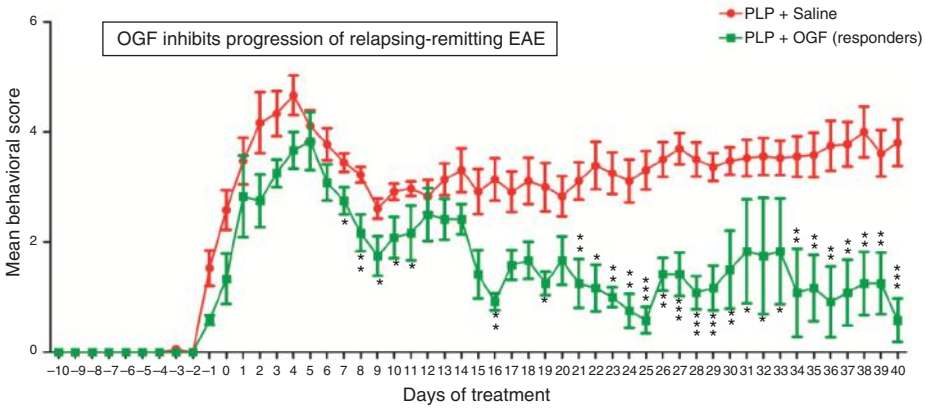

Figure 2 Clinical behavioral scores in SJL/J mice immunized with $PLP_{135–151}$ to induce relapsing-remitting EAE and treated daily at the time of established disease with either saline (PLP+Saline) or 10 mg/kg OGF (PLP+OGF). Values represent behavioral scores (scale of 0–10) ± S.E.M. for at least 12 mice per group. Significantly different from saline controls at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Unpublished data.

was markedly reduced in comparison to saline-treated animals with RR-EAE. In a second series of investigations on RR-EAE, mice were immunized and, following the appearance of clinical disease, were injected with 0.1 mg/kg naltrexone (LDN) or saline daily for 40 days. Clinical behavior was markedly reduced in the RR-EAE mice receiving LDN relative to those mice on saline. Moreover, the length of complete remission was markedly elevated for mice receiving LDN, and the length of relapses was significantly decreased. These studies provide preclinical evidence that elevated enkephalins induced by either direct OGF injections or LDN therapy could provide positive changes in behavior and possibly extend periods of remission for individuals with MS.

OGF REDUCTION OF T-LYMPHOCYTES AND B-LYMPHOCYTES

The mechanism by which animal models for MS are derived involves the basic properties of immunization. Mice are inoculated with adjuvant containing myelin proteins and within days T-lymphocytes and B-lymphocytes are stimulated in peripheral tissues (e.g., spleen and lymph nodes) and begin to proliferate and then migrate to the central nervous system. Several studies were undertaken to examine the role of enkephalins, and the OGF–OGFr regulatory pathway, in T-cell and B-cell proliferation during each of these events (33–36). Initial studies investigated *in vitro* stimulation of T-cells and B-cells (33, 34). While this model falls short of mimicking clinical reality, the studies revealed that direct application of OGF or LDN to activated splenocytes inhibited T-cell and B-cell proliferation without requiring intervention from other immune system mediators.

Animal studies using both models of EAE confirmed our findings that OGF, exogenously or endogenously stimulated following LDN, inhibited T-cell and

B-cell replication *in vivo* (35, 36). Examination of peripheral lymphocyte dynamics following immunization of mice with MOG antigen and treatment with OGF or LDN was conducted over a 2-week period following immunization (35, 36). Isolated lymphocytes from spleens and draining inguinal lymph nodes were counted by flow cytometry, and the subpopulations of CD4+ and CD8+ T-cells, as well as B-lymphocytes, were noted. Within 5 days of treatment with exogenous OGF or LDN, the number of CD4+ and CD8+ T-lymphocytes in MOG-injected mice (no evidence of disease at this early time point) treated with OGF or LDN were reduced on average by 30% from immunized, saline-treated mice. After 12 days of injections, mice receiving OGF or LDN had 32–37% reduction in the number of CD4+ T-cells, and 35–42% reduction in CD8+ T-cells isolated from the spleen relative to cell number for saline-injected mice. As expected following immunization, B-cell number was elevated 2-fold in MOG-immunized mice relative to nonimmunized normal mice. OGF and LDN treatments markedly reduced the number of B220+ B-cells by approximately 29% from the saline-injected MOG mice (35).

Additional investigations on the intracellular distribution of CNS-derived lymphocytes from lumbar spinal cord tissue were conducted on material collected on day 15 of OGF or LDN treatment. Cell homogenates were labeled with markers for CD4+ T-cells, as well as for cytokines that were expressed on Th1, Th2, and Th17 subsets of T-cells (35). OGF treatment resulted in approximately a 2-fold increase in the percentage of total lymphocytes that were CD4+ T-cells relative to the number recorded for saline-treated, MOG-immunized mice, as well as increasing the percentage of Th1-cell and Th17-cell subpopulations compared with saline-treated mice. LDN treatment did not alter the number of Th1, Th2, and Th17 subsets within 15 days (35), and no further studies have been pursued. In conclusion, exogenous enkephalins (i.e., OGF) or endogenous OGF following LDN suppressed T-lymphocyte and B-lymphocyte proliferation in the spleen and inguinal lymph nodes in the chronic progressive model of EAE specifically repressed replication of CD4+ and CD8+T-cells and B220+ B-lymphocytes in the spleen and lymph nodes of immunized mice within a week of immunization.

To examine the effects of enkephalins on the RR-EAE, autoreactive $CD4^+$ T-cells were followed as they migrated from peripheral tissues into the CNS (36). Immunohistochemical studies demonstrated that CNS-infiltrating $CD3^+$ T-cells are diminished with exogenous OGF or LDN administration. Investigation of Th effector responses in CD4+ T-lymphocytes in the CNS suggested that modulation of the OGF–OGFr axis did not result in changes to Th1 or Th17 pro-inflammatory cytokines IFNγ and IL-17, respectively, nor were there changes in the activity of anti-inflammatory Th2, IL-4 secreting cells. Overall, cell number was diminished, supporting the concept that enkephalins are immunomodulatory because of their anti-proliferative action.

Clinical Studies

Substantial progress has occurred in the treatment of MS over the last several years. At least 12 disease-modifying therapies (DMTs) have received FDA approval, and a few have been developed as oral medications (37–39). However, the financial

burden of individual therapy can range upward to $60K annually (37), and side effects still reduce compliance and thereby overall efficacy (38). Randomized clinical trials of enkephalins or LDN are limited (40–43), possibly because use of LDN has been reported to have a few side effects, and large pharmaceutical companies are not interested in sponsoring studies on a repurposed drug (i.e., LDN) that is already FDA approved at substantially higher dosages. Nonetheless, there remains a need for safe, effective treatments that are alternatives to the β-interferon products. With the widespread use of LDN (42–44) and the information available on many websites devoted to LDN, physicians are cautiously prescribing LDN.

Our findings in animal studies suggest that the endogenous opioid system is a worthwhile target for designing novel therapeutic interventions for MS. Two studies utilizing patient data from the Penn State Hershey Neurology Clinic revealed that individuals diagnosed with MS and offered LDN had no discernible side effects over extended periods of time (45, 46). A chart review performed through RedCap database focused on 215 MS patients who were provided a prescription for oral LDN (45). The study found that a significant number of patients benefit with LDN and an immodulating agent. Some patients preferred to take LDN as a monotherapy. The LDN did not cause any unexpected side effects. A second retrospective study was conducted at the Penn State Hershey Medical Center in patients who were diagnosed with RR-MS for up to a 9-year period (46). One group of patients (n = 23) were initially prescribed LDN the first time they visited the medical center. A second group of patients (n = 31) were treated with glatiramer acetate (Copaxone) and offered LDN as an adjunct therapy to their DMT. Patient visits after 1–50 months were evaluated in a retrospective manner. Data were obtained from patient charts that included laboratory values from standard blood tests, timed 25-feet walking trials, and changes in magnetic resonance imaging (MRI) reports. Statistical analyses between the groups and for each patient over time indicated no significant differences in clinical values, timed walking, or changes in MRIs following LDN alone. These data suggested that the inexpensive, nontoxic, biotherapeutic is safe and if taken alone did not exacerbate the disease symptoms.

Extension of this work has resulted in studies that have shown that animals with EAE (47, 48) or individuals with MS (48) have decreased enkephalin levels. Treatment with OGF or LDN restored serum enkephalin levels to normal and often correlated with reduced clinical behavior and restored sensitivity to pain and heat in mice. The animal work facilitated measurement of serum enkephalins in a longitudinal manner and was able to demonstrate that normal animals inoculated with $MOG_{35–55}$ antigen expressed decreased enkephalins as the disease progressed (47, 48). This work is the first to suggest that OGF (chemically termed [Met^5]-enkephalin) may be a specific marker for the onset of MS. Larger clinical trials measuring the serum enkephalins beginning at the time of first diagnoses, clinically isolated syndrome, are needed to confirm these observations. Nonetheless, the reports of aberrant enkephalin levels are not surprising given that exuberant proliferation of immune cells (e.g., T-cells and B-cells) are associated with MS, and that often administration of enkephalin to animal models was "immunosuppressant." While the end result was accurate (i.e., fewer T-cells and B-cells), the mechanism was not immunomodulatory, but rather inhibited cell replication related to the interaction of OGF and OGFr.

Conclusion

The role of endogenous opioids in the cause and treatment of autoimmune diseases is at its infancy. Our focus on OGF and blockade of OGF action with naltrexone has provided a platform for preclinical studies of enkephalins and their role in MS. OGF is an inhibitory growth factor that downregulates replication of immune cells in response to antigens. OGF also inhibits gliosis that leads to the release of cytokines and inflammatory markers that facilitate demyelination and neurodegeneration. While there is no confirmatory data yet that low levels of enkephalins are suitable markers of other autoimmune diseases, there are a growing number of basic science and clinical reports that enkephalins, either exogenously administered or endogenously stimulated following receptor blockade with LDN, are effective treatments for progressive and RR-EAE and RR-MS.

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9 Immunomonitoring Lymphocyte Subpopulations in Multiple Sclerosis Patients

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Abstract: Advances in the understanding of pathogenic mechanisms of diseases have led to the defining of new biomarkers for diagnosis, prognosis, and therapy response. In this context, flow cytometry has been positioned as one of the most useful technologies for monitoring immune-mediated diseases, such as multiple sclerosis (MS), allowing a detailed analysis of lymphocyte subpopulations in peripheral blood. The autoimmune inflammatory response in MS results in changes in lymphocyte subpopulations that might be useful as surrogate markers for the evaluation of disease activity, progression, and monitoring of therapy response. This chapter discusses the role of T-lymphocyte and B-lymphocyte subpopulations in MS pathogenesis, the effect of MS treatments on these subsets, and their potential usefulness as biomarkers of treatment response.

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Introduction

There is evidence of patients with the same disease responding differently to the same treatment. Thus, it is necessary to define biomarkers to stratify patients, monitor the course of the disease, and predict response to treatment. Peripheral blood leukocytes play an important role in the pathogenesis of autoimmune diseases. It has been demonstrated that immunomodulatory treatments decrease the percentage of these cell populations, alter the expression of their surface markers, and modify their functionality (i.e., cytokine production, proliferation, and induction of apoptosis). For these reasons, it has been hypothesized that systematic analyses of peripheral blood immune cells could serve as surrogate biomarkers of activity of the disease and/or response to therapy, leading to the development of personalized medicine (1–4).

FLOW CYTOMETRY, A TOOL FOR IMMUNE-MONITORING

Flow cytometry enables the analysis of a panel of surface molecules at single-cell level that not only determines the percentages of peripheral lymphocytes but also their differentiation stage. In addition, the activation state of peripheral lymphocytes and their memory or effector functions can be measured. Recent advances in the development of multiparametric flow cytometry have made detailed characterization of lymphocyte subsets possible in whole blood or isolated peripheral blood mononuclear cells (PBMC) of healthy donors and patients, and it has been presented as a powerful tool for immunomonitoring of response to treatment (5, 6). Concurrent to this development, several international consortia have been created to standardize immune-monitoring using flow cytometry for immune-mediated diseases, transplantation, and hematological diseases, for potential use in clinical settings (7–9).

Pathogenic Mechanisms of Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, inflammatory demyelinating disease of the CNS, characterized by infiltration of T-lymphocytes, B-lymphocytes, macrophages, NK cells, demyelination, and axonal damage (10–12). The etiology of MS remains unknown; however, it has been proposed that there is a selective autoimmune response against myelin autoantigens causing damage to the CNS. However, like the majority of autoimmune diseases, the triggers of this response are unknown. Both environmental and genetic factors have been postulated. A 40% concordance in monozygotic twins as well as association with HLA-DRB1*1501 and DQB1*0602 alleles have been described (11, 13). GWAS studies in MS patients have shown the involvement of several loci related to the immune system, of which the HLA locus presents the highest association (14–16).

The existing evidence on the induction and perpetuation of the disease points to an important role of autoreactive CD4+ T-cells (2). Studies in the animal model of MS, experimental autoimmune encephalomyelitis (EAE), have shown that the effector CD4+ T-subpopulations, Th1 and Th17, play an important role in the pathogenesis of the disease. These subpopulations have been found increased in the CNS of patients with MS, mainly in CSF and the perivascular space (3, 4). In addition, oligoclonal expansions of activated CD8+ T-cells in CNS lesions of MS patients have been described, indicating their participation in CNS damage (5, 6). The involvement of B-lymphocytes in the pathogenesis of MS is better understood: they produce autoantibodies; induce, maintain, and reactivate CD4+ T-cells; act as antigen-presenting cells; and produce pro-inflammatory cytokines (7). Impairment in the immunoregulatory function of NK cells in MS patients has also been decribed (12). A schematic overview of the roles of immune cells in MS pathogenesis is represented in Figure 1.

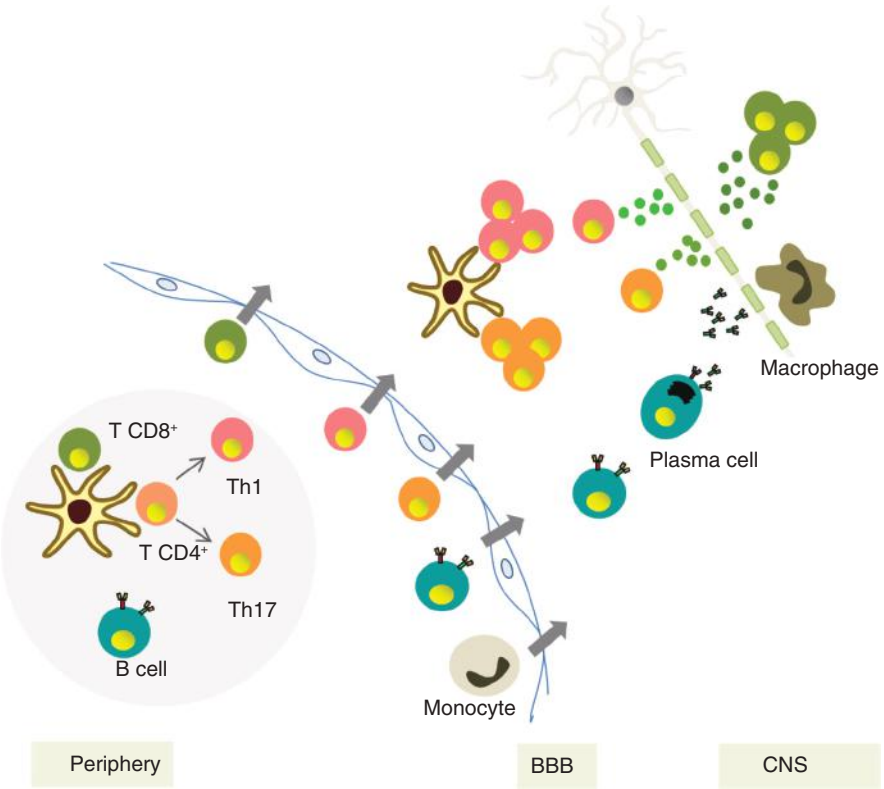

Figure 1 Pathogenic mechanisms of multiple sclerosis. Autoreactive T-cells and B-cells are activated in peripheral lymph nodes where they are differentiated into effector cells, CD8+ T-cells, and CD4+ T-cells (Th1 and/or Th17). Activated cells migrate through the blood–brain barrier (BBB) where they are further activated by local antigen-presenting cells. These processes induce cytokine and chemokine production, facilitating the entry of other cell types from peripheral blood. At the central nervous system (CNS), macrophages and activated T-cells attack myelin components and release cytokines that activate B-cells which mature to antibody-producing plasma cells. This increases the inflammatory response and causes demyelination and axonal damage.

Lymphocyte Subpopulations in MS

The autoimmune inflammatory response in MS results in changes in lymphocyte subpopulations of peripheral blood (17–20). These changes might be useful surrogate markers for the evaluation of disease activity, progression, and monitoring of therapy response.

T-CELL SUBPOPULATIONS

T-cell subpopulations can be divided into naïve, central memory, effector memory, and other minor effector subsets such as terminally differentiated effector cells (T_{EMRA}), based on the expression of CD45RA, CCR7, and CD27 (7, 21). Studies published until now regarding T-cell subpopulations in MS patients are discrepant. Differences among studies might be due to different genetic backgrounds, stages of the disease, analysis of small groups of patients, and also different monoclonal antibodies used to define T-cell subpopulations. These discrepancies are particularly relevant in studies regarding $CD8^+$ T-subpopulations. Whereas some authors report an increase of effector $CD8^+$ T-cells (22, 23), other authors describe a decrease in effector memory and T_{EMRA} $CD8^+$ T-cells in peripheral blood (24). Analysis of the cellularity of the CNS infiltrates show enrichment in the number of effector memory and T_{EMRA} $CD8^+$ T-cells in patients with MS and other inflammatory neurological diseases (25, 26). In these studies, the increase in central memory and effector memory $CD8^+$ T-cells in peripheral blood, and in CSF, were related to active disease or early-stage disease. In contrast, in patients with less active disease, no changes in central memory $CD8^+$ T-cells or the percentages of $CD8^+$ early effector memory in peripheral blood were found, although a decrease in absolute counts of $CD8^+$ early effector memory T-cells could be observed, which would suggest that in MS patients these cells migrate to the CNS (17).

TH17 AND TREG SUBPOPULATIONS

The increased percentage of Th17 in the peripheral blood of RRMS patients has been widely reported and a pathogenic role for these cells postulated (27, 28). Moreover, Th17Th1 cells, a subpopulation which secretes both IL-17 and IFN-γ, have also been related to MS pathogenesis (29). Regarding Treg subpopulations, most of the reports found a similar percentage of Tregs in MS patients compared with healthy donors, although a functional impairment has been found in *in vitro* assays (30–32). In this context, an increase of the Th17/Treg balance has been associated with higher disease activity and severity (20, 33).

B-CELL SUBPOPULATIONS

Although the involvement of B-lymphocytes in the pathogenesis of MS has been a focus in recent years, a full characterization of B-cell subpopulations in peripheral blood of MS patients is still lacking (34, 35). Most of the studies on B-cells are focused on their changes in response to treatments (36, 37).

Current Therapies for MS and Their Effect on Lymphocyte Subpopulations

Even though a number of new drugs have been developed to treat MS, a treatment that can cure the disease has not been developed as yet. Approved treatments reduce the frequency of relapses and decrease inflammation but fall short of stopping CNS degeneration. Current treatments can be divided basically into two groups: those that treat acute relapses (megadoses of metilprednisolone) and disease-modifying therapies (DMTs). DMTs include classic injectable drugs (interferon-β and glatiramer acetate (GA)), oral substances (fingolimod, terifunamide, and dimethyl fumarate (DMF)), and monoclonal antibodies—anti-CD49d (natalizumab) and anti-CD52 (alemtuzumab). Other monoclonal antibodies such as anti-CD25 (daclizumab) and anti-CD20 (ocrelizumab) that cause depletion of B-cells are expected to be in the clinics soon. DMT treatments have broad immunemodulatory/immunosuppressive effects affecting peripheral blood subpopulations (38–41). The major changes in lymphocyte subpopulations in response to DMT treatments are summarized in Table 1.

INTERFERON β (1A AND 1B)

Interferon β (IFN-β) was the first treatment approved for MS. It decreases the number of relapses, progression of disability, and disease activity (measured by MRI). The mechanism of action of IFN-β, although extensively studied, is not fully understood. The known mechanisms include a decrease in lymphocytes activation and proliferation, a reduction in pro-inflammatory cytokines production, and an increase in anti-inflammatory cytokines. IFN-β has a nonspecific immunomodulatory effect on various immune cells, and it has been demonstrated that it interferes with the transmigration of leukocytes through the blood–brain barrier (BBB). This treatment induces a weak leukopenia, an increase of IL-10 that has been associated with an increase of both $CD4^+$ and $CD8^+$ T regulatory cells, and $CD56^{bright}$ NK cells (42–44). Moreover, some studies described a decrease of IL-17 production, and Th17 cells, in peripheral blood in MS patients under IFN-β treatment (45, 46). It has also been described that the effect of IFN-β causes a decrease of activated and memory T-cells (44, 47); on the other hand, it induces an increase of B-cells production—an increase in transitional (immature) Bccells and k-deleting recombination excision circles (KRECs), thereby supporting its use for increasing B-cell release from bone marrow (17, 48). Its effect on thymic egress of recent thymic emigrants (RTEs) is still unclear, but it seems that IFN-β may induce a decrease of RTEs and TCR recombination excision circles (TRECs) in peripheral blood (48, 49).

GA OR COPOLYMER-1

It is a polymer composed of the most frequent aminoacids in the myelin basic protein (L-tyrosine, L-glutamate, L-alanine, and L-lysine) (13). Its mechanism of action is poorly understood, but it is postulated that GA acts by binding the major histocompatibility complex class II molecules, competing with other antigens as

TABLE 1 Main changes in lymphocyte subpopulations induced by DMT treatments

Drug	ACL	T-cells	CD4+ T-cells	CD8+ T-cells	T-Cells Memory Subsets	RTEs	Th17	Tregs	B-Cells	B-Cells Memory Subsets	B Transitional/ Immature	Bregs
Interferon	↓	↓			↓ memory and activated	NC/↓	↓	↑			↑	
Glatiramer acetate					↑ Th2			↑				
Natalizumab	↑	↑	↑	↑	NC	↑	=	NC	↑	↓ naive ↓ memory	↑	
Fingolimod	↓	↓	↓	↑	↓ naive/CM ↑ EM/TEMRA	↑	NC	↑	↓	NC	↑	↑
Dimethyl fumarate	↓				↑naive ↓EM ↑Th2			=/↑			↑	↑
Alemtuzumab	↓	↓	↓	↓	↑ EM ↑ TEMRA		=	↑	↑			
Teriflunomide	↓				↓ activated*				↓	↓ activated*		

ACL = absolute count lymphocytes, CM = central memory, EM = effector memory, TEMRA = terminally differentiated effector cells, RTEs = recent thymic emigrants; NC = nonconclusive.
*Inhibition synthesis of rapidly dividing lymphocytes.

myelin basic protein, and inhibiting the activation of myelin basic protein-specific T-cells (50, 51). GA has a nonspecific effect on the immune system because no specific changes have been described in peripheral blood of patients under treatment. Some studies describe that GA induces a shift in the CD4 T-cells' response to a Th2 profile. Moreover, it has been proposed that it induces an increase in Treg subpopulation (50, 52).

DIMETHYL FUMARATE

DMF is an oral drug of the fumaric acid ester. It induces activation of the transcription factors Nfr2 (decreasing inflammation) and NF-κB (modifying cytokines production), and diminishes neuroinflammation by promoting the cytoprotection of CNS cells against oxidative stress (41). DMF induces a pronounced lymphopenia that has been associated with the occurrence of rare and fatal cases of progressive multifocal leukoencephalopathy (PML) associated with JC virus infection (53, 54). DMF reduces the number of lymphocytes with a decrease of B-cells and $CD4^+$ and $CD8^+$ T-cells. A decrease of central and effector memory T-cells with a concomitant expansion of naïve T-cells in peripheral blood of patients under treatment with DMF have been reported. Moreover, a shift in T helper (Th) subpopulations (a decrease in Th1 and Th17, and an increase in Th2 and regulatory T-cells) has been reported (55–58). Regarding B-cell subpopulations, an increase of a subset of B-cells with regulatory capacity has been described (59).

TERIFLUNOMIDE

Teriflunomide is an active metabolite of leflunomide, an approved treatment for other autoimmune diseases. It inhibits dihydroorotate dehydrogenase, blocking the *de novo* pyrimidine synthesis that is required by rapidly dividing lymphocytes, resulting in a reversible cytostatic effect that limits the expansion of stimulated T-cells and B-cells. It is administered orally (60–62). Teriflunomide impairs the production of activated lymphocytes (inhibiting their proliferation). Specific changes in lymphocyte subpopulations have not been reported.

FINGOLIMOD

Fingolimod is the first oral drug approved for MS treatment. It is a structural analogue of sphingosine and its phosphorylated metabolite, sphingosine 1-phosphate (S1P). S1P and its receptor ($S1P_1$) mediate the circulation of T-cells and B-cells between blood and lymph nodes (LNs). In physiological conditions, the interaction between S1P and $S1P_1$ promotes their egress from LNs by overcoming retention signals as the chemokine receptor CCR7. Naive and central memory T-cells as well as B-cells express CCR7. In contrast, effector memory T-cells and terminally differentiated effector T-cells (T_{EMRA}) are $CCR7^-$ and may egress from LNs independently of $S1P_1$ receptor. Fingolimod binds to four of the five subtypes of S1P receptors, causing the internalization and degradation of these receptors, and consequently blocking the egress of $CCR7^+$ lymphocytes from LNs (21, 63, 64). The main effect of fingolimod is a decrease of $CCR7^+$ cells in peripheral blood, specifically of naïve and central memory T-cells (65–68). In contrast to T-cells,

B-cell subsets have not been extensively studied in patients under fingolimod treatment. Literature on the effect of fingolimod in naïve and memory subset subpopulations is scarce and equivocal (69–71). An increase in immature and transitional B-cells (71, 72) and Treg cells has been reported in peripheral blood of MS patients under fingolimod treatment (67, 70, 73–76), supporting the conclusion that fingolimod can exert an alternative immunomodulatory mechanism inducing the production of Treg cells, as previously suggested by *in vitro* and *ex vivo* experiments (77–79). Results regarding the effect of fingolimod on Th17 cells are inconclusive and contradictory (67, 72, 75, 80). This is probably a consequence of the diversity in surface markers used to define this T-cell subset. Specifically, CCR7 (a clue marker for cells homing to LNs) can differentiate effector Th17 cells ($CCR7^-$) from central memory or pre-Th17 cells ($CCR7^+$). In a longitudinal study (72), we detected an increase in the percentages of effector Th17 cells, defined as $CD4^+CCR7^-CCR6^+CCR4^+$ following the international consensus of 2008 (21), in accordance with other studies (67). In contrast, Mehling et al. observed, in a cross-sectional study, that Th17 lymphocytes of MS patients were predominantly central memory Th17 and that their percentages were decreased in patients under fingolimod treatment compared with untreated MS patients and healthy donors. These authors did not analyze the effector Th17 subpopulation (80).

ALEMTUZUMAB

It is a humanized monoclonal antibody against CD52, recently approved for MS treatment (previously approved and widely used in the treatment of leukemia). It is administered via intravenous route (13, 41). As CD52 is a panleucocitary molecule, it promotes a rapid, marked, and sustained depletion of T-lymphocytes and B-lymphocytes, NK cells, monocytes, and some granulocytes. Studies performed in a transgenic mouse model postulated that the mechanism of lymphocyte depletion is predominantly antibody-dependent cytolysis (81). A decrease in the percentage of T-cell subpopulations at day 7 posttreatment with the onset of reconstitution 1 month after treatment has been described (82). Although $CD4^+$ and $CD8^+$ T-cell depletion lasts for months after treatment, there is a selective delayed reconstitution of some $CD4^+$ T-cells subsets that remain decreased for up to 24 months after treatment (82, 83). In contrast, there is an increase in the percentages of Tregs with an increase of suppressive activity. No differences in Th1 and Th17 percentages have been reported after reconstitution of the $CD4^+$ T-cell pool (83).

$CD8^+$ T-cell pool reconstitution is faster than $CD4^+$, normalized at the third month after treatment with the dominance of effector subsets (T_{EMRA}) for at least 24 months (82, 84). These results indicate that T-cell recovery is due to homeostatic expansion. In contrast to T-cells, the repopulation of $CD19^+$ B-cells reaches percentages above baseline in the first 12 months of treatment (85). Interestingly, in B-cell reconstitution, there is an output from bone marrow reflected in a significant frequency of immature B-cells in the first months after treatment. The B-cell pool is dominated by memory B-cells at 12 months after treatment; however, they remain below the baseline levels (86). The efficacy of alemtuzumab has been found to last longer than the lymphocyte depletion, probably due to the fact that after treatment there is a reconstitution with a different lymphocyte repertoire (87).

Furthermore, the selectively delayed CD4+ T-cell repopulation can contribute to the suppression of the disease activity (82). The main adverse effect of alemtuzumab is autoimmunity, the most frequent being thyroid autoimmunity, that appears in 30% of patients after treatment (84, 85, 87). The development of autoimmunity could be explained by the homeostatic expansion that occurs in the T-cell pool reconstitution (84).

NATALIZUMAB

Natalizumab is a humanized monoclonal antibody against CD49d (subunit α4 of VLA-4 integrin). The strong adhesion between VLA-4 of lymphocytes and VCAM-1 of the endothelium is very important for the migration of leucocytes through the BBB and entry to the CNS. Natalizumab is administered intravenously, and it binds to CD49d, blocking the transmigration of leucocytes through the BBB. This treatment decreases the occurrence of relapses by up to 90%, inducing a decrease of disease progression and MRI activity. The main side effect of natalizumab is the risk of developing PML caused by JC virus infection, which is associated with high mortality. As natalizumab blocks the transmigration of leukocytes through the BBB, in the peripheral blood of MS patients under treatment with natalizumab, there is an increase in the absolute numbers of B, T CD4+, T CD8+ (without alterations in CD4/CD8 ratio), and NK cells (88–90). The effect of natalizumab on lymphocyte subpopulations is not fully defined, although it has been described that memory T-cells would be increased in peripheral blood and would induce changes in memory B-cells (90–92). Moreover, natalizumab treatment interferes with the mechanisms of bone marrow egress of hematopoietic stem cells, inducing an increase of CD34+ cells in peripheral blood, specifically lymphoid progenitors, transitional B-cells, and RTEs (17, 91, 93–97).

Changes in Lymphocyte Subpopulations as Biomarkers of Therapy Response

Immunomonitoring of peripheral lymphocyte subpopulations may be useful to assess treatment response. In DMF treatment, patients with stable disease had lower numbers of CD4+, CD8+ T, and B-cells than those with active disease (98). Moreover, percentages of CD8+ T-cells and B-cells at 6 months after treatment could predict response to treatment (98). Regarding response to fingolimod treatment, Song et al proposed that percentages of central memory CD4+ T-cells could predict relapse (76). In a pilot study, our group described that the baseline percentage of RTEs and transitional B-cells are lower in responder patients. Therefore, immunomonitoring their percentages could be a tool for predicting which patients would be good candidates to receive fingolimod treatment. Moreover, the percentage of late effector memory CD4+ T-cells and RTEs could provide information on the response to therapy as early as 1 month after starting this therapy (72). Using quantitative flow cytometry as a tool for immune-monitoring, a method for immunomonitoring CD49d receptor occupancy in MS patients under natalizumab therapy has been reported. Using this method, it is possible to determine

the percentage of CD49d molecules bound to natalizumab and identify those patients with low receptor occupancy (suboptimal doses), which in a long-term sustained therapy context would show a decrease in treatment efficacy (99).

Conclusion

DMTs induce changes in lymphocyte subpopulations that can be detected in peripheral blood using flow cytometry. Treatment with monoclonal antibodies (natalizumab and alemtuzumab), fingolimod, and DMF induces a clear effect on different peripheral blood lymphocyte subpopulations. In contrast, IFN-β, GA, and teriflunomide produce nonspecific changes. Immunomonitoring lymphocyte subpopulations allows to define biomarkers of therapy response and opens up the opportunity to initiate a personalized therapy in MS treatments, enabling clinicians to choose the best treatment for each patient and predict which patients are the most suitable for receiving a specific therapy.

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Novel Approaches of Oxidative Stress Mechanisms in the Multiple Sclerosis Pathophysiology and Therapy

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Abstract: It is suspected that the development of multiple sclerosis (MS) can be affected by oxidative stress (OS). In the acute phase of the disease, OS is responsible for initiating inflammation, whereas in the chronic phase it sustains neurodegenerative process. Redox processes in MS are related to dysregulation of axonal bioenergetics, cerebral iron accumulation, mitochondrial dysfunction, impaired oxidant/antioxidant balance, and OS memory. This chapter gives an overview of the role of OS in MS.

Key words: Antioxidants; Antioxidative enzymes; MS biomarkers; Multiple sclerosis; Oxidative stress

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Introduction

Multiple sclerosis (MS) is a multifactorial disease of the central nervous system (CNS), characterized by inflammation, demyelination, and axonal loss. MS is considered a biphasic disease with inflammatory relapsing-remitting (RR) and degenerative secondary progressive (SP) phases (1). The ultimate causative factors of these processes remain unknown. Emerging evidence suggests a role for oxidative stress (OS) in demyelination (1–3). This chapter summarizes the role of OS in the pathology of MS and the potential of oxidant scavengers as therapeutics for the treatment of MS.

Mechanisms of OS

An imbalance between the production of free radicals and the antioxidative defense leads to OS and nitrosative stress (4, 5). Free radicals are defined as unstable, short-lived, and highly reactive molecules - containing one or more unpaired electrons in the valence shell or the outer orbit.

As a result of the high reactivity, free radicals can abstract electrons from other molecules which lose their electron and the molecule becomes a free radical itself, initiating a chain reaction cascade which finally damages the living cell (4). Free radicals, that is, the reactive oxygen species (ROS) and reactive nitrogen species (RNS), may have an influence on crucial classes of biological molecules, which results in multiple lipid and protein damage due to peroxidation and nitration processes (4, 6). ROS and/or RNS are involved in many essential physiological functions such as immune regulation (i.e., defense against pathogens), mitogenic response, cellular signaling, and redox regulation (4, 7). Both ROS and RNS can be grouped into two subgroups: radicals and nonradicals (4, 8) (Figure 1). Superoxide radical, hydrogen peroxide, hydroxyl radical anion, nitric oxide (NO), and peroxynitrite are thought to be involved in the development of MS (8, 9). The superoxide radical exists in two forms: superoxide and hydroperoxyl radical anion. It is mostly produced in the mitochondria. Under physiological pH, superoxide is the most common ROS that reduces iron complexes such as cytochrome c and ferric ethylene diaminetetraacetic acid, and oxidizes ascorbic acid and tocopherol (4). The hydroperoxyl radical can easily enter the phospholipid bilayer of cell membranes (4).

The enzymes that can produce superoxide include xanthine oxidase (10), lipoxygenase, cyclooxygenase (11), and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidase (12). Hydrogen peroxide is formed *in vivo* in a dismutation reaction catalyzed by superoxide dismutase (SOD). It can cross biological membranes and damage DNA by forming hydroxyl radical, which can react with organic and inorganic molecules (13). It is formed during the Fenton reaction, between hydrogen peroxide and metal ions (Fe or Cu). It is often bound to ferritin and ceruloplasmin or other molecules. Under stress conditions, the superoxide anion radical releases free iron from ferritin. The released free iron participates in the Fenton reaction to form the hydroxyl radical (4).

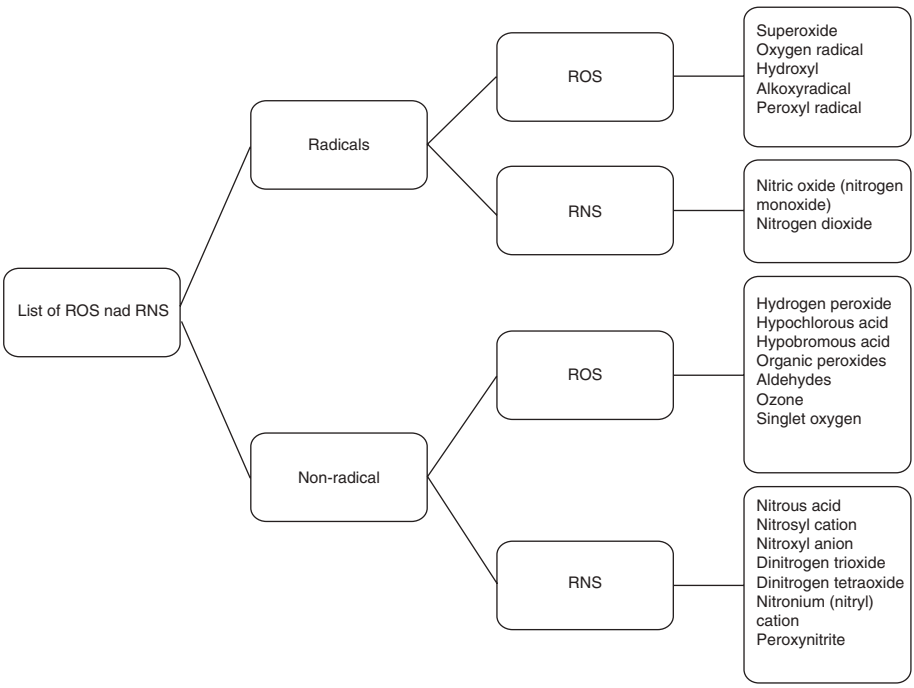

Figure 1 Reactive oxygen species (ROS) and reactive nitrogen species (RNS) (5, 9–11). The classification of ROS and RNS depended on having an unpaired electron. Nonradial species exists without an unpaired electron.

Nitric oxide is produced by nitric oxide synthases (NOSs). NOS isoforms include neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO is a crucial intracellular second messenger involved in many biological activities such as blood pressure regulation, smooth muscle relaxation, neurotransmission, cellular defense, and immune regulation (4). Peroxynitrite, which is a very toxic compound, is formed during the reaction between superoxide radical and NO (nitrogen monoxide) (14), with subsequent new reactive compounds (nitroso-peroxo-carboxylate or peroxynitrous acid) leading to oxidation of lipids, proteins (methionine and tyrosine), and DNA (15).

The Mitochondrial Dysfunction Theory in MS

Mitochondria play a significant role in synthesizing adenosine triphosphate and providing energy to the cells. They possess their own DNA and are genetically independent organelles. Moreover, they are involved in apoptosis and metabolism of fatty acids (16–18). An oxidative energy metabolism is required for the lifespan of neurons while the large amount of adenosine triphosphate is produced during oxidative phosphorylation. In this reaction, the greatest amount of

harmful ROS and RNS is formed. In the case of the disturbed mitochondrial antioxidant production, the following are observed: decreased adenosine triphosphate synthesis, impaired Ca^{2+}, and elevated ROS and RNS (16, 19). Mitochondrial dysfunction plays a particular role in inflammatory processes. In the case of mitochondrial dysfunction, an overproduction of toxic ROS and RNS is observed (20). It plays a pivotal function in myelin and oligodendrocyte loss which is detrimental to neurons and glia (14, 21). Mitochondrial disturbances cause many neurodegenerative processes, including DNA damage, insufficient mitochondrial enzyme activity, abnormal mitochondrial gene expression, and defective DNA repair mechanism (22). As a result, mitochondrial damage in MS was considered to play an important role in disease progression (23, 24).OS leads to mitochondrial damage, thus disrupting transport of adenosine triphosphate along axons, resulting in neurodegeneration (25–27). Faulty mitochondrial DNA was reported as the consequence of oxidative and nitrosative stress (28). It was found that peroxynitrite, superoxide, and NO can destroy mitochondria in experimental autoimmune encephalomyelitis (EAE) and inhibit aconitase, creatine kinase, manganese, and SOD. These reactions lead to increased mitochondrial proton permeability, damage to mitochondrial DNA, and lipid peroxidation (29). In addition, recent findings in EAE suggest that mitochondrial dysfunction occurs in the early stage of MS (30). Interestingly, mitochondrial damage seems to develop before the inflammatory process in the disease (31). Mitochondria have a variety of antioxidant enzymes, including antioxidants peroxiredoxin-3 and thioredoxin-2 as well as their regulator *PGC-1*α. Increased astrocytic *PGC-1*α in active MS lesions might be an endogenous protective mechanism to reduce oxidative damage. Activation of *PGC-1*α represents a promising therapeutic strategy (32).

Inflammatory Mediators and Antioxidants

New findings suggest that chemokine 11 (CCL11) in the serum and in the cerebrospinal fluid (CSF) released from activated astrocytes promote OS via microglial NOX1 activation and glutamate-mediated neurotoxicity. These findings proposed using inhibitor of NOX1 in therapy (33, 34). The modulation of glutamate release and transport may also become a new therapeutic target (35). Another study explained how tumor necrosis factor-alpha (TNF-α) inhibits the accumulation of progenitor cell differentiation. It depends on a number of factors such as increased ROS production, altered mitochondrial calcium uptake, mitochondrial membrane potential, and respiratory complex I activity. The accumulation of progenitor cells at the lesion sites is observed in MS patients (36) and suggests that failed remyelination is a consequence of the inhibition of differentiation (37). In another study, authors presented the possibility of using a *TNFR2* agonist as a factor protecting microglia against OS (38). Enhanced astrocytic peroxisome proliferator–activated receptor gamma coactivator1-alpha (*PGC-1*α) levels reduce the production of pro-inflammatory mediators such as IL-6 and chemokine (C-C motif) ligand 2, and antioxidant enzymes such as peroxiredoxin-3 and thioredoxin-2, in human primary astrocytes. Activation of *PGC-1*α may be a protective factor for neurons (32).

The results from the study of Andaloussi et al. presented the use of exosomes, biologically active nanovesicles (30–120 nm) that can be easily delivered across the blood–brain barrier (BBB) (39), to increase remyelination post-injury. They stimulated primary dendritic cell cultures with a low level of IFNγ. Exosomes (IFNγ-DC-Exos) contain microRNA species which are involved in oligodendrocyte development pathways and can increase baseline myelination, reduce OS, and improve remyelination. IFNγ-DC-Exos also increased oxidative tolerance, antioxidant levels, and anti-inflammatory miRNAs. Furthermore, IFNγ-DC-Exos, nasally administered to animals, increased CNS myelination *in vivo* (40).

Such therapy may involve supplementation of melatonin which can scavenge the hydroxyl, carbonate, alkoxyl, peroxyl, and aryl cation radicals, and stimulate the activities of antioxidative enzymes (GPx, SOD, etc.). Oxidative process may also be inhibited by NOS (41). It was reported that melatonin (10 mg daily/30 days) caused a statistically significant increase in antioxidative enzymes such as SOD and GPx and a decrease in malondialdehyde (MDA) in erythrocytes of SPMS patients (42). However, the relationship between the Expanded Disability Status Scale (EDSS), Gd + and SOD concentration in erythrocytes in clinically isolated syndrome (CIS) and RRMS patients is not clear and requires further investigation (42, 43). Melatonin also plays an important role in improving the antioxidant defense in MS through upregulation of sirtuin1 (*SIRT1*) and its target genes for MnSOD and CAT (44). Moreover, melatonin is selectively taken up by mitochondrial membranes, which makes it a potential therapeutic tool in treating neurodegenerative disorders (45).

Genetics seems to play a significant role. The GSTP1 polymorphism and quinone oxidoreductase 1 (NQO1) variant genotypes in MS patients suggest that a defective function of detoxification enzymes could be a determinant of susceptibility and the clinical presentation of the disease (46, 47). α(alpha)-lipoic acid (ALA) is a natural, endogenous antioxidant that acts as a peroxisome proliferator–activated receptor-γ (*PPAR-γ)* agonist to counteract OS (48, 49). Another data provided the first evidence that ALA may increase the production of *PPAR-γ in vivo* in EAE and may reveal antioxidative and immunomodulatory mechanisms for the application of ALA in humans with MS (48).

Emami Aleagha et al. indicated that a decreased concentration of Klotho, an antiaging protein, in the CSF of patients with RRMS showed a significant negative correlation with the EDSS and a positive correlation with total antioxidant capacity (TAC). Klotho concentrations may play an important role in the regulation of the redox system (50). Glutathione is an antioxidant in the brain which might be a marker of the oxidative line of defense in MS patients and might serve to monitor the disease progression (51). Furthermore, an impaired iron metabolism plays a major role in the pathogenesis of MS (4). In the saliva of patients with MS, ferric reducing ability (FRA) was reduced by 38% as compared to the control. The same study also demonstrated a decrease in the antioxidant status in the serum such as TAC (52). A study on 30 female patients showed lower TAC levels and higher TOS levels compared with the controls indicating a decreased endogenous antioxidants and increased OS (53). Another study showed that an expression of antioxidant power such as plasmatic FRA and thiol group dosage was significantly lower in patients with active disease (54).

Ferroxidase (FeOx) activity of ceruloplasmin prevents OS by promoting the connection of free radicals from iron ions to transferrin. A reduced serum FeOx

activity was noted in 69 RRMS patients and in 62 patients with other inflammatory neurological disorders (55). Serum uric acid (UA) concentrations in 30 MS patients and 20 controls with noninflammatory neurological diseases support the significance of UA in the pathogenesis of MS. Serum UA concentrations were found to be significantly lower in MS patients as compared to the controls (56). Recent reports indicated that urine aMT6s levels significantly correlated with MS functional composite score but not with the EDSS. These authors believe that there might be some new hope in developing a quantitative and objective measure to assess the severity of MS (57).

Antioxidants: Enzymatic and Nonenzymatic

Antioxidants, which are divided into enzymatic and nonenzymatic, are substances that protect the body against free radicals (Table 1). Among enzymes, the most important include catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), SOD, serum paraoxonase, arylesterase (53), and δ-aminolevulinate dehydratase (δ-ALA-D) (48). SOD has three isoforms, namely, copper/zincSOD (SOD-1), manganeseSOD (SOD-2), and extracellular EC-SOD (58). It needs to be stressed that in serum, the major antioxidant enzymes that can eliminate the hydrogen peroxide include CAT, GPx, and peroxiredoxins (4). Furthermore, glutathione-S-transferases (GSTs) and nitrite reductase NAD(P)H quinone oxidoreductase 1 (NQO1) are detoxifying enzymes that prevent cells from oxidative

TABLE 1 The Types of Antioxidants

Enzymes Oxidants (28, 46, 47, 51, 55)	Nonenzymatic Antioxidants (12)	
CAT	Low molecular weight antioxidants	Antioxidant elements
GPx	Uric acid	Ions: Cu, Fe, Zn, Mn
GR	Vitamin C	
SOD	Vitamin D	
Paraoxonase	Vitamin E	
Arylesterase	Glutathione	
GSTs	Coenzyme Q	
NQO1	B-carotene	
Peroxiredoxin-3	AU	
Thioredoxin-2, 6		
FeOx		
δ-ALA-D		

The types of antioxidants depend on molecular structure. The table lists the most important barrier antioxidant enzymes and other compounds and ions which are not enzymes.

CAT = Catalase, GPx = Glutathione peroxidase, GR = Glutathione reductase, SOD = Superoxide dismutase, GSTs = Glutathione-S-transferases, NQO1 = NAD(P)H:quinone oxidoreductase1, FeOx = Ferroxidase, δ-ALA-D = δ Aminolevulinate dehydratase, UA = Uric acid.

damage (46). The concentration of these enzymes in serum may reflect the status of an antioxidant line of defense.

Nonenzymatic antioxidants may be classified into low molecular weight and antioxidant elements (ions). Low molecular weight antioxidants include UA; vitamins C, D, and E; glutathione; coenzyme Q; and b-carotene (9). Other tissue antioxidants include ceruloplasmin and ferritin. Iron (Fe), copper (Cu), zinc (Zn), and manganese (Mn) are the most important ions with antioxidant properties. The general and nonprotein thiol groups represent a nonenzymatic segment of the antioxidant defense system (59). The total glutathione and reduced glutathione can be assessed in the serum and are substrates for enzymes such as GPx and GR (60). UA is a natural nonenzymatic endogenous antioxidant, neutralizing overproduction of peroxynitrite (9).

The Importance of OS in MS

The inflammatory component in the course of MS is significant not only due to neuronal and axonal loss but also due to the initiation of the degenerative cascade in MS in the early stage (2). The activation of microglia and macrophages constitutes a major factor responsible for the production of ROS (8) due to high oxygen consumption (2, 4). Microglia activated by T-lymphocytes release proteolytic enzymes, cytokines, oxidative products, and free radicals. However, microglia also have many protective properties (61), such as neuroprotection, lowering of inflammatory response, and stimulation of tissue repair (62). Neurodegeneration in the course of MS is influenced by two processes, namely, OS (63) and excitotoxicity. Pathomechanisms of excitotoxicity are associated with glutamate overload (16), calcium overload, ionic channel dysfunction, mitochondriopathy, proteolytic enzyme production, and activation of apoptotic pathways.

Interestingly, persistent hyperactivation of oxidative enzymes suggests an "OS memory" in chronic neuroinflammation (64). Dysregulation of axonal bioenergetics plays a significant role in OS and axonal injury (27, 65). CSF examination during the exacerbation of MS demonstrated a bioenergetic failure related to an increased mitochondrial proton leak as well as an increased expression of genes that are involved in oxidative damage (66). Furthermore, the presence of proinflammatory cytokines in the CSF and pro-oxidative markers (e.g., nitrotyrosine) leads to cytokine-induced synaptic hyperexcitability and also glutamate-dependent neurotoxicity (67, 68). Recently published studies stress the significant role of ceramides in the CSF as the signaling molecules causing mitochondrial dysfunction. Short-chain ceramides stimulate the production of OS and lead to neuronal death (69). Cerebral iron accumulation is also significant. This process causes chronic cell stress, contributing to axonal and neuronal death (70). The excessive accumulation of iron was detected in MS plaques. Extracellular hemoglobin oxidizes and leads to local OS by the globin radical which may be responsible for myelin basic protein oxidative cross-linking and heme involved in the peroxidation of lipids (71). Neurodegeneration is related to iron liberation from the myelin sheath at the time of demyelination (72). Diffuse neurodegenerative process is

connected with high iron concentration in the basal ganglia (73). Ferrous iron may intensify oxidative injury in the presence of oxygen radicals (74, 75). Mitochondrial injury, OS, and energy failure may be connected to the formation of plaques and neurodegeneration in white and gray matter lesions (17, 76). Neurodegeneration in the course of MS is related to chronic subclinical extravasation of hemoglobin into lesions, the dysfunction of various cellular protective mechanisms against extracellular hemoglobin reactivity, and OS (77). Another study stressed that changes in the oxidant and/or antioxidant balance played a role in the pathophysiology of the disease. Attention was paid to the balance between the concentration of compounds such as lipid peroxidation levels; carbonyl protein content; DNA damage and SOD; CAT activities; vitamins E and C; and nonprotein thiol content (78). Also, the presence of free radicals in the nervous tissue may be toxic; for example, peroxynitrite increases the inflammatory response, thus leading to such a high concentration in the chronic phase that it may result in neurodegeneration (9).

The Impact of Antioxidants on the Course of MS

OS at each stage of MS is a key element in the pathogenesis of the disease. At the time of relapse, all these processes are intensified, leading to neuronal loss. Current treatment is focused on decreasing inflammation, but not on preventing neurodegeneration. It is possible that a new target of treatment will focus on neutralizing free radicals. The course of the disease is affected by the use of antioxidants and substances that affect antioxidant pathways that reduce the severity, cause faster remission, and result in less pronounced course of neuroinflammation and neurodegeneration (79). The process, known as "remote damage," may have a significant effect on neurodegeneration. This process can damage neurons functionally related to the primary focus. The therapeutic window that occurs between the primary and secondary damage can be used to implement new neuroprotective treatment (80).

New Possibilities in the Treatment of MS—Neuroprotection

A number of substances have been tested for a possible ability to protect the brain against neurodegeneration; however, the identification of neuroprotective drugs has been problematic (2). The limited response to the application of ROS scavengers results from their short half-life, in the order of milliseconds, and the degree of instability of ROS (61, 81, 82). Hydralazine may become a potential therapy due to the fact that it protects cells from the damaging effects of acrolein (61, 83, 84). The following agents could offer help in preventing mitochondrial dysfunction and in improving neurodegeneration: CDDO-ethyl amide, CDDO-trifluoroethylamide, pioglitazone, rosiglitazone, resveratrol, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), and bezafibrate (85).

Other findings suggest that neural stem cells (NSCs) exposed to 125 μM H_2O_2 for 30 min, and pretreated with different doses of lovastatin for 48 h, were protected

against OS-induced cell death by the expression of *PGC-1*α, which is a master regulator of mitochondrial function controlling energy metabolism and *Nrf2*. It is possible that in the future lovastatin may be used to promote the survival rate of NSCs (86). The compounds that can readily cross the BBB include:simvastatin, atorvastatin, cerivastatin, pravastatin and rosuvastatin (87). Exendin-4 and GLP-1 have been shown to reduce inflammation, demyelination and cytokine release in various animal models of MS (88). Most glucagon-like peptide-1 (GLP-1) mimetics such as exendin-4, liraglutide, and lixisenatide cross the BBB and show neuroprotective effects in many studies. However, further studies are needed to clarify the relationship with OS.

Polymerized form of nano-curcumin (PAP) has been shown to exert anti-inflammatory and antioxidative effects, and also repair myelin in EAE, a mouse model of MS (89). Nontoxic inhibition of myeloperoxidase may restore the BBB integrity and limit migration of myeloid cells into the CNS (90). The antioxidant protein peroxiredoxin 6 (PRDX6) can reduce the inflammation in the CNS and potentiate oligodendrocyte survival (91).

The Relationship between Immunomodulatory Therapy, OS, and Antioxidants

Immunomodulatory therapies protect from relapses whereas corticosteroids treat relapses. However, their effect is only partial and further search for new therapeutic options is needed. The transcription factor *Nrf2* is a key regulator of antioxidative defense (92, 93). Oral dimethyl fumarate (DMF) activates anti-inflammatory and antioxidative pathways to upregulate the expression of this molecule (94, 95). A differential expression is involved in the defense against OS, predominantly in actively demyelinating white matter lesions (58, 94, 96).

DMF and monomethyl fumarate (MMF) activate Nrf2 transcriptional pathways (97). Target genes of *Nrf2* include heme oxygenase-1, glutamate cysteine ligase transcription factor1, and NAD(P)H oxidoreductase-1. Furthermore, MMF impedes the activation and migration of lymphocytes; however, it does not have an impact on the function of macrophages. It is a potential novel mode of action differentiating this drug from other immune-modifying drugs (98). It was also shown that therapies aimed at stimulating endogenous antioxidant pathway, for example, the induction of the Nrf2 pathway, may demonstrate positive effects in a situation of moderate OS such as the one in the classical EAE models (27). On the other hand, they might be counterproductive in the case of extensive oxidative injury; it has been proposed that the amplification of oxidative injury in MS is only minimal in the studied rodent models (99).

T-cell-secreted IFNγ stimulates OS and demyelination in MS. However, induction of physiological levels of IFNγ protects against demyelination and OS. Therefore, it is important to apply phasic and pulsed IFNγ to the brain (100). Combination therapy with immunomodulatory drugs antioxidants, for example, IFN-β and glatiramer acetate, significantly reduced TNF-α; however, it did not affect other ROS/NRS biomarkers or disease progression (101). In another study, the level of protein carbonyls was elevated in RRMS patients treated with interferon

β-1b and glatiramer acetate whereas, serum protein thiol groups were decreased; in the absence of immunomodulatory drug, the same markers of OS were significantly elevated (102). Sadowska–Bartosz et al. demonstrated an increase in oxidation parameters in serum of RRMS patients treated with IFNβ-1a and IFNβ-1b. However, this increase was less significant compared with untreated RRMS patients or SPMS patients treated with mitoxantrone (103). It should be borne in mind that mitoxantrone is associated with an increased level of OS (104). On the other hand, the study demonstrated that mitoxantrone did not have an effect on the activity of paraoxonase 1 (a type of enzyme that protects cells from OS) (104).

Arnold et al. evaluated the suicidal erythrocyte death induced by mitoxantrone. The study showed that mitoxantrone triggered cell apoptosis, partially due to the formation of ROS and ceramide, thus increasing OS. In addition, the authors assessed the effect of the antioxidant N-acetylcysteine, which significantly reduced the effect of mitoxantrone (105). Due to the fact that the studies are not conclusive, it appears that treatment with IFN-β and mitoxantrone does not reduce OS (103). Another study demonstrated that melatonin supplementation at a dose of 5 mg over 90 days resulted in a significantly decreased MDA concentration in IFN-β and glatiramer acetate–treated groups but not in the group treated with mitoxantrone. In turn, a significant increase in SOD activity was observed only in the group treated with glatiramer acetate as compared to the controls (106).

Interestingly, melatonin may also have implications for the treatment of severe MS. One of the studies indicated that the TAC level was significantly lower in the mitoxantrone-treated group, and it increased after melatonin supplementation (107). Therefore, a combined use of immunomodulatory therapies with antioxidants may prove beneficial. IFN-β and C-phycocyanin, a biliprotein from *Spirulina platensis* with antioxidant, anti-inflammatory, and cytoprotective properties, improved the redox status and ameliorated clinical deterioration of mice with EAE (108). Fingolimod reduced hyperoxia-induced OS, activation of microglia, and associated pro-inflammatory cytokine expression in neonatal oxygen-induced brain injury (109).

Attempts were also made to explain some of the beneficial effects of natalizumab and its antioxidant capacity. Researchers studied serum melatonin levels in 18 patients with RRMS treated with natalizumab and noted that it caused significant increases in serum melatonin concentrations (87). In one of the studies, 22 MS patients were assigned to the treatment with 300 mg of natalizumab. After 14 months, it was observed that natalizumab prompted a decrease in oxidative damage biomarker levels and induced nuclear translocation of *Nrf2*, which is responsible for the activation of the antioxidant pathway, and a fall in serum vascular cell adhesion molecule-1 levels (60). In addition, a decrease in carbonylated protein levels was found in patients with the highest levels of severity (EDSS>5) (110). To conclude, it appears that most of the drugs used in MS are directly or indirectly modulate OS.

Corticosteroids in Relapses—The Importance of OS and Antioxidants

The role of corticosteroids in OS is poorly understood. Wang et al. examined levels of MDA and TAC in peripheral blood and in the CSF of RRMS patients 7 days before

methylprednisolone (MP) treatment and 1 month after MP treatment. They found that the increase in OS markers precedes inflammatory response in MS patients and MP treatment reduces the neuroinflammatory attack by decreasing brain antioxidant enzymes (111). Ozone autohemotherapy is an emerging therapeutic technique that can change brain metabolism. It was shown that MS patients demonstrated a marked increase in cytochrome-c-oxidase (CYT-c) activity and concentration about 40 min after autohemotherapy, possibly revealing a reduction of the chronic OS level typical of MS patients (112) A protective effect of ozone (O3) therapy was reported in EAE in rats either alone or in combination with corticosteroids. Such a combination allows to reduce the dose of MP due to a decrease in the level of brain glutathione, paraoxonase 1 enzyme activity, brain MDA, TNF-α, IL-1β, IFN-γ, Cox-2 immunoreactivity, and p53 proteins (113). The study showed that adding compounds that modulate redox pathways in the cell could increase the effectiveness of the therapy and reduce the dose of corticosteroids.

Conclusion

The role of OS in MS is of great importance as it has a pivotal role throughout the duration of the disease. In the acute phase it initiates inflammatory processes and in the chronic phase it sustains neurodegeneration. Increased levels of OS markers and decreased levels of antioxidant molecules have been observed in patients with MS independently of the course of the disease. The use of antioxidants offers hope for a better prognosis, particularly in conjunction with immunomodulatory therapy and corticosteroids. MS patients may benefit from antioxidant supplementation.

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11 Experimental *In Vivo* Models of Multiple Sclerosis: State of the Art

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Abstract: Multiple sclerosis is a multifactorial and heterogeneous neurological disease; hence, several experimental animal models had to be developed to mimic the different features of human pathology. Three main classes of animal models have been developed:experimental autoimmune encephalomyelitis (EAE), cuprizone intoxication, and Theiler's murine encephalomyelitis virus (TMEV) infection. The EAE model is the most versatile as it allows the reproduction of different patterns of multiple sclerosis; it is mostly relevant for relapsing-remitting multiple sclerosis and has allowed the development of several first-line, disease-modifying drugs for the treatment of multiple sclerosis. The other two models are less flexible than the EAE model and, to date, have not led to the discovery of any clinically relevant therapies. The cuprizone model mostly mimics the acute and chronic courses of multiple sclerosis, and it may represent a useful tool to develop novel therapies to protect oligodendrocytes and stimulate remyelination. Finally, the TMEV infection is the reference model to specifically study viral-mediated mechanisms of acute and primary progressive multiple sclerosis.

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Introduction

Multiple sclerosis is a complex and heterogeneous neurological illness with regard to its pathological phenotype (e.g., primary progressive, secondary progressive, and relapsing-remitting) (1) and etiology (e.g., autoimmune-dependent and autoimmune-independent) (2, 3). Although many conflicting hypotheses exist about the nature of the primary hit triggering this pathology (e.g., multiple genetic predisposing factors in interaction with different environmental factors) (4), multiple sclerosis is characterized by the concomitant manifestation of a wide range of specific biological alterations. For instance, demyelination, inflammation, astrogliosis, microglia activation, macrophage and lymphocyte infiltration, and axonal damage represent common hallmarks of this pathology (5–8). Due to the large number of molecular mechanisms, variability of this disease among patients, and uncertain etiology, the following three experimental animal models, each reproducing different features of human pathology, have been developed: the experimental autoimmune encephalomyelitis (EAE) model, the cuprizone intoxication model, and the Theiler's murine encephalomyelitis virus infection (TMEV) model. In this chapter, the characteristics of these animal models, the procedures of induction, the main biological features, and their relevance in multiple sclerosis research are described.

The EAE Model of Multiple Sclerosis

Since 1947, when Walt and colleagues suggested that the EAE is a suitable experimental model for multiple sclerosis, many research projects have employed this model to investigate the pathophysiological mechanisms underlying human multiple sclerosis and to test new therapies (9). EAE is characterized by an autoimmune reaction against the myelin proteins in the central nervous system. Two distinct protocols are used to induce EAE, the administration of activated T-lymphocytes that act specifically against myelin antigens or, more frequently, the administration of myelin-derived peptides, which, in turn, cause an immune reaction against specific antigenic myelin proteins. Different types of peptides, such as the myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and several of their encephalitogenic epitopes are used to induce EAE (10). The peptides are generally administered via subcutaneous injection, solubilized in complete Freund's adjuvant solution, which functions as a depot of antigens for a prolonged and continuous release of the active peptides. However, in 2002, it was pointed out that this adjuvant exerts some inhibitory activities on EAE pathology, suggesting that it should be used with caution (11). More recently, it has been shown that EAE can be induced even without the Freund's adjuvant (12).

Three lymphocytic cell populations mediate the induction of EAE, Th1, and Th17 types of the $CD4^+$ cells, and $CD8^+$ T-lymphocytes, with the $CD4^+$ lymphocytes being the main mediators; after entering the central nervous system, these

cells target myelin proteins and mature oligodendrocytes causing myelin degradation, axonal damage, and oligodendrocyte apoptosis (13–16). The addition of the pertussis toxin to the injection mixture facilitates the migration of the lymphocytes across the blood–brain barrier (17). The migration of T-cells into the brain is typically accompanied by monocyte and/or macrophage infiltration and activation (18). Moreover, resident microglia and astrocytes actively respond to the insult and undergo activation as well. All these cell types have been shown to produce and release inflammatory mediators, such as chemokines and cytokines, thus contributing to the axonal damage and demyelination (18, 19).

In the EAE model, the peak of demyelination is reached after 10–15 days from the injection, primarily confined to the spinal cord, although a certain degree of demyelination is also detected in the optic nerve, cerebral cortex, and cerebellum (20, 21). Moreover, axonal damage and generalized paralysis are progressively developed with demyelination (8). Specifically, the paralysis starts from the tail, then affects the hind limbs, and ultimately compromises the forelimbs.

The pathological characteristics of EAE are not uniform as they considerably vary depending on the type of the epitope and the type of the animal used. For instance, in C57BL/6 mice, encephalitogenic epitopes of MOG induce a chronic progressive disease, whereas in NOD/Lt and SJL mice and Lewis rats they cause a chronic relapsing-remitting disease with variable severity (17, 22, 23). Susceptibility to EAE is modulated by genetic factors that influence the response to myelin antigens. For instance, B6 and SJL mice are resistant to MBP immunization, but they respond well to MOG treatment. This variability seems to be modulated by some polymorphic regions within the major histocompatibility complex genes (24–25). In PL/J mice, the epitope injection induces a noncanonical form of relapsing-remitting disease (26). Interestingly, in SJL mice, a spontaneous relapsing-remitting EAE can be induced if the mice have been previously engineered to carry a specific T-cell receptor for myelin oligodendrocyte glycoprotein (27). Finally, the disease course differs between genders; for example, SJL, ASW, and NZW females show a higher incidence of EAE, resembling the higher prevalence of multiple sclerosis in women when compared to men (28).

Lewis is the most commonly used rat strain for EAE. Lewis rats develop brain pathology without the need of pertussis toxin that represents an artifact with regard to human pathology. However, inducing EAE in Lewis rats presents several drawbacks, as the obtained pathological phenotype lacks fundamental hallmarks of human multiple sclerosis. In particular, different to the human pathology, demyelination is not clearly detected and inflammation is not widespread in the whole brain, but mostly localized in the spinal cord. Even though rats have been considered valid experimental animals to study the activity of the immune cells in the central nervous system, they have been gradually supplanted by mice for multiple sclerosis research. Mice are easier to handle and particularly convenient for genetic manipulation (29). In addition to mice and rats, EAE can be induced in many other animal species like primates, rabbits, and guinea pigs (30–33). In summary, EAE reproduces many aspects of multiple sclerosis in terms of disease course, pathogenic mechanisms, and pathological features. In particular, myelin degradation and axonal damage are prominent in the spinal cord, consequent to autoimmune processes primarily mediated by the infiltrating $CD4^+$ T-lymphocytes. EAE is broadly deemed to be a good model to test immunosuppressive therapeutic agents, as demonstrated by the fact that it led to the establishment of several clinically relevant therapies (34, 35).

The Cuprizone Model of Multiple Sclerosis

The intoxication models of demyelination are based on the administration to laboratory animals of bioactive molecules that specifically target oligodendrocytes causing their degeneration and death, ultimately leading to severe demyelination in the brain. Several toxins such as ethidium bromide, lysolecithin, and cuprizone have been shown to efficiently trigger demyelination in the central nervous system (36). Of these, cuprizone is widely used in multiple sclerosis research. Cuprizone, bis-cyclohexanone oxaldihydrazone, is a neurotoxic copper chelator agent. Its deleterious effects on rodent brain were discovered by the pioneering work of Carlton in 1966 (37). Administered in the past, in addition to Swiss, CD1, and ICI mice (38), to other species, like guinea pigs, today cuprizone is prevalently used in mice (37, 39). It has been suggested that rats do not develop demyelination with cuprizone as consistently and reproducibly as mice do and that several rat brain areas remain completely unaffected (40). However, recent studies show that Wistar rats, in response to cuprizone, develop widespread demyelination of the cortex, corpus callosum, and cerebellum (41, 42) suggesting that rats, similarly to mice, are suitable for longitudinal studies. Indeed, rats could be a better choice for imaging studies due to their larger size (42).

C57BL/6 is the most widely used strain of mice for the induction of the cuprizone-mediated multiple sclerosis. In this strain, a minimal dosage of the compound is sufficient to cause highly reproducible brain pathology with limited peripheral side effects, such as weight loss and liver toxicity. As established by Hiremat and colleagues in 1998, cuprizone is administered per os by using a 0.2% w/w powdered rodent standard chow ad libitum for 5–6 weeks to C57BL/6 mice aged 8–10 weeks (43). After 6 weeks of cuprizone diet, a maximum of demyelination is reached within the gray and white matter, especially in the corpus callosum area (43) and the superior cerebellar peduncles (44, 45), but not in the spinal cord (46); motor disabilities become prominent (43). The demyelination process is characterized by selective and progressive apoptosis of mature oligodendrocytes, axonal pathology, activation of astrocytes and microglia, infiltration of macrophages and inflammation (43–45, 47–49). The inflammatory burden is characterized by the production of cytokines, interleukins, tumor necrosis factor, and arachidonic acid metabolizing enzyme, and by the consequent production of lipoxins, thromboxane, and proinflammatory prostaglandins that play an active role in the severity of demyelination (47, 48, 50, 51). An intact blood–brain barrier with no signs of lymphocyte infiltration have been observed in the cuprizone model (52, 53).

The interruption of cuprizone feeding after 6 weeks of continuous intoxication, immediately after peak demyelination has reached, allows for a spontaneous remyelination of the brain and a complete recovery in a time lapse of six additional weeks (47). For this reason, the cuprizone model is also used to investigate the mechanisms of remyelination. Prolonged administration of cuprizone, for 6–7 months, impairs remyelination as in progressive multiple sclerosis (54). Cuprizone can also be administered in repeated doses mimicking the course of relapsing-remitting multiple sclerosis (55). In summary, cuprizone allows an experimental reproduction of different pathological courses, such as the acute, chronic, and relapsing-remitting forms of multiple sclerosis.

Given these characteristics, the cuprizone model allows investigators to selectively study demyelination and remyelination processes, independently from the effects of the immune system. It is mostly used to test new pharmacological treatments to counteract demyelination and to favor remyelination. Remyelination, in fact, can be severely impaired in multiple sclerosis, because of dysfunctional and inefficient maturation of oligodendrocyte precursors. However, the recommended pharmacological therapies, currently used in clinics, have no specific activity on remyelination; thus, the need to develop novel therapies in this direction makes the cuprizone model a useful tool.

Theiler's murine encephalomyelitis virus

Viral infections have been hypothesized to be directly or indirectly implicated in the initiation of multiple sclerosis (56). The TMEV infection method was developed by Theiler in 1934 (57, 58) and later established as a model of multiple sclerosis by Lipton (59). This model is induced only in mice. When compared to TMEV, the rat TEV is not as highly virulent. With the exception of evidence published in 2005, rats do not seem to develop brain demyelination (60). In mice, susceptibility to TMEV is modulated by genetic factors. Several susceptibility polymorphic loci have been identified in the mouse genome within the major histocompatibility complex genes and the gene that codes for the beta-chain of the T-cell receptor. These loci modulate the severity of TMEV infection and the length of viral persistence in the brain (61, 62).

In mice, the pathology is induced via an intracerebral injection of *Picornaviridae*, which is a family of single-stranded RNA viruses belonging to the *Cardiovirus* genus. Two main types of TMEV are known, one highly aggressive that causes an extremely severe neuropathology leading to death within 1 week (induced by GDVII and FA strains of TV), and the other, less aggressive and not fatal (induced by DA and BeAn strains) (63). The latter can induce either a monophasic or a biphasic disease, depending on the mouse strain. The monophasic disease is inducible in most of the murine strains, whereas the biphasic form is inducible only in specific susceptible strains (64). The monophasic type and the first phase of the biphasic type are characterized by acute apoptosis of neurons in gray and white matter, appearing 1 week after the injection of the virus. The monophasic disorder clears out within three weeks and the biphasic disease (usually from 1-month post injection) sets the stage for chronic and progressive inflammation, and demyelination begins. This phase is characterized by the activation of glial cells and macrophages, apoptosis of oligodendrocytes, demyelination, and axonal damage, mostly in the spinal cord. The peak demyelination is reached from the third month of virus injection (65). In parallel with the worsening of the pathology, motor disabilities are observed (66). The neurological effects of TMEV seem to be mediated by the activation of T-lymphocytes, such as the $CD8^+$ T-cells, rather than by a direct interaction of the virus with the myelin proteins; moreover, the permanence of the virus in the central nervous system seems to depend on the astrocyte activity that supports viral replication (67). In summary, TMEV is useful to reproduce acute or chronic/progressive phases of the disease (64, 68).

From Animal Models to Human Pathology: Critical Issues

The EAE model is the most widely used model in multiple sclerosis research. This model is particularly useful to test disease-modifying agents with potential immunomodulatory activity; however, out of the hundreds of drugs tested in the EAE model, only a few have been approved for human use. Indeed, some drugs that attenuate EAE pathology in animals, like anti-tumor necrosis factor (TNF) drugs, actually worsen multiple sclerosis symptoms in humans (20, 69). Nevertheless, none of the recommended clinical medications for multiple sclerosis comes from pharmacological experimentations on the two other types of animal models. Despite the undeniable utility of EAE model to test novel medications, the consent of scientific community is not unanimous. For example, one of the main criticisms of the EAE model is that it fails to mimic some important features of multiple sclerosis, especially those concerning the immune system activation: EAE is mainly mediated by $CD4^+$ T-cells, whereas, in multiple sclerosis, the $CD8^+$ T-cells play a predominant role (70). To get around this limitation, researchers have developed a $CD8^+$ T-cell-mediated EAE (71), thus making this model more suitable for the study of CD8-mediated pathology. In addition, EAE is usually characterized by spinal cord demyelination, and in contrast to human pathology, cortical lesions are nearly absent. Cortical demyelination is a prominent marker of chronic multiple sclerosis. This major limitation can actually be overcome by stereotaxic injection of the MOG directly into the rat cerebral cortex (72). Another critical point is the enormous variability of EAE pathology, due to the different activities of the available antigenic peptides, and to the variable immune responses by the different animal species and strains. For these reasons, the choice of the peptide and of the animal species/strain is critical for study design and data interpretation.

Cuprizone, although it efficiently and consistently reproduces the demyelination and remyelination processes, it cannot be interpreted as an actual model of multiple sclerosis. Nevertheless, it can be used to investigate the molecular mechanisms implicated in oligodendrocyte degeneration and remyelination, in order to identify biological markers for the development of new pharmacological treatments to protect mature oligodendrocytes and to prompt oligodendrocyte precursor maturation.

In contrast to the other two models, the TMEV can be considered an actual model of the pathogenic mechanisms of multiple sclerosis, as the virus infection probably plays a role in the onset of the human disease. In general, when translating from animal models to the human pathology, it is relevant to take into account and investigate why some animals, within the same experimental group, neither develop the disease nor respond to therapies. Most literature does not present negative data, and exclude the "nonresponder" animals from the statistical analysis as outliers. The number of "nonresponders" should also be reported and the origin of this usual variability investigated, as it might be helpful in understanding the human variability with respect to susceptibility to multiple sclerosis, the clinical course, the severity of the disease, and the response to treatment (73).

Conclusion

Taking into account the intrinsic limitations of each animal model, we can summarize that the EAE model is mostly relevant for relapsing-remitting multiple sclerosis, which affects the majority of patients (about 80%).The EAE model is extremely versatile and can be designed to mimic acute and chronic disease courses. The cuprizone intoxication model, although less flexible than the EAE model, is mostly relevant to the acute and chronic courses of disease, but it can be manipulated also to recreate a relapsing-remitting pathology. The TMEV infection is the reference model to study viral-mediated mechanisms of acute and primary progressive multiple sclerosis. Finally, data on animals that do not respond to the disease induction, or treatment, are also essential to explain the variability usually observed in multiple sclerosis patients.

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