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Antibody cross-reactivity between casein and myelin-associated glycoprotein results in central nervous system demyelination

Rittika Chunder[a,b], Alicia Weier[a,b], Hannah Mäurer[b], Nicolas Luber[b], Michael Enders[a,b], Gabriele Luber[c], Thorsten Heider[d], Alfred Spitzer[e], Sabine Tacke[b], Janine Becker-Gotot[f], Christian Kurts[f], Radhika Iyer[g], Peggy P. Ho[h], William H. Robinson[g], Tobias V. Lanz[g,i], and Stefanie Kuerten[a,1]

[a]Institute of Neuroanatomy, Medical Faculty, University of Bonn, 53115 Bonn, Germany; [b]Institute of Anatomy and Cell Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91054 Erlangen, Germany; [c]Practice for Neurology, Psychiatry and Psychotherapy, 90491 Nürnberg, Germany; [d]Clinic for Neurology, Klinikum St. Marien Amberg, 92224 Amberg, Germany; [e]Neurological Medical Practice Erlangen, 91054 Erlangen, Germany; [f]Institute of Molecular Medicine and Experimental Immunology, Rheinische-Friedrichs-Wilhelms University of Bonn, 53127 Bonn, Germany; [g]Division of Immunology and Rheumatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305; [h]Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA 94305; and [i]Department of Neurology, Medical Faculty Mannheim, University of Heidelberg, 68167 Mannheim, Germany

Edited by Lawrence Steinman, Stanford University, Stanford, CA; received September 15, 2021; accepted January 26, 2022

Multiple sclerosis (MS) is a neuroinflammatory demyelinating disease of the central nervous system (CNS) with a high socioeconomic relevance. The pathophysiology of MS, which is both complex and incompletely understood, is believed to be influenced by various environmental determinants, including diet. Since the 1990s, a correlation between the consumption of bovine milk products and MS prevalence has been debated. Here, we show that C57BL/6 mice immunized with bovine casein developed severe spinal cord pathology, in particular, demyelination, which was associated with the deposition of immunoglobulin G. Furthermore, we observed binding of serum from casein-immunized mice to mouse oligodendrocytes in CNS tissue sections and in culture where casein-specific antibodies induced complement-dependent pathology. We subsequently identified myelin-associated glycoprotein (MAG) as a cross-reactive antigenic target. The results obtained from the mouse model were complemented by clinical data showing that serum samples from patients with MS contained significantly higher B cell and antibody reactivity to bovine casein than those from patients with other neurologic diseases. This reactivity correlated with the B cell response to a mixture of CNS antigens and could again be attributed to MAG reactivity. While we acknowledge disease heterogeneity among individuals with MS, we believe that consumption of cow's milk in a subset of patients with MS who have experienced a previous loss of tolerance to bovine casein may aggravate the disease. Our data suggest that patients with antibodies to bovine casein might benefit from restricting dairy products from their diet.

antibodies | casein | cross-reactivity | multiple sclerosis | myelin-associated glycoprotein

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) in genetically susceptible individuals. It is a clinically heterogenous disease, with the classic variants of MS being relapsing–remitting MS (RRMS) which accounts for the majority of all MS cases, secondary progressive MS which follows up to 70% of RRMS cases after 10 y of disease onset, and primary progressive MS which is the rarer variant (1, 2). Although there is a definite pathogenic role of inflammation in MS (3, 4), the precise roles played by the different cell populations remain controversial. Moreover, another level of complexity is added by the diverse mechanisms driving the development of the disease not just between the different subtypes but also between patients within one type of MS (5, 6). Evidently, the pathophysiology of MS—which is both complex and incompletely understood—involves genetic and environmental factors that interact to disrupt immunological self-tolerance to components of CNS myelin (7). Specific patterns in the epidemiology of MS suggest environmental determinants, including insufficient sun exposure, smoking, and dietary intake (8), play an important role in disease initiation and modulation. Mounting evidence implies that food habits and the gut microbiome, in particular, influence the disease course of MS (9, 10). While the impact of gut microbiota as a potential triggering factor in MS has been widely discussed (11–13), how certain dietary factors may be related to brain autoimmunity remains poorly investigated.

One of the reports as early as in the 1970s suggested milk consumption as an etiological factor in MS (14). Furthermore, epidemiological evidence from another study indicated a correlation between cow's milk consumption and the prevalence of MS (15). Yet, how milk consumption might trigger autoimmune responses to CNS antigens and contribute to disease development remains obscure. Stefferl et al. (16) have previously reported sequence homologies between a CNS myelin-specific

Significance

Multiple sclerosis (MS) is the most prevalent autoimmune disease of the central nervous system (CNS), leading to irreversible deficits in young adults. Its pathophysiology is believed to be influenced by environmental determinants. As far back as the 1990s, it had been suggested that there is a correlation between the consumption of cow's milk and the prevalence of MS. Here, we not only demonstrate that a high percentage of MS patients harbor antibodies to bovine casein but also that antibody cross-reactivity between cow's milk and CNS antigens can exacerbate demyelination. Our data broaden the current understanding of how diet influences the etiology of MS and set the stage for combining personalized diet plans with disease-modifying treatment strategies.

Author contributions: R.C., C.K., W.H.R., T.V.L., and S.K. designed research; R.C., A.W., H.M., N.L., M.E., S.T., J.B.-G., R.I., P.P.H., T.V.L., and S.K. performed research; G.L., T.H., and A.S. contributed new reagents/analytic tools; R.C., A.W., H.M., M.E., J.B.-G., T.V.L., and S.K. analyzed data; and R.C. and S.K. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: stefanie.kuerten@uni-bonn.de.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2117034119/-/DCSupplemental.

Published March 2, 2022.

antigen, namely, myelin oligodendrocyte glycoprotein (MOG), and butyrophilin (BTN), a protein of milk fat. In a follow-up study, it was further demonstrated that antibodies specific to the extracellular domain of MOG cross-reacted with bovine milk protein BTN in a mouse model of MS (17).

In the present study, we provide evidence of how an immune response against casein—another common protein component from bovine milk—can aggravate the demyelinating pathology of MS as a result of sequence homology with myelin-associated glycoprotein (MAG). Furthermore, our data suggest an antibody- and complement-dependent mechanism.

Together, these results identify how consumption of milk and milk products may exacerbate the autoimmune response in MS.

Results

Mice Immunized with Bovine Casein Show Spinal Cord Pathology in a Time-Dependent Manner. Mice immunized with bovine casein were killed at different time points (i.e., day 13, day 20, and day 40) after immunization (n = 6 to 9 in each cohort). Mice that had been immunized with either α-lactalbumin or β-lactoglobulin were killed on day 40. Every cohort was observed daily for the development of any clinical signs like cramping and hind limb weakness. While none of the mice immunized with noncasein milk antigens developed any signs of physical impairment, casein-immunized mice exhibited a range of symptoms that were broadly grouped under "weakness" (including grabbing and gait abnormalities and weakness of the limbs) and "disorientation" (including running around in circles, moving backward, or hesitant movement). A more detailed list of the signs and symptoms, the scoring system, and the clinical score progression of the different cohorts of casein-immunized mice are shown in Table 1 and *SI Appendix*, Fig. S1.

Subsequently, sections of the lumbar region of the spinal cord from each mouse of the different cohorts were processed for electron microscopy (EM) (Fig. 1*A*). Prominent changes were observed only in those cohorts that had been immunized with casein (Fig. 1 *A* and *B*). Furthermore, time kinetics studies of the different casein-immunized cohorts revealed progressive myelin degeneration characterized by swelling of the myelin sheaths with the myelin lamellae diverging widely from each other. In some mice, particularly those killed at day 40, there was severe detachment of the myelin sheath from its axon, indicating maximum pathology (Fig. 1*C*).

To focus on cellular infiltrates that could possibly explain the EM pathology, we subsequently performed hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining for infiltrating T and B cells on five to eight sections per spinal cord tissue per mouse of every casein-immunized cohort. However, despite the identification of sites of demyelination by EM, no corresponding immune cell clusters or perivascular infiltrates in the spinal cord of casein-immunized mice at day 40 were observed (*SI Appendix*, Fig. S2). These findings indicated that casein immunization can provoke demyelinating pathology in a time-dependent manner in the absence of any obvious immune cell infiltration.

Pathology Is Accompanied by IgG Deposition in Casein-Immunized Mice. The lack of cellular infiltration spatially associated with sites of spinal pathology in casein-immunized mice suggested that the observed pathology was not caused by a direct effect of inflammatory T or B cells, but, possibly, by an antibody-mediated mechanism.

Accordingly, we stained spinal cord sections of mice killed at the three time points after immunization to visualize immunoglobulin (Ig) deposition. While we observed only minimal amounts of IgG around the axonal tracts of the spinal cord in casein-immunized mice killed at earlier time points, there was evidence of marked IgG deposition in the spinal cord of animals killed at day 40 (*SI Appendix*, Fig. S3*A*).

To confirm that the pathology observed in casein-immunized mice was mediated by an antigen-specific Ig, IgHEL mice (n = 6) were similarly immunized with casein and killed 40 d after immunization. In these mice, B cells express a transgenic B cell receptor for hen egg lysozyme (HEL) and are therefore unable to generate Igs of any other specificities (18, 19). Accordingly, there were no signs of myelin pathology in casein-immunized IgHEL mice when analyzed by EM compared to casein-immunized wild-type (WT) mice (P = 0.0022; Mann–Whitney U test) (*SI Appendix*, Fig. S3*B*).

We also determined the casein-specific serum IgG and IgM titers in both IgHEL and WT mice. IgM was used as additional control Ig isotype, as the IgHEL mice produce only IgM. In accordance with the IHC data, IgG titers to casein were significantly higher in mice killed 40 d after casein immunization than in mice killed on day 20 ($P < 0.0001$; Mann–Whitney U test) (*SI Appendix*, Fig. S3*C*). In contrast, we detected no casein-specific IgG titers in IgHEL mice and no casein-specific IgM response in the WT or IgHEL mice (*SI Appendix*, Fig. S3*C*).

IgG from Casein-Immunized Mice Recognizes Antigens Expressed in the CNS. The occurrence of high titers of IgG to casein in casein-immunized mice prompted us to investigate binding of serum IgG from these mice to CNS tissue. We hypothesized two possibilities: either direct binding of serum IgG to endogenously expressed mouse casein or cross-reactivity to other antigenic structures in the CNS, resulting in spinal cord pathology observed in mice immunized with bovine casein.

To test the first possibility, we checked the expression of casein by CNS-resident cells. Although there are five functional casein genes in the common house mouse (*Mus musculus*) (20), we looked only at the expression of *CSN1S1*, *CSN2*, and *CSN3* (encoding αS1-, β-, and κ-casein, respectively), because these

Table 1. Clinical scoring strategy in mice immunized with milk antigens

Score	Balance score	Orientation score	Other signs
0	No problems balancing	No signs of spatial disorientation	
0.5	Difficulties in grabbing the cage mesh	No signs of spatial disorientation	
1		Signs of spatial disorientation	
1.5	Slipping off the cage mesh		
2.0			
2.5		Moving around in circles or moving backward	
3.0	Falling off the cage mesh		Cramping

For the balance test, mice were allowed to walk on their cage mesh while the mesh was gently rotated in a 360° manner. Mice that displayed no signs of balance problems were able to turn along as the mesh was rotated. Those which slipped, were hesitant with their grabbing, or fell were scored accordingly. Mice also displayed signs of disorientation, when they were placed on the cage mesh, that ranged from running around in circles to moving backward. A combination of the disorientation problems and the balance problems was used for the complete scoring of mice.

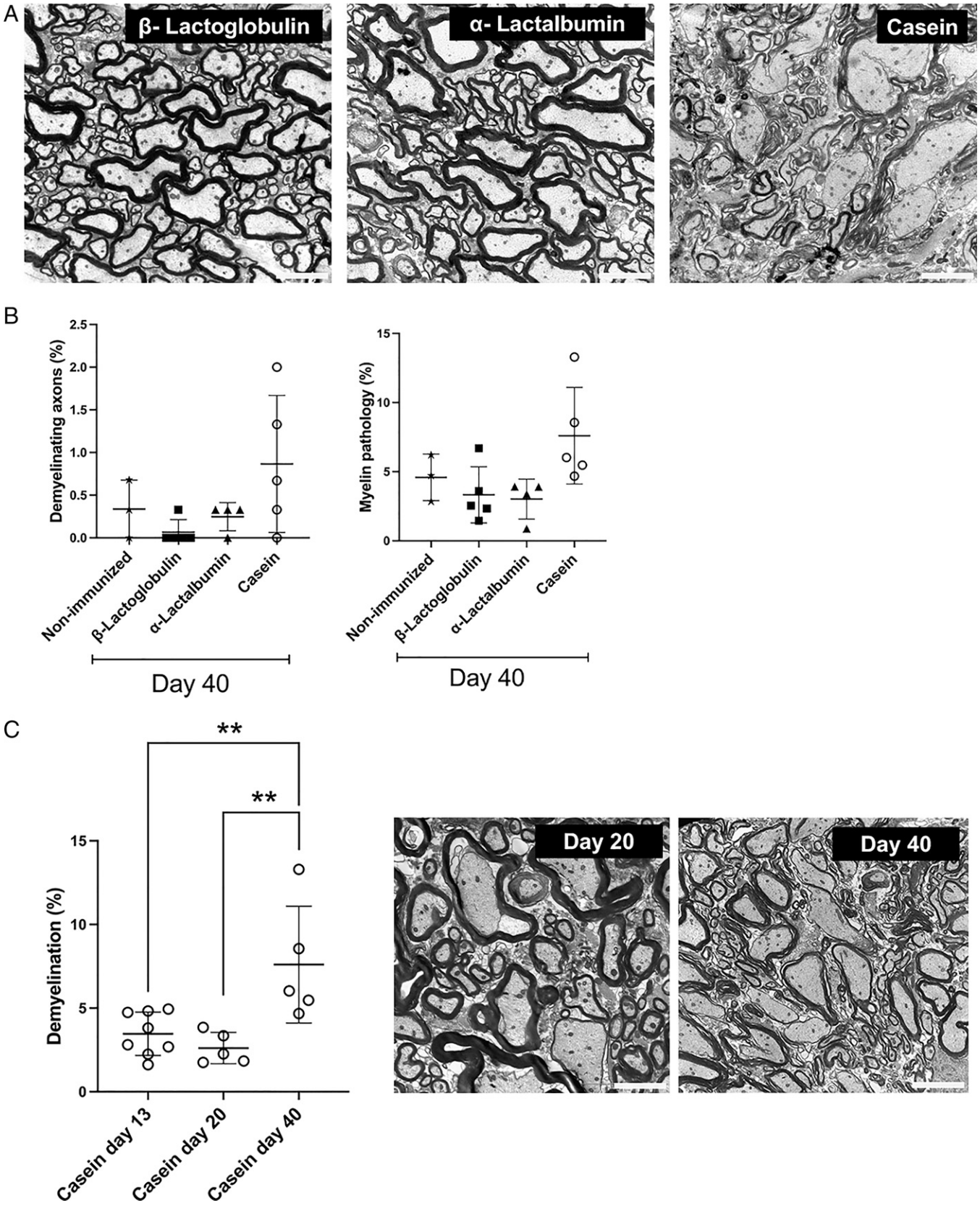

Fig. 1. Spinal cord pathology in casein-immunized WT B6 mice is time-dependent. (*A*) Representative EM images of lumbar regions of the spinal cord of casein-immunized, α-lactalbumin–immunized, and β-lactoglobulin–immunized WT mice. (Scale bar, 10 μm.) (*B*) Myelin pathology in the different cohorts of mice (killed at day 40). (*C*) Time-dependent exacerbation of demyelination (**$P = 0.0045$ and **$P = 0.0082$ for groups day 40 vs. d 20 and day 40 vs. d 13, respectively; one-way ANOVA). Each data point represents the mean ± SD for each mouse. Myelin pathology was quantified by counting the number of demyelinating axons per mouse (from a total of 10 representative images per spinal cord per mouse) as a proportion of the total number of axons. (Scale bar, 10 μm.)

are expressed in cattle breeds originating from Europe. No expression of the casein genes was detected at the messenger RNA level in the mouse CNS under physiological conditions as shown by endpoint and qPCR (Fig. 2*A* and *SI Appendix*, Table S1, respectively).

As this argued against caseins as an antigenic target, we investigated the possibility of alternative target(s) for the binding of serum IgG from casein-immunized mice. As previously discussed, IgG titers to casein were significantly higher in mice killed 40 d versus 13 and 20 d after immunization, which was also associated with more pronounced spinal cord pathology in the mice killed at the later time point. We therefore proposed a "cross-reactive" IgG response in casein-immunized mice as the etiologic agent of the aggravated pathology.

Serum from casein-immunized mice was incubated on spinal cord tissues from nonimmunized B6 mice, revealing an

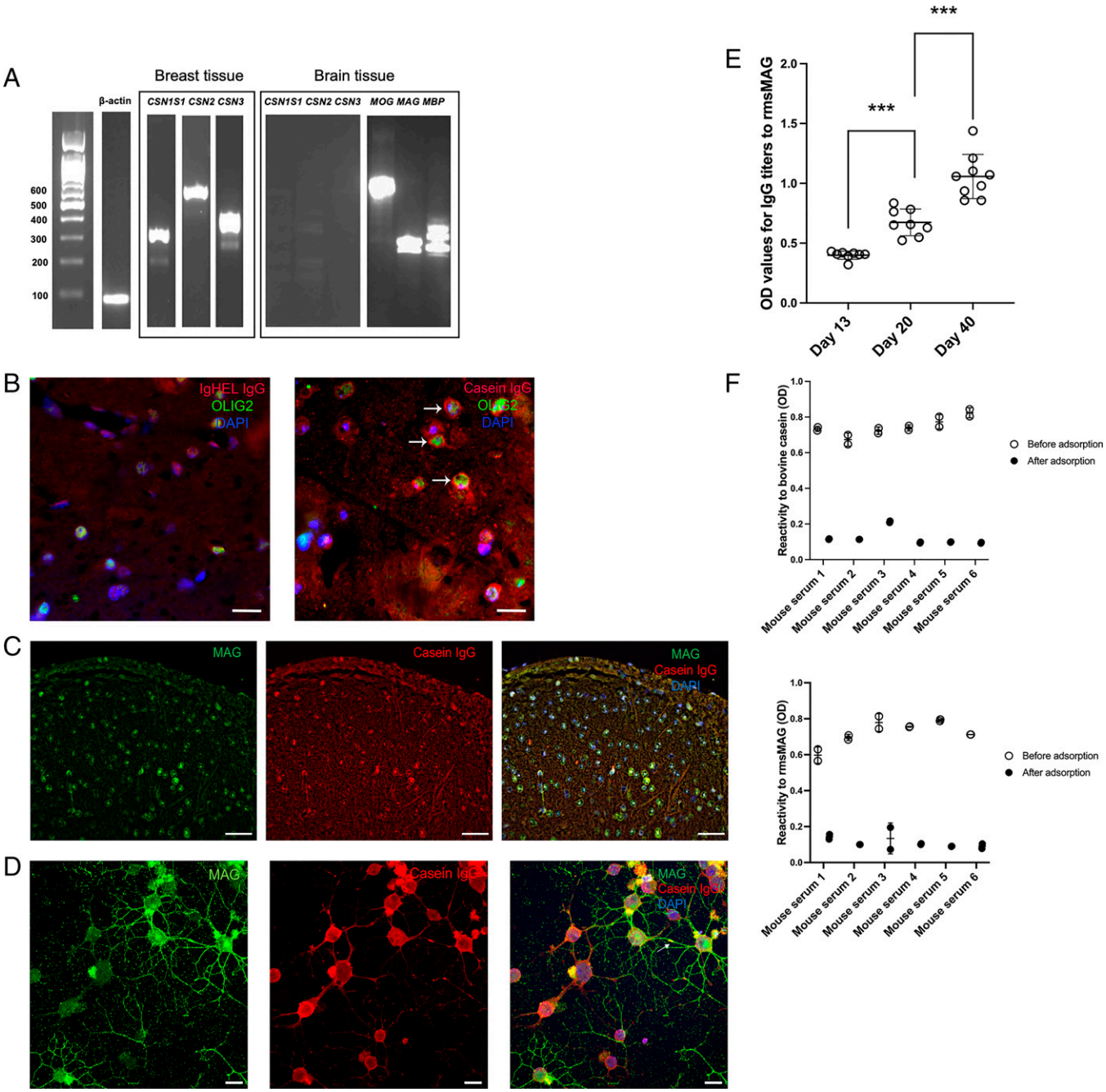

Fig. 2. Serum IgG from bovine casein-immunized mice recognizes MAG expressed by oligodendrocytes. (*A*) Total RNA was extracted from whole brain tissue lysate of 8- to 9-wk-old healthy WT B6 mice ($n = 3$), and RT-PCR was conducted for the detection of casein genes (*CSN1S1*, *CSN2*, and *CSN3*), as well as an array of CNS-related genes. Lysate from breast tissue was used as positive control for the casein genes. (*B*) Incubation of serum IgG from casein-immunized mice on murine spinal cord sections, counterstaining for $OLIG2^+$ oligodendrocytes. (Scale bars represent 10 μm.) (*C*) Colocalization of MAG and anti-casein sera on spinal cord sections from mice. (Scale bars, 50 μm.) (*D*) Double staining of the Oli-Neu cell line with an anti-MAG antibody and purified Ig from casein-immunized WT mice. (Scale bars, 10 μm.) (*E*) The rmsMAG reactivity of serum samples from casein-immunized mice killed on days 13, 20, and 40 analyzed by ELISA. Mean (± SD) OD values are shown. ***$P < 0.0001$. (*F*) Adsorption analysis of serum from casein-immunized WT mice with bovine casein. Mean OD ± SD values for binding to recombinant MAG and casein are shown.

array of staining patterns. We detected homogenous cytoplasmic staining of some cells and a more granular and dotted staining of others. This range of antigen–antibody binding patterns was, however, only seen with serum from casein-immunized mice that had been killed at the later time point (*SI Appendix*, Fig. S4).

To further characterize these individual patterns, the reactivity of serum IgG from mice killed at day 40 to murine spinal cord tissue was analyzed. Although there was a certain degree of heterogeneity in the staining patterns, serum from three out of six mice (3/6) bound to oligodendrocyte transcription factor-2 $(OLIG2)^+$ cells in spinal cord sections. OLIG2 was used as a marker to screen for oligodendrocytes. In contrast, no reactivity to glial fibrillary acidic protein $(GFAP)^+$ astrocytes was detected. This binding of serum IgG to $OLIG2^+$ cells was our first line of evidence indicating a "cross-reactive response" to oligodendrocytes (identified as $OLIG2^+$ cells) in at least half of the casein-immunized mice that were killed at a later time point (Fig. 2*B*).

In order to identify a specific cross-reacting antigen on oligodendrocytes, we costained murine spinal cord with MAG. MAG was selected as a potential cross-reactive target because of the shared sequence homologies between bovine αS1-casein, β-casein, κ-casein, and mouse MAG. Regions of amino acid sequence homologies between mouse MAG and the different bovine caseins are detailed in *SI Appendix*, Table S2. Accordingly, as shown in Fig. 2*C*, a colocalization of the transmembrane protein MAG and sera from casein-immunized mice was observed.

To further underscore the binding of anti-casein IgG to oligodendrocytes on a cellular level, specifically, to its cross-reactive antigen MAG, purified Ig from pooled serum of an additional cohort of casein-immunized mice ($n = 10$) killed on day 60 was tested on Oli-Neu cells, a murine oligodendroglial precursor cell line (21). Oli-Neu cells were cultured for 48 h in the presence of PD174265, which is a selective inhibitor of epidermal growth factor receptor that has been shown as a differentiating agent in these cells (22). When incubated with anti-MAG antibody and purified Ig from casein-immunized mice, differentiated Oli-Neu cells revealed a staining colocalization as shown in Fig. 2*D*.

Following these sets of observations, we tested the reactivity of casein-specific serum samples to recombinant mouse (rms)MAG. Similar to the increasing trend of IgG titers to bovine casein, we observed that the titers to rmsMAG also increased over the time course of the casein immunization, from lower titers at day 13 to high titers in mice killed at the later time point, that is, 40 d after immunization (Fig. 2*E*).

In a last set of experiments to confirm cross-reactivity of casein-specific antibodies with MAG, we adsorbed sera from casein-immunized mice killed after 40 d to bovine casein and measured the degree of IgG binding (using an enzyme-linked immunosorbent assay [ELISA]) when the sera were exposed to casein and rmsMAG before and after adsorption. Casein-adsorbed serum presented significantly reduced optical density (OD) values to both casein ($P < 0.0001$; paired t test) and MAG ($P < 0.0001$; paired t test) (Fig. 2*F*).

Casein-Specific Antibodies Are Cytotoxic to Oligodendrocytes and Cause Morphological Changes in the Presence of Complement. Having identified that spinal cord pathology in casein-immunized mice was accompanied by IgG deposition and serum from these mice cross-reacted with myelin antigens, we further explored the mechanism by which these antibodies can mediate damage. Antibody-induced pathology can depend on a number of mechanisms, including activation of the complement system. Accordingly, we tested the pathogenicity of IgG from mice immunized with casein on differentiated Oli-Neu cells in the presence and absence of 1% rat serum as the source of complement. A lactate dehydrogenase assay was performed on the supernatant of differentiated Oli-Neu cells in the presence of IgG purified from casein-immunized mice to determine the cellular toxicity levels to oligodendrocytes. As shown in *SI Appendix*, Fig. S5*A*, casein-specific IgG in the presence of 1% rat serum was significantly more cytotoxic to Oli-Neu cells compared to serum only ($P = 0.0052$; one-way ANOVA) or the combination of random IgG and serum ($P = 0.02$; one-way ANOVA). Furthermore, to show whether casein antibody-mediated complement-dependent oligodendrocyte cell death followed an apoptotic pathway, we conducted a flow cytometric staining of cells treated with anti-casein IgG and 1% rat serum using annexin V and propidium iodide. Results, as shown in *SI Appendix*, Fig. S5*B*, indicate that Oli-Neu cells underwent apoptosis as a mechanism of cell death in the presence of complement and casein-specific IgG.

Additionally, immunocytochemistry (ICC) done on PLP^+ differentiated Oli-Neu cells (also positive for MAG) in the presence of casein IgG and 1% rat serum revealed morphological changes, including fewer and shorter branchings of the oligodendroglial processes as well as cytoplasmic shrinkage. Cells that were treated with a combination of rat serum and a random anti-mouse IgG did not show any difference in their morphology when compared to medium-only control (*SI Appendix*, Fig. S5*C*). Quantitative analysis of the ratio between cell cytoplasm and nucleus demonstrated that there was a significant amount of cytoplasm shrinkage ($P = 0.0082$; unpaired t test) in oligodendrocytes treated with purified casein IgG and complement (*SI Appendix*, Fig. S5*C*). Together, the data demonstrate that IgG purified from casein-immunized mice that binds oligodendrocytes (shown in Fig. 2*D*) in the presence of complement not only is cytotoxic to the cells but also causes observable changes to the cell morphology.

We also repeated the experiments using primary oligodendrocyte precursor cells (OPCs) which were first checked for their differentiation into oligodendrocytes using neural/glial antigen 2 (NG2) and MOG as maturation markers on culture days 1, 6, and 14 (*SI Appendix*, Fig. S6*A*). OPCs that were in culture with platelet-derived growth factor (PDGF)-AA and fibroblast growth factor (FGF)-2 for 1 or 6 d revealed a significantly higher expression of NG2 compared to day 14 ($P = 0.0065$ for day 1 vs. d 6 and $P = 0.0021$ for day 6 vs. 14; unpaired t test) with an opposite effect observed for MOG comparing days 1 and 6 ($P < 0.0001$; unpaired t test). Additionally, OPCs were treated either with casein- or MOG-specific IgG and 1% rat serum, with casein-specific IgG alone, or with 1% serum alone for 24 h on culture day 1 vs. 14. NG2 staining of these differently treated cells revealed morphological changes between the conditions as shown in *SI Appendix*, Fig. S6*B*. The data indicate that casein-specific IgG preferentially affects mature oligodendrocytes in a complement-dependent manner.

In addition to our in vitro cell culture experiments, we immunized a new cohort of WT B6 mice with casein ($n = 12$). For transient depletion of complement, half of these mice received a single injection of cobra venom factor (CVF) on day 10 after casein immunization. A control group ($n = 6$) received phosphate-buffered saline (PBS) only. On day 6 following treatment with CVF or PBS, mice were killed, and the lumbar regions of their spinal cords were analyzed by EM for demyelination and axonal damage. CVF-injected mice showed less spinal cord pathology, characterized by a trend toward fewer numbers of demyelinated axons and significantly less axonal pathology compared to PBS-injected mice (*SI Appendix*, Fig. S7). The results highlight the involvement of the complement cascade in casein-immunized mice. Taken together, our results provide evidence of a pathogenic role for the humoral arm of a casein-specific immune response generated in the WT mice.

Antibody Repertoire Analysis Confirms Cross-Reactivity between Casein- and MAG-Specific Antibodies. As another approach to investigate the B cell response against casein and cross-reactivity to MAG, an additional group of mice was immunized twice with casein ($n = 4$), and a control group was immunized twice with HEL ($n = 3$). Plasmablasts were sorted by flow cytometry 10 d after the second immunization, and the single-cell antibody repertoire was sequenced. Plasmablast counts did not differ significantly between the groups (casein: mean [SD] 0.17% [0.04%]; HEL: mean [SD] 0.24% [0.05%]). Repertoire analysis, however, revealed that casein potently induced class switching to IgG, whereas HEL generated an IgA-dominated B cell response (Fig. 3 *A* and *B*; and *SI Appendix*, Fig. S8*A*). Clonality and mutation counts were elevated in the respective dominant Ig classes (Fig. 3 *C–F* and *SI Appendix*, Fig. S8*B*). The repertoire in the casein group was skewed toward the preferential use of a few heavy chain (HC) V genes (IGHV), most significantly IGHV9-3 and IGHV8-8 (*SI Appendix*, Fig. S8*C*).

To identify anti-casein antibodies that might cross-react with MAG, we aligned all HC and light chain (LC) complementarity-determining regions 3 (CDR3), and then identified clusters that contained sequences from casein-immunized but not from HEL-immunized or nonimmunized mice (Fig. 3*G* and *SI Appendix*, Fig. S8*D*). A total of 17 representative antibody sequences from 14 casein clusters were selected and expressed as recombinant monoclonal antibodies (mAbs) (Fig. 3 *G*, *i–iv*). Three of the 17 expressed mAbs bound strongly to casein (Fig. 3*H*) and two of those showed significant cross-reactivity to recombinant human MAG (rhuMAG) (Fig. 3*I*). The cross-reactive antibodies originated in several mice, indicating that casein/MAG cross-reactivity might be a commonly occurring phenomenon. The antibodies shared the same HC with high similarity in all three CDR regions, indicative of binding to the same epitope (*SI Appendix*, Fig. S8*E*). The LCs were substantially different (*SI Appendix*, Fig. S8*F*), suggesting that specificity was mostly determined by the HC.

Patients with MS Harbor Bovine Casein-Specific Antibodies and B Cells. To translate our findings to humans, we investigated whether patients with MS have a high titer of antibodies to bovine casein. We selected a group of 39 patients with MS and 23 patients with other neurological diseases (ONDs) to test for their serum IgG reactivity to casein (Table 2 and *SI Appendix*, Tables S3 and S4). Mean IgG titers to casein in patients with MS were significantly higher than in patients with ONDs ($P = 0.032$; Mann–Whitney U test) (Fig. 4*A*).

To confirm the specificity of serum IgG to casein in patients with MS, we performed an adsorption assay using a selected number of serum samples ($n = 10$) and tested the IgG titers before and after adsorption on bovine casein. Although our selected cohort of MS serum samples had varying IgG titers to casein before adsorption, an ELISA showed that all sera had minimal baseline reactivity to casein after adsorption ($P = 0.0008$; paired t test) (Fig. 4*B*). This indicated that a subset of patients with MS harbor casein-specific antibodies. To provide evidence of cross-reactivity of anti-casein antibodies with MAG in patients with MS, we used the bovine casein-adsorbed serum samples ($n = 10$) and tested their reactivity to rhuMAG by ELISA before and after adsorption. In general, all patients with MS from our selected cohort displayed low levels of humoral immunity to MAG in comparison with casein. Although the serum samples showed inconsistent results, an overall significant decrease was observed in OD values (corresponding to the IgG titers) after adsorption with bovine casein ($P = 0.0142$; paired t test).

To further complement our data, we tested the CNS- and casein-specific B cell response in patients with MS ($n = 45$) and those with ONDs ($n = 35$) (Table 2 and *SI Appendix*, Tables S3 and S4) by performing B cell enzyme-linked immunosorbent spot (ELISPOT) assays. Polyclonally stimulated peripheral blood mononuclear cells (PBMCs) from patients were seeded onto ELISPOT assay plates in which B cells producing antibodies to CNS antigens and bovine casein could be detected. While the number of casein-specific B cell spots/patient was similar in the two patient groups, the number of patients positive for casein-specific B cells was higher in the MS (19/45; 42%) than in the OND group (10/35; 28%) ($P = 0.0379$; χ^2 test). In addition, a Pearson's correlation coefficient of $r = 0.605$ ($P < 0.0001$) was obtained when the numbers of CNS- and casein-specific B cell spots were plotted against each other for the patients in the MS group (Fig. 4*C*). Taken together, these findings corroborated the link between casein- and MAG-specific antibodies and MS.

Discussion

Alterations of the immune repertoire by environmental and dietary factors have been implicated in the etiology of several diseases with an underlying autoimmune component, including autoimmune uveitis and type I diabetes (10, 23–26). In this study, we propose that an immune response to casein from bovine milk can contribute to the pathology of MS.

The allergenicity and antigenicity of bovine casein has been well documented in different reports (23, 27–29). While oral tolerance to casein may be the default response following gastrointestinal exposure to bovine milk, a breakdown of this tolerance in MS patients can be initiated by disturbance in antigen uptake and presentation of the antigenic epitopes in individuals with a genetic predisposition (30, 31). The exact mechanisms by which oral tolerance is broken are not well understood. However, exposure early in life to opioid peptides from casein, which are a major component of bovine milk (27), could act as a destabilizer for oral tolerance (32, 33). In general, milk proteins are suspected of having the potential to trigger autoimmune responses (16, 34), with humans being the only species that consumes milk products from other species through adulthood.

We speculate that, once tolerance to an otherwise harmless food antigen like casein is broken, it might exacerbate ongoing autoimmunity like in MS, as a result of cross-reactivity to self-antigens (17). In the present study, we have identified MAG as a potential cross-reactive self-antigen, an important myelin component localized in the periaxonal membrane of the CNS and peripheral nervous system (PNS) myelin sheaths. Despite the wider distribution of MAG in Schwann cell membranes than in oligodendroglial membranes, the total amount of MAG in the CNS is much greater than in the PNS (35). This would be an argument for why consumption of bovine milk could specifically aggravate CNS myelin pathology in the event where antibodies against bovine casein cross-react with human MAG. Nonetheless, pathophysiological consequences of drinking milk by MS patients are difficult to predict, as they will be influenced by multiple factors, including an individual's immunological repertoire as well as the health state of the gastrointestinal tract (16). However, it may also be interesting to note that, while one of the patterns of demyelination (pattern II) in MS lesions is characterized by antibody and complement deposition, a second type of pattern, pattern III, can be described by its preferential loss of MAG (36). In our cohort of casein-immunized mice, we have demonstrated that antibodies to casein cross-react with MAG and that the pathology is complement-dependent. One could speculate whether, in MS patients with a loss of tolerance to milk antigens, there is anti-casein antibody-mediated loss of MAG within the lesion.

Our current study has focused on immunization experiments in B6 mice. Typically, in mice susceptible to developing experimental autoimmune encephalomyelitis, immunization with complete

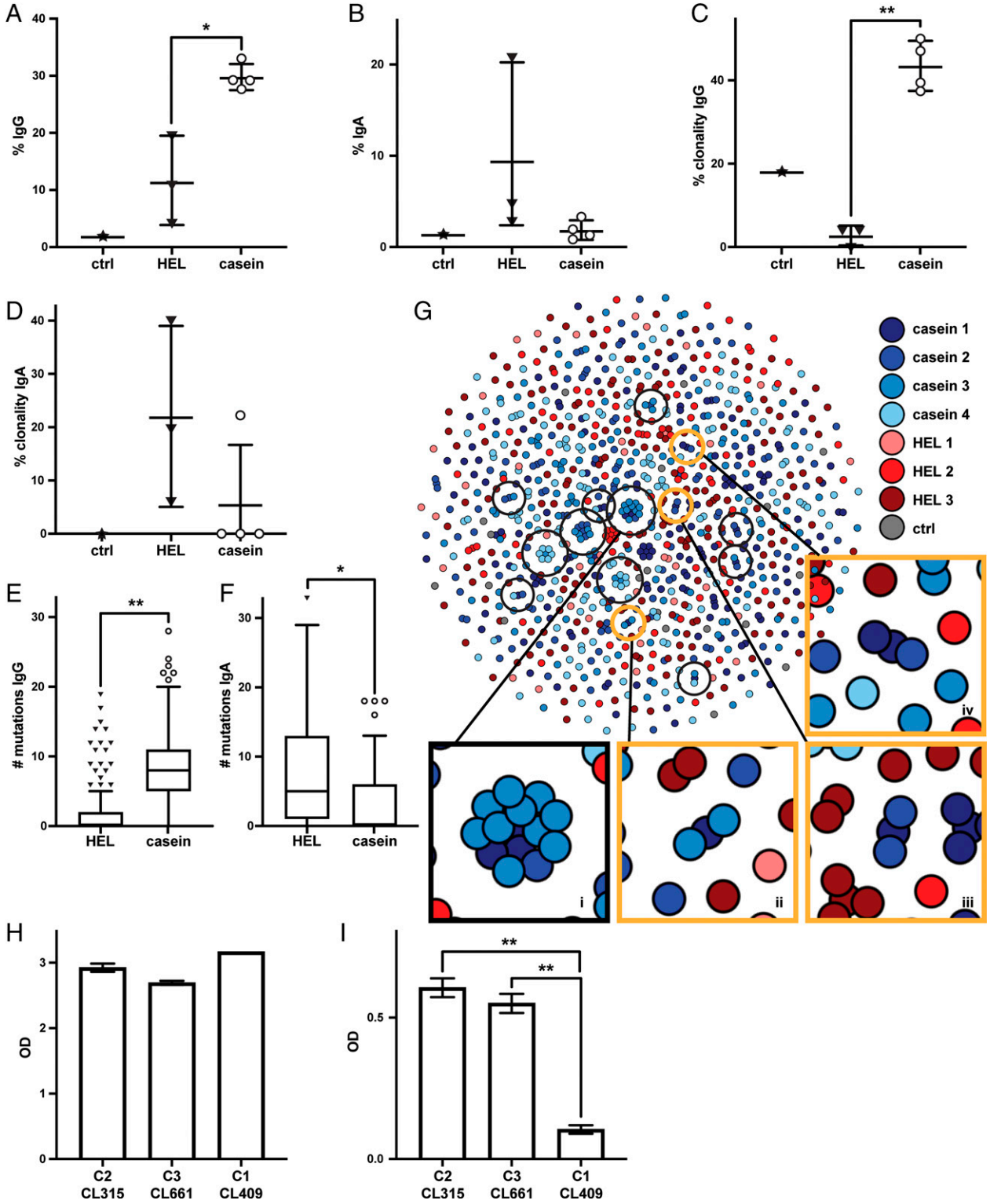

Fig. 3. B cell repertoire analysis and identification of casein/MAG cross-reactive antibodies. (*A–F*) Repertoire analyses, for the groups of casein-immunized ($n = 4$) and HEL-immunized ($n = 3$) mice, and the control mouse ($n = 1$). Number of (*A*) IgG and (*B*) IgA sequences, represented as percentage of all antibody sequences; $*P < 0.05$; Student's *t* test. Clonality of (*C*) IgG and (*D*) IgA sequences; $**P < 0.005$; unpaired *t* test. (*A–D*) Bar and whiskers represent mean ± SD for each group; each data point represents mean of all respective sequences from one mouse. (*E* and *F*) HC and LC V-gene mutation counts in (*E*) IgG-expressing and (*F*) IgA-expressing B cells. Mean ± SD are shown for all sequences of all mice in each group; $*P < 0.05$, $**P < 0.005$; unpaired *t* test. (*G*) Visualization of clustering of HC and LC CDR3 sequences of all IgG and IgA. Blue tones represent antibody sequences from casein-immunized mice, red represent antibody sequences from HEL-immunized mice, and gray represent antibody sequences from control mouse. Circles indicate the clusters that were selected for mAb expression. (*i–iv*) Magnifications of (*i*) the largest cluster, and (*ii–iv*) the clusters containing casein-binding antibodies (*ii*, cluster containing C3_CL661; *iii*, cluster containing C2_315; *iv*, cluster containing C1_CL409). (*H* and *I*) ELISA data of the three highly reactive mAbs reacting to (*H*) casein and (*I*) MAG. Mean ± SD of triplicate measurements are shown from one representative of three independent experiments; $**P < 0.005$; unpaired *t* test.

Table 2. Demographics and disease characteristics of patients

Characteristic	Patients with ONDs	Patients with MS
Total, *n*	35	45
ELISA	23	39
ELISPOT	35	45
Female sex, %	60	67.5
Age, median (range), y	56 (24–88)	48 (20–70)
Time since diagnosis, median (range), y	NA	11 (0.75–33)
EDSS score (range)	NA	2.5 (1–5.5)
Consumption of milk,* median (range), mL/d	42.85 (0–400)	53.35 (0–1,000)
Consumption of cream,* median (range), g/d	0 (0–35)	0 (0–28)
Consumption of cheese,* median (range), g/d	14.28 (0–100)	14.28 (0–71)
ELISPOT casein responders, %†	28	42
ELISA OD value > 1.0, %†	21.7	41.02

EDSS, expanded disability status scale; NA, not applicable.
*Data for 35 patients with ONDs and 39 patients with MS.
†The table indicates values for the ELISA and ELISPOT assays that were both directed against bovine casein.

Freund's adjuvant (CFA) emulsified with different myelin antigens along with pertussis toxin (PTx) overcomes the tolerant/anergic state and induces an inflammatory response in the CNS (37–39). Here we immunized B6 mice with antigens from bovine milk and CFA and Ptx to potentiate a milk antigen-specific proinflammatory/humoral immune response. Serum IgG from mice that were immunized specifically with casein presented a diverse and unique range of immunohistological staining patterns when incubated on murine spinal cord sections or human brain sections. This heterogeneity of response can be explained by the nature of immunization where the immunizing antigens include all casein types (α-, β-, κ-casein), the hydrolyzed products of casein and partially dephosphorylated casein. Because of the complexity of the casein proteins/immunizing antigens, differences in antigen processing and presentation between individual mice could essentially lead to the release of multiple and dissimilar epitopes (40–42). This immune response to one or multiple casein proteins or their hydrolyzed products could induce cross-reactive antibodies to more than one epitope or candidate antigen. While further analysis needs to be done to carefully dissect this diverse response to casein, these observations can be translated to the human disease itself, where patients who were seropositive for casein but negative for titers to MAG could potentially have higher titers to other casein cross-reactive myelin or neuronal antigens.

To summarize, we hypothesize that, in the event of ongoing CNS inflammation, there is a loss of tolerance to the group of milk protein casein(s), one or several of which share sequence similarities with CNS antigens such as MAG, resulting in antibody cross-reactivity between the two. While there is also an epidemiological correlation involving milk consumption and the prevalence of MS (14, 15, 43), whether this is related to the cross-reactivity between casein and MAG remains a matter of speculation. We acknowledge that, in addition to the experiments performed, further studies need to be conducted, including isolation of casein-specific antibodies from MS patients with high seropositivity to bovine casein, to provide stronger evidence that anti-casein antibodies cross-react with MAG. Nevertheless, our observations call for the need to consider personalized dietary restrictions in the treatment of individual patients with MS.

Materials and Methods

Mice and Immunizations. Eight-week-old female WT B6 mice (Charles River) were kept in specific pathogen-free conditions at the animal facility of the Franz-Penzoldt-Zentrum, Erlangen, Germany (approval by the Regierung von Unterfranken, RUF-55.2.2-2532-2-575-5). All animal experiments complied with the German Law on the Protection of Animals, the "Principles of laboratory animal care" (44) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (45). For immunization, incomplete Freund's adjuvant (IFA) was prepared by mixing paraffin oil and mannide monooleate (both Sigma Aldrich) in a 9:1 ratio. CFA was subsequently obtained by adding 5 mg/mL *Mycobacterium tuberculosis* H37 Ra (Difco) to IFA. A total of six cohorts of B6 mice ($n = 6$ to 10 per cohort) were immunized subcutaneously in both sides of the flank with casein solution from bovine milk (Sigma Aldrich) emulsified (1:1) in CFA, resulting in a total injection of 200 μg of antigen per mouse. Additionally, 200 ng PTx (List Biological Laboratories) was injected intraperitoneally on the day of immunization and 48 h later. One cohort each of casein-immunized mice was killed at day 13 or day 20, three cohorts were killed at day 40, and the last cohort was killed at day 60.

The three control groups comprised nonimmunized mice or mice immunized with β-lactoglobulin ($n = 5$) (Sigma Aldrich) or α-lactalbumin ($n = 4$) from bovine milk (Sigma Aldrich). The same protocol and amount of protein (i.e., 200 μg antigen per mouse) was used as for casein immunization.

Additionally, another cohort of casein-immunized ($n = 18$) WT B6 was used for complement depletion studies. A summary of the different cohorts of mice and the respective immunizations are mentioned in *SI Appendix*, Table S5. A clinical scoring system was developed to check the mice of the different cohorts for any signs of weakness or disability, as shown in Table 1.

IgHEL Mice. IgHEL mice carry the *IghelMD4* transgene, that is, Igkc (κ chain constant region) and rearranged Ighm (IgM) and Ighd (IgD) HC specific for HEL, integrated at chromosome 17. Over 90% of splenic B cells derived from the transgene are specific for HEL and predominantly express IgM; therefore, these mice are unable to generate Igs of any other specificities (18, 19). All mice were bred in specific pathogen-free conditions at the central animal facility of the University Clinic of Bonn, Bonn, Germany, and were used at 8 to 12 wk of age. All animal experiments were approved by a government ethics board of the German state of North Rhine-Westphalia, with approval from the Bezirksregierung Köln of the German state of North Rhine-Westphalia (File # 8.87-50.10.31.09.027) and were performed in strict accordance with the recommendations of the Federation of Laboratory Animal Science Associations. To confirm that the pathology observed in casein-immunized WT mice was IgG-mediated, IgHEL mice ($n = 6$) were immunized with bovine casein as above and killed 40 d later. Spinal cords from casein-immunized IgHEL mice were examined by EM.

Electron Microscopic Assessment. Tissue preparation and subsequent EM analysis of the spinal cord were performed as previously described (46). Briefly, immunized mice and controls were killed by CO_2 asphyxiation and perfused with 4% paraformaldehyde (PFA) (wt/vol) in PBS. The lumbar region of the spinal cord was removed from the vertebral canal and post-fixed overnight at 4 °C in 4% glutaraldehyde/4% PFA/0.2% picric acid in phosphate buffer/cacodylate buffer. Following fixation, the specimens were rinsed in PBS and postfixed in 1% (vol/vol) osmium tetroxide and 1.5% (vol/vol) potassium ferricyanide at 4 °C. The tissues were rinsed again in PBS and dehydrated through an ascending ethanol series. The samples were infused with an ethanol/acetone mixture, pure acetone, an acetone/Epon 812 substitute mixture, and pure Epon 812 substitute (Carl Roth). After addition of 2% (vol/vol) 2,4,6-Tris(dimethylaminomethyl)phenol (Carl Roth) to the final volume of the Epon 812 substitute, tissue specimens were placed in resin-filled BEEM® capsules and polymerized at 60 °C and 80 °C. Single ultrathin sections were cut, mounted on single Pioloform-

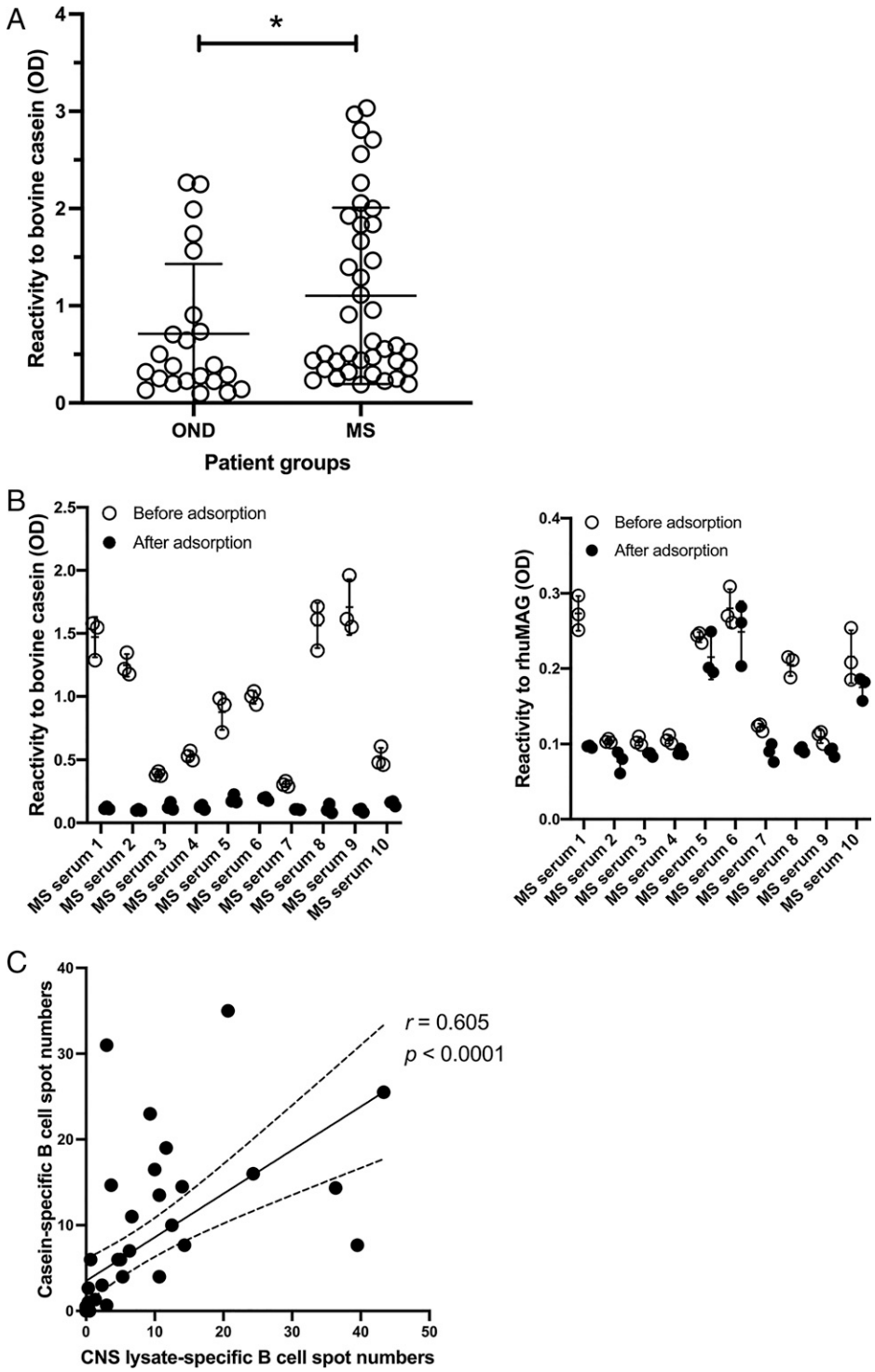

Fig. 4. Patients with MS show elevated numbers of B cell responses and IgG titers to bovine casein in the blood. (*A*) Casein-specific IgG reactivity in patients with MS versus patients with ONDs, measured by ELISA. Each data point for the OD represents the mean ± SD of triplicates for every serum sample. $*P < 0.05$ (Mann–Whitney *U* test). (*B*) IgG titers to bovine casein and MAG before and after adsorption against bovine casein of serum samples from patients with MS. Mean values ± SDs are shown for the individual samples. (*C*) Correlation analysis between B cell reactivity to whole normal human brain lysate and bovine casein in the MS group; the dashed lines are the 95% CIs to the correlation (solid line) (Pearson's correlation coefficient $r = 0.605$; $P < 0.0001$).

coated copper slot grids (Plano), and stained with lead citrate and uranyl acetate. Ultrathin sections of 50 nm were examined with a Zeiss 906 electron microscope (Carl Zeiss). Ten images were acquired per mouse, and the percentage of nerve fibers showing myelin pathology was quantified using ImageJ according to previously published criteria (47). Analysis was performed blinded.

IHC. Immunized and control mice were killed by CO_2 asphyxiation and transcardially perfused with 4% PFA (wt/vol) in PBS. For histological analysis and IHC, spinal cord tissue from the lumbar region was carefully dissected out and fixed overnight in 4% (wt/vol) PFA, following which it was processed according to standard protocols for paraffin embedding. Paraffin-embedded spinal cord sections were cut to 5-μm thickness using a semiautomated microtome (Leica),

rehydrated in a descending series of ethanol solutions, and stained with H&E according to standard histologic protocols. For IHC, heat-induced antigen retrieval of murine spinal cords was performed in 10 mM sodium citrate buffer (pH 6.0). For all IHC stainings, a technical negative control was included where the tissue section was incubated with secondary antibody only.

Staining for IgG Deposition. To confirm the deposition of IgG, sections of spinal cord tissues from immunized WT and IgHEL mice were blocked in 5% normal goat serum (vol/vol) in Tris-buffered saline (TBS) with 0.05% (wt/vol) Tween 20 (TBS-T) for 1 h at room temperature and then incubated with either a fluorescein isothiocyanate-conjugated goat anti-mouse IgG F(ab′)2 antibody (Invitrogen) or biotinylated goat anti-mouse Igs (Agilent Dako) (dilution 1:1,000) in 0.5% (vol/vol) blocking buffer for 2 h to 3 h at room temperature. Sections incubated with goat anti-mouse Igs (biotin) were developed using streptavidin conjugated with Alexa Fluor 647 (Invitrogen).

Mouse ELISA for Bovine Casein and Recombinant MAG. ELISA Microlon medium binding plates (Greiner Bio-One) were coated overnight at 4 °C with casein solution from bovine milk (Sigma Aldrich) or rmsMAG (Sino Biological) in PBS. The concentration of the coating antigens was optimized at 10 μg/mL. Plates were blocked with 10% (vol/vol) fetal bovine serum (FBS) (BD Biosciences) in 0.05% (wt/vol) Tween 20 in PBS (PBS-T). Serum samples were diluted in 0.5% FBS in PBS-T, and secondary antibodies were diluted in PBS-T. Biotinylated goat anti-mouse IgG (Abcam) or biotinylated mouse anti-mouse IgM (BD Biosciences) served as the secondary antibody at dilutions of 1:10,000 and 1:1,000, respectively. For development, plates were incubated with streptavidin–alkaline phosphatase (ALP) (Mabtech) diluted in PBS-T for 2 h before paranitrophenyl phosphate (pNPP) ELISA substrate (Mabtech) was applied. The OD in the wells was read at 405 nm on an MRXII microplate reader (Dynex Technologies) with Dynex Revelation software (version 4.22).

RNA Extraction, PCR, and qPCR for Casein Gene Expression in Mice. Total RNA was isolated from unfixed snap-frozen homogenized murine tissues (brain and breast, where breast tissue was used as a positive control for the detection of casein genes) from $n = 3$ nonimmunized WT B6 mice using TRIzol reagent (Invitrogen). The lysate was incubated in chloroform (Sigma Aldrich) at room temperature and centrifuged at 4 °C. The aqueous phase was precipitated by isopropanol (Sigma Aldrich) at room temperature; 75% (vol/vol) ethanol was added to the RNA pellet, mixed well, and centrifuged. The pellet was dried, resuspended in diethylpyrocarbonate-treated water (Invitrogen), and incubated in a heat block at 57 °C, before quantification using a photometer. The RNA was reverse transcribed to complementary DNA (cDNA) using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was used as a template for subsequent endpoint and qPCR analysis. β-actin was used as a loading control and housekeeping gene control for the endpoint and qPCRs, respectively. The qPCR runs included a no-template control for every primer set, and data were analyzed using the ΔΔcycle threshold method. A list for the primer pairs used for the different types of PCR is provided in *SI Appendix*, Table S6.

Detection of Casein-Specific Serum Reactivity to Mouse CNS Tissue. A Mouse-on-Mouse (MOM) Immunodetection Kit (Vector Laboratories) was used to identify antigen–antibody binding patterns on mouse spinal cord tissue using sera from casein-immunized mice. Briefly, antigen retrieval was performed as described above, following which mouse spinal cord sections were treated as per the instructions in the MOM kit. Dilutions of serum from immunized mice were used as the primary antibody. For fluorescence detection, streptavidin conjugated with Alexa Fluor 647 (Invitrogen) was used following incubation with MOM biotinylated anti-mouse IgG reagent.

To confirm binding of serum IgG to specific cell populations, costaining was performed using either anti-GFAP or anti-OLIG2 antibodies (both Abcam), which recognize astrocytes and oligodendrocytes, respectively. Additionally, a MAG antibody (Abcam) was used to confirm colocalization of serum IgG and its cross-reactive antigen, MAG, on oligodendrocytes. Following use of the MOM kit, spinal cord sections were incubated with either anti-GFAP, anti-OLIG2, or anti-MAG antibodies in TBS-T. Donkey anti-chicken Cy2 or donkey anti-rabbit Cy3 (both Jackson ImmunoResearch) were used as the corresponding secondary antibodies for the detection of GFAP or OLIG2 and MAG, respectively. All sections were counterstained with DAPI (Sigma Aldrich). Images were acquired using a Leica DM6 B fluorescence microscope equipped with Las X software (Leica), a Nikon D-Eclipse C1 confocal microscope, or a Leica DMi8 inverted microscope (Thunder Imager, Leica).

Cell Line and ICC. To further confirm the cross-reactive antigen that might be responsible for the binding of anti-casein IgG to oligodendrocytes, purified Ig from serum samples of mice killed at day 60 ($n = 10$) was tested on cultured Oli-Neu cells. Ig from casein-immunized mice was purified using a commercially available mouse antibody purification kit (Abcam) following the manufacturer's protocol. The oligodendroglial precursor cell line Oli-Neu, derived from mouse brain (20), was cultured and maintained at 37 °C and 5% CO_2.

For immunocytochemical staining, Oli-Neu cells were seeded at a density of 25,000 cells on poly-L-ornithine (Sigma Aldrich)-coated coverslips and cultured at 37 °C, 5% CO_2. Cell culturing was done in Bottenstein–Sato medium supplemented with 2% horse serum (ThermoFisher), 5 μg/mL insulin (Sigma), 1% penicillin/streptomycin (ThermoFisher), 1% N2 supplement (ThermoFisher), 5 ng/mL sodium selenite (Sigma S-9133), 25 μg/mL gentamicin (Sigma), 400 nM T3 (Sigma), and 520 nM T4 (Sigma) in Dulbecco's Modified Eagle Medium (GlutaMAX) (Thermo Fisher). After 48 h in culture, they were treated with 1 μM of PD174265 (Abcam) for their differentiation and arborization into oligodendrocytes (22). Cells were fixed with 4% (wt/vol) PFA and blocked with 10% (vol/vol) bovine serum albumin (BSA) in PBS. For double staining, the fixed cells were sequentially incubated with 40 μg/mL of IgG purified from casein-immunized mice (killed at day 60), followed by rabbit anti-MAG antibody (Abcam). Alexa Fluor 488-conjugated goat anti-mouse IgG (Abcam) and donkey anti-rabbit Cy3 (Jackson ImmunoResearch) were used as the corresponding secondary antibodies for the purified mouse IgG and anti-MAG antibody, respectively. After washing, cells were counterstained with DAPI (Sigma Aldrich). Immunofluorescent images were acquired using a Leica DMi8 inverted microscope (Thunder Imager, Leica). Images at a higher magnification were taken using a Nikon A1R laser scanning confocal microscope (Nikon).

Adsorption Assay. For casein adsorption experiments, 20 μL to 30 μL of mouse serum or 100 μL of human serum was incubated overnight with 0.3 mg or 1 mg of powdered casein from bovine milk (Sigma Aldrich) in 150 μL or 500 μL of PBS, respectively, at 4 °C. Casein and bound Igs were pelleted by centrifugation, and the resulting supernatant was tested for residual titers to casein from bovine milk by ELISA.

Plasmablast Sort and Repertoire Sequencing. For plasmablast sort and B cell receptor repertoire sequencing, an additional cohort of mice was immunized with either casein ($n = 4$) or HEL ($n = 3$, control group). Immunizations were performed on day 0 and day 21, as described above, and mice were killed on day 31. One control mouse remained nonimmunized. Splenocytes, blood, and lymph node cells were singularized by mechanic disruption and stained with antibodies against CD19 (BD Biosciences), B220 (ebioscience), CD38 (Miltenyi Biotec), CD138, CD27, MHCII, CXCR4, IgA, IgM (BioLegend), and Sytox Green (ThermoScientific). The Sytox$^-$ CD19$^+$ B220$^{int/low}$ CD38$^+$ CD138$^+$ CXCR4$^+$ MHCII$^+$ population was sorted on a FACSAria II sorter (BD Biosciences) (48). Two thousand sorted cells per sample were used for preparing single-cell immune profiling libraries with 5′-transcription (10× Genomics), yielding 712 to 995 cells per sample, of which 463 to 622 passed filter thresholds. Variable sequences were aligned to the International ImMunoGeneTics (IMGT) database (https://www.imgt.org) using IMGT/HighV-QUEST (49). Data were analyzed with Loupe V(D)J Browser (10× Genomics) as well as R, version 3.6.1 (50), and GraphPad Prism, version 8.4. Clonality was defined as usage of the same HC and LC V and J genes as well as 70% overlap in CDR3 regions (Levenshtein distance). Mutation load is the summed HC and LC V-gene mutation load, as assessed by IMGT. For clustering of CDR3 regions, full-length sequences of HC and LC CDR3 were concatenated with a 10-amino acid spacer between the two regions, and clustered with Cluster Database at High Identity with Tolerance (CD-HIT) using a 90% identity cutoff (51). Visualization of clusters was done in R version 3.6.1 using the iGraph package. Antibodies for recombinant expression were selected on the basis of being a representative sequence of a cluster consisting of sequences from casein-immunized mice but not from HEL-immunized mice or the control mouse. HC and LC variable sequences were cloned into pFUSEss-CHIg-hG1 and pFUSE2ss-CLIg-hK/hL2 vectors (Invivogen), respectively, expressed in HEK293 cells, and purified with protein A according to standard protocols. Phylogenetic analysis was performed as previously described (52). Briefly, individual sequences were binned according to their HC usage, then concatenated and aligned with multiple sequence alignment (MUSCLE) (53). They were clustered using maximum-likelihood clustering in PhyML (54). All individual phylogenetic trees were arranged by their HC V-gene families, generating the displayed phylogenetic trees. Trees were visualized with iTol (55). The CDR alignment visualization was generated with Geneious Prime, version 2020.1.2 (Biomatters).

Patient Serum ELISA. Using ELISA, sera from 39 patients with MS and 23 patients with ONDs (as control) were tested for their IgG reactivity to casein. The research protocol was approved by the Ethics Committee of the University of Erlangen-Nürnberg, Germany (file 185_18B). The study used pseudonymized

data, and informed written consent was obtained from all patients. To investigate cross-reactivity of anti-casein antibodies with MAG in patients with MS, a subset of serum samples ($n = 10$) was tested for their reactivity to rhuMAG before and after adsorption with bovine casein.

ELISA Microlon medium binding plates (Greiner Bio-One) were coated overnight at 4 °C with 10 μg/mL casein from bovine milk (Sigma Aldrich) or 10 μg/mL rhuMAG (Sino Biological) in PBS. Plates were blocked with 10% FBS in PBS-T (Carl Roth). Patient serum samples were diluted in 0.5% (vol/vol) FBS in PBS-T, and secondary antibodies were diluted in PBS-T. All patient sera were plated in triplicate at 1:100 dilution for both casein- and rhuMAG-coated plates. Biotinylated anti-human IgG (Mabtech) diluted in PBS-T at a concentration of 0.1 μg/mL served as the secondary antibody. For development, plates were first incubated with streptavidin–ALP (Mabtech) diluted in PBS-T before pNPP substrate (Mabtech) was applied. The OD in the wells was read at 450 nm on a MRXII microplate reader (Dynex Technologies) with Dynex Revelation software (version 4.22). To measure the reactivity of recombinant monoclonal antibodies against bovine casein and rhuMAG, the same protocol was followed, with the modification that the primary antibodies were diluted at a concentration of 5 μg/mL.

Patient Sample ELISPOT. PBMCs from 45 patients with MS and 35 patients with ONDs were tested by ELISPOT assay. The research protocol was approved by the Ethics Committee of the University of Erlangen-Nürnberg, Germany (files 185_18B and 74_18B). The study used pseudonymized data, and informed written consent was obtained from all patients. The protocol for this assay has been previously described by our group (56). Briefly, PBMCs were isolated from patients' blood by Biocoll (Merck) density gradient centrifugation. For polyclonal stimulation of B cells, PBMCs were cultured for 6 d before the ELISPOT assay at a concentration of 3×10^6 cells/mL in complete Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2.5 μg/mL R-848 (Enzo Life Sciences), 15 ng/mL interleukin-2 (Peprotech), and 1 μM mercaptoethanol (Sigma Aldrich). Ninety-six-well ELISPOT plates (Merck) were coated overnight with whole normal human brain tissue lysate (Novus) or with casein solution from bovine milk (Sigma Aldrich) at a final concentration of 30 μg/mL diluted in PBS. Wells coated with 10 μg/mL anti-human κIgG1 (SouthernBiotech) served as a positive control. Plates were blocked with 10% (vol/vol) FBS in sterile PBS at room temperature for 2 h. Each patient sample was subsequently plated in triplicate at a density of 1×10^6 polyclonally stimulated PBMCs per well. Biotinylated anti-human IgG (Mabtech) at 0.2 μg/mL in 1% (wt/vol) BSA in PBS solution was used as a detection antibody. All plates were developed with Vector Blue substrate (Vector Laboratories). Spots were counted on an ImmunoSpot Series 6 Analyzer (Cellular Technology Limited). The background reading was calculated from the negative control and was subtracted from the raw sample reading to provide the test sample measurement.

Statistical Analysis. GraphPad Prism 8.0 (GraphPad Software) was used for statistical analysis. A Shapiro–Wilk normality test was used to confirm whether the dataset followed a Gaussian distribution. Accordingly, differences between two parametric groups were assessed using an unpaired *t* test or paired *t* test while ANOVA was used for greater than two groups. For nonparametric datasets, a Mann–Whitney *U* test was used. For both parametric and nonparametric datasets, a significance level of 5% was chosen. Pearson's correlation coefficient was used to assess the correlation between OD values in ELISAs and between spot numbers in ELISPOT assays.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank Professor Winfried L. Neuhuber and Clemens Wagner for help with confocal imaging. We thank Birgit Blanck, Marion Michels, Anita Hecht, Andrea Hilpert, and Stephanie Link for their excellent technical assistance. This research project was sponsored by Bärbel and Berthold Pohl and also funded by the Interdisziplinäres Zentrum für klinische Forschung - Anschubfinanzierung program of the Friedrich-Alexander-Universität (Project P019, grant to S.K.). S.K. is also supported by Deutsche Forschungsgemeinschaft IRTG2168 Grant 272482170. S. K., J.B.-G., and C.K. are members of the Excellence Cluster "ImmunoSensation" EXC2151-390873048.

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Current Journal of Neurology

Original Paper

Curr J Neurol 2020; 19(4): 180-9

Impact of supplementation with "multivitamin-mineral" specially formulated to improve fatigue and inflammatory state in patients with multiple sclerosis: A triple-blind, randomized, placebo-controlled trial

Received: 24 May 2020
Accepted: 01 Aug. 2020

Sama Bitarafan[1], Elmira Karimi[2,3], Abdorreza Naser Moghadasi[4], Razieh Sadat Kazemi-Mozdabadi[4], Zinat Mohammadpour[5], Mohammad Ali Sahraian[4]

[1] Iranian Center of Neurological Research, Neuroscience Institute, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Community Nutrition, School of Nutritional Sciences and Dietetics, Tehran University of Medical Sciences, Tehran, Iran
[3] Students' Scientific Research Center, Tehran University of Medical Sciences, Tehran, Iran
[4] Multiple Sclerosis Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
[5] Adelaide Medical School, Faculty of Health and Sciences, University of Adelaide, Adelaide, Australia

Keywords
Multiple Sclerosis; Fatigue; Multivitamin Mineral; Cytokine; Randomized Clinical Trial

Abstract

Background: Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) with the most common complaint of fatigue. A high number of patients with MS are interested in taking dietary supplements as a complementary therapy. We propose a specially formulated supplement for patients with MS and aim to evaluate its effects on fatigue.

Methods: This study was a triple-blind, randomized, placebo-controlled trial using a stratified randomization method according to sex. 46 eligible patients participated in the study, 23 in the placebo group and 23 in the intervention group. The intervention group received two capsules of multivitamin-mineral (MVM) daily for 3 months.

How to cite this article: Bitarafan S, Karimi E, Naser Moghadasi A, Kazemi-Mozdabadi RS, Mohammadpour Z, Sahraian MA. Impact of supplementation with "multivitamin-mineral" specially formulated to improve fatigue and inflammatory state in patients with multiple sclerosis: A triple-blind, randomized, placebo-controlled trial. Curr J Neurol 2020; 19(4): 180-9.

Corresponding Author: Mohammad Ali Sahraian
Email: sahraian1350@yahoo.com

Measurements of fatigue and cytokines were performed in all patients at the baseline and after the 3-month intervention

Results: Finally, information of 41 participants was used for data analysis. However, fatigue was decreased after supplementation than before, in the intervention group (P = 0.005). There was no significant difference (P = 0.090) between the change of fatigue score in the MVM group (-3.00 ± 4.42) and the control group (-0.40 ± 5.14). Among cytokines, Interleukin 4 (IL-4) significantly increased in the intervention group compared to the placebo (P = 0.030).

Conclusion: Our study showed that the present MVM probably could improve the inflammatory state and fatigue in patients with MS.

Introduction

Multiple sclerosis (MS) is an immune-mediated inflammatory disease (IMID) causing demyelination and axonal loss in the central nervous system (CNS). The relapsing-remitting MS (RRMS) is the dominant and benign form of the disease with relapse and remission phases.[1,2] Another form is secondary progressive MS (SPMS), a slowly worsening phase with more disability, which occurs in patients with RRMS over a long period.[3]

However, the pathogenesis of MS is still under debate, evidence suggests an underlying role for activated lymphocytes including CD4+ T helper, lymphocytes Th1 and Th17, and cytotoxic CD8+ to stimulate inflammation and autoimmunity in the CNS.[4] Studies have shown that activated immune cells initially disturb the blood-brain barrier (BBB) and consequently migrate to the CNS. Imported immune cells release several pro-inflammatory mediators, resulting in structural and functional disruption to the CNS.[5-8] Numerous studies have shown that RRMS progress to SPMS due to increased pro-inflammatory cytokines. While pro-inflammatory cytokines released from Th17 (IL-17) and Th1 (IFN-γ, TNF-α, and IL-2) have been implicated as mediators of MS progression, the anti-inflammatory cytokines such as IL-4 and IL-10 produced by regulatory T cells (T-regs) have an antagonist effect on inflammatory cells and can control MS progression.[9-12]

One of the most common problems due to the aforementioned inflammatory states leading to impaired daily life activities and quality of life (QOL) in patients with MS is the overwhelming feeling of physical or psychological exhaustion called fatigue.[13] Despite using medical treatments, most patients report mild to severe fatigue. In addition, many patients with MS experiencing any degree of fatigue have an interest in consuming different types of dietary supplements with various contents of vitamins and minerals as complementary therapy.[14]

Some studies have shown the effects of supplementation with vitamins or minerals on clinical and laboratory outcomes in patients with MS.[15-17] Numerous studies have reported that patients with MS improved after separate supplementations with vitamins (A, B, C, D, and E) and minerals (Calcium, Magnesium, and Selenium). These supplements may reduce the biological synthesis of pro-inflammatory and oxidative compounds.[18-25] However, it is well known that excessive intake of Iron, Zinc, and Copper must be controlled to avoid increasing inflammatory and oxidative stress processes due to their cumulative effect in patients with MS. Therefore, many studies have yet to be performed to precisely find out the dosage of vitamins and minerals needed to achieve optimal therapeutic response in patients with MS. Based on the evidence available, it appears that a routine use of multivitamin-mineral (MVM) including Iron, Copper, and Zinc is inappropriate for these patients due to high dose intake of these minerals results in detrimental effects.[26-28] Currently, there is no such a suitable supplement appropriate to decrease fatigue in patients with MS. Here, we conducted a clinical trial to investigate whether administration of the specially proposed MVM to MS affects the degree of fatigue and inflammatory state in patients with MS.

Materials and Methods

Study design: The present study was a triple-blind and randomized clinical trial to compare the state of fatigue and inflammatory factors in patients with MS receiving MVM supplements specialized for fatigue treatment with the placebo group. The study was registered on the Iranian Registry of Clinical Trials (IRCT) with code IRCT2016022026658N1. Additionally, the ethical approval was received from the Ethics Committee, Tehran University of Medical Sciences, Tehran, Iran (IR.TUMS.REC.1394.873).

Participants: Randomization was performed using a stratified randomization method according to sex to select eligible patients among those referred to MS Research Center, Sina Hospital, Tehran between December 2018 and June 2019. All the colleagues that were involved in the study were blind, and only one person who coded the drug packs was aware of the intervention who had no further involvement in the study (Figure 1).

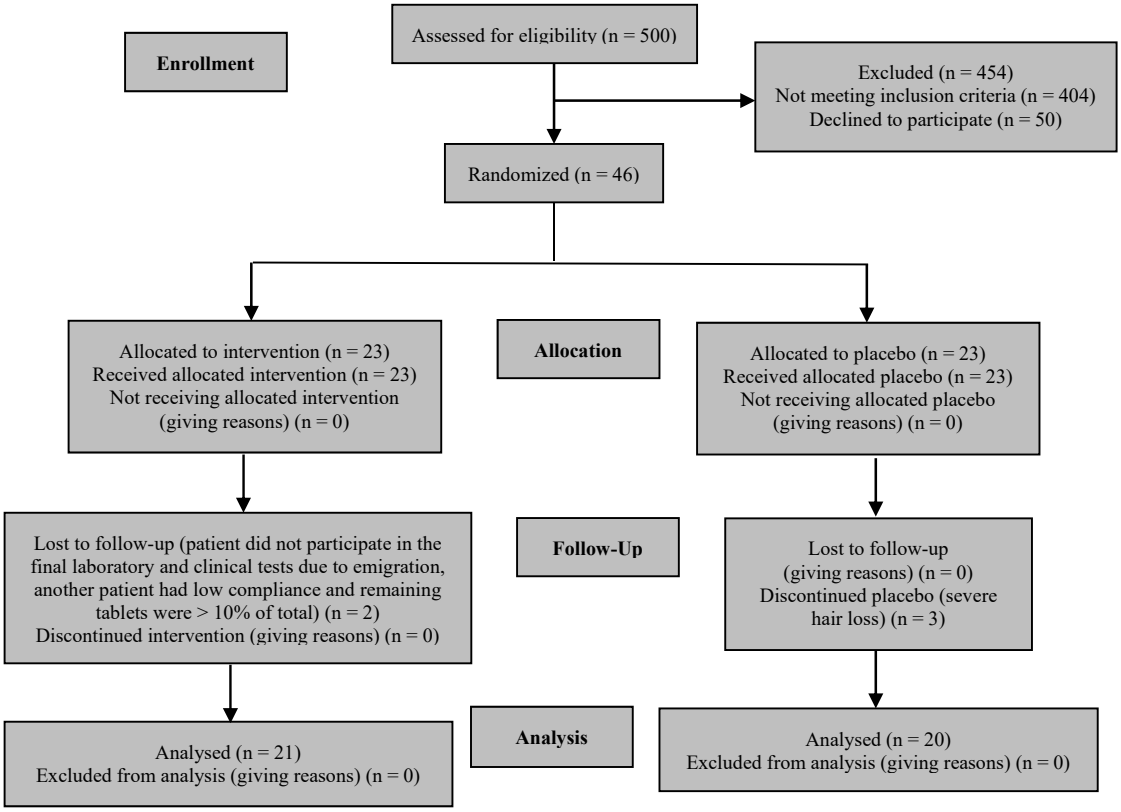

Figure 1. CONSORT 2010 flow diagram for multivitamin-mineral (MVM) intervention study

The authors checked the inclusion and exclusion criteria, generated the random allocation sequence, and assigned participants to the groups. All participants were informed and signed informed consent forms and they were enrolled with the definite diagnosis of MS (RRMS subtype), according to the 2010 McDonald criteria.[29]

To control the selection bias, the study was designed to recruit both male and female patients aged 18 to 45 years, which were not in the acute phase of MS at the time of screening. The patients were followed up every month during the study to determine the adhesion to using the supplement. Furthermore, patients were recruited for study if they had all the following inclusion criteria:

- Suffering from RRMS;[29]
- Receiving interferon within at least 3 months prior to taking part in the study;
- Patients with the same protocol for fatigue treatment [they all received selective serotonin reuptake inhibitors (SSRIs)];
- Lack of taking complementary supplements at least within 3 months prior to taking part in the study;
- Having an Expanded Disability Status Scale (EDSS) score between 0 and 6;

Patients were excluded from the study if they had one or more of the following criteria:

- Pregnant participants;
- Presenting acute forms of liver disease and biliary system and pancreas disease;
- A history of viral illnesses, asthma, and other autoimmune diseases that have an impact on Th1/Th2 balance, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type 1 diabetes (T1D) and inflammatory bowel disease (IBD);
- Patients with Iron deficient;
- Participants who had a special diet or changed their diet during the study;
- Consumption of any nutritional supplements or new drugs during the study;
- Forgetfulness to use the supplement for more than 12 days (10% of treatment period);
- Obese patients [body mass index (BMI) ≥ 30 kg/m^2), malnourished patients (BMI < 18.5 kg/m^2) and substance, cigarette, or alcohol dependence;

Intervention: Our intervention group received

a daily intake of two MVM capsules for 3 months. The MVM and placebo capsules were similar in appearance to inhibit the possible information bias.

Each two MVM capsules consisted of 350 µg of vitamin A, 15 µg of vitamin D,[30,31] 7.5 mg of vitamin D, 75 mg of vitamin C, 1.1 mg of vitamin B1, 1.5 mg of vitamin B6, 400 µg of vitamin B9, 2.4 µg of vitamin B12, and also 250 mg of calcium, 160 mg of magnesium, 27 µg of selenium, 200 mg of Q10, and 100 mg of L-carnitine.

The dose of vitamins and minerals was recommended according to the Recommended Dietary Allowance (RDA) intake for vitamins D, C, B1, B6, and B12, and preventing toxicity for vitamins A and E, preventing gastrointestinal complications for calcium, magnesium, and selenium. In the case of L-carnitine and Q10, the recommended dose was based on the amounts suggested in published articles.[32] The MVM capsules were designed to have zero amounts of Zinc, iron, and copper and placebo capsules consisted of sunflower oil.

Assessment of outcomes

Primary outcomes: We measured disability using EDSS.[33] The EDSS test was performed for each subject to obtain a physical measure of neurological impairment before and at the end of the study. The Fatigue Severity Scale (FSS) measured tiredness or fatigue score.[34] The cytokines including Interferon Gama (INF-γ), IL-17 (interleukin), IL-4, and Tumor Necrosis Factor alpha (TNF-α) were performed in all patients at the baseline and after the 3-month intervention. The serum levels of cytokines were measured with enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory, China).

Secondary outcomes: Demographic characteristics including age, sex, smoking, and alcohol use were documented based on self-reported information. Moreover, measurements of anthropometric indices including weight, height, and BMI were evaluated.

To offset any changes in energy and macronutrient consumption (protein, fat, and carbohydrate) that could alter outcomes, standardized 24-hour dietary recalls were recorded through interviews at the baseline and on the same day of the week following the final assessment. Mean daily intakes of energy and nutrients were calculated with computerized Nutri4 software.

The Beck Depression Inventory (BDI) was used to evaluate the degree of depression.[35] The FSS and BDI questionnaires were completed for each participant at the time of enrollment and at the end of the study. Measurements of serum levels of vitamin D, alanine transaminase (ALT), aspartate transaminase (AST), Zinc, Ferritin, and high-sensitivity C-reactive protein (hs-CRP) were performed in all patients at the baseline and after the 3-month intervention, as follows:

Measurement of hs-CRP in serum was performed with turbidimetric assay by specific ELISA kits and serum concentrations of ALT and AST were measured by enzymatic spectrophotometry using specific ELISA kits (Pars Azmoon, Tehran, IRAN and Autoanalyzer BT 1500, Medsystem, USA). Serum Zinc was determined by enzymatic spectrophotometry with specific ELISA kits (Zist Shimi, Tehran, IRAN and Autoanalyzer BT 1500, Medsystem, USA). Serum 25 (OH) vitamin D and ferritin levels were estimated using electrochemiluminescence (ECL) and an ELISA kit provided by Roche Diagnostics GmbH (06506780160, Mannheim, Germany) by an automated device (Cobas e411; Roche Diagnostics GmbH, Mannheim, Germany).

The data were screened for normality through the one-sample Kolmogorov-Smirnov (KS) test. Furthermore, parametric and non-parametric tests were applied to analyze the data with normal and non-normal distributions, respectively. A paired sample t-test was employed to compare the intragroup discrepancies in fatigue state and biochemical markers, before and after the intervention. An independent sample t-test was also used for assessing differences of the mentioned outcomes between groups before and after the intervention. All statistical analyses were performed using Statistical Package for Social Science (version 18.0, SPSS Inc., Chicago, IL, USA) and P-values < 0.050 were considered statistically significant.

Results

Recruitment and baseline characteristics: 46 eligible patients with MS were enrolled in the study, 23 patients to the MVM group, and 23 patients to the placebo group. 2 patients were excluded from the intervention group; 1 did not participate in the follow-up tests due to emigration and the other patient had low compliance (remaining tablets > 10% of the total). Moreover, 3 patients in the placebo group withdrew from the study due to severe hair loss. Finally, information of 41 remaining participants was used for data analysis. The primary characteristics of participants are presented in table 1. There was no significant difference between the two groups in age, sex, duration of illness, energy intake, and BMI (Table 1).

Table 1. Self-explanatory characteristics of patients

Characteristic	Groups		P
	Supplementation	Placebo	
Sex (female/male) [n (%)]	21 (17.4)	20 (17.3)	0.530[#]
Age (mean ± SD)	35.14 ± 5.39	35.35 ± 5.73	0.910[*]
Disease duration (mean ± SD)	7.10 ± 3.18	6.70 ± 3.88	0.720[*]
BMI (mean ± SD)	24.49 ± 1.90	23.76 ± 1.65	0.200[*]
Energy intake[**] (mean ± SD)	1930.29 ± 111.20	1958.85 ± 112.70	0.410[*]

BMI: Body mass index; SD: Standard deviation
[*]Independent sample t-test, [**]Kilocalorie/day, [#]Chi-square

Blood biochemical outcomes: The serum levels of biochemical factors including ALT, AST, Zinc, vitamin D, Ferritin, and CRP in the two groups before and after the study are summarized in table 2. There were no significant differences in these biochemical markers between the two groups, but there was a significant reduction in ALT (P = 0.040) and hs-CRP (P = 0.006) in the intervention group after the study. In addition, a significant decrease was observed in ferritin concentration in both groups after the study (Table 2).

BMI and energy intake: Comparisons of BMI and intake calories between intervention and control groups before and after the study are summarized in Table 3. The change in BMI had a significant reduction in the intervention group compared to the placebo group. However, the change in calorie intake was not significantly different between the two groups (Table 3).

Clinical outcomes: However, there was no significant difference between the groups (P = 0.090); the intervention group reported less fatigue experience after supplementation compared to before the supplementation (P = 0.005). Furthermore, neither depression (P = 0.180) nor disability rates (P = 0.110) were not different between the groups (Table 3).

Serum levels of cytokines: The present findings showed an increase in IL4 in the intervention group compared to the placebo group (P = 0.030). Serum levels of the other cytokines did not differ between the groups. However, after the 3-month intervention, the INF-γ level in the intervention group decreased significantly after the intervention compared to before (P = 0.040) (Table 4).

Table 2. Comparison of biochemical parameters between the two groups of study and between before and after supplementation in each group

Biochemical parameters	Time	Groups[¥]		P[*]
		Supplementation	Placebo	
Aspartate transaminase	Before	22.95 ± 8.60	21.10 ± 8.73	0.370
	After	21.29 ± 6.89	20.90 ± 8.03	0.870
	Change	-1.67 ± 7.48	-0.20 ± 4.81	0.520[#]
	P[***]	0.480	0.610	
Alanine transaminase	Before	21.33 ± 13.38	21.35 ± 17.70	0.570[#]
	After	17.05 ± 7.66	21.95 ± 11.95	0.130
	Change	-4.29 ± 9.128	0.60 ± 10.97	0.130
	P[**]	0.040	0.810	
Zinc	Before	146.80 ± 34.65	144.48 ± 18.84	0.790
	After	134.44 ± 14.51	142.64 ± 15.55	0.090
	Change	-12.36 ± 28.41	-1.84 ± 5.74	0.490[#]
	P[***]	0.110	0.190	
Vitamin D	Before	61.78 ± 26.96	53.27 ± 25.91	0.310
	After	61.78 ± 25.79	54.55 ± 25.85	0.380
	Change	-0.01 ± 8.08	1.29 ± 5.14	0.550
	P[**]	0.990	0.270	
Ferritin	Before	125.28 ± 86.57	105.75 ± 80.87	0.460
	After	94.89 ± 65.37	94.78 ± 72.76	0.980
	Change	-30.40 ± 37.46	-10.96 ± 15.63	0.137[#]
	P[***]	< 0.001	0.002	
CRP	Before	2.82 ± 2.27	2.80 ± 1.97	0.880
	After	2.31 ± 1.84	2.44 ± 1.86	0.690
	Change	-0.51 ± 0.77	-0.37 ± 0.88	0.570
	P[**]	0.006	0.080	

SD: Standard deviation CRP: C-reactive protein
[¥]Mean ± SD, [*]Independent sample t-test, [**]Paired sample t-test, [***]Wilcoxon signed-rank test, [#]Mann-Whitney U test

Table 3. Comparison of clinical outcomes between the two groups of study and between before and after supplementation in each group

Clinical outcomes	Time	Groups[¥]		P*
		Supplementation	Placebo	
BMI	Before	24.49 ± 1.90	23.76 ± 1.65	0.200
	After	24.08 ± 1.84	23.74 ± 1.64	0.540
	Change	-0.42 ± 0.48	-0.02 ± 0.24	0.002
	P[**]	0.001	0.620	
Calorie[€]	Before	1930.29 ± 111.20	1958.85 ± 112.70	0.420
	After	1937.81 ± 110.95	1939.15 ± 127.11	0.970
	Change	7.52 ± 95.93	-19.70 ± 63.21	0.290
	P[**]	0.720	0.180	
Fatigue	Before	38.52 ± 8.01	36.20 ± 6.46	0.310
	After	35.52 ± 8.23	35.80 ± 6.72	0.910
	Change	-3.00 ± 4.42	-0.40 ± 5.14	0.090
	P[**]	0.005	0.730	
Depression	Before	18.14 ± 3.32	17.60 ± 3.49	0.610
	After	17.48 ± 7.09	19.25 ± 5.25	0.370
	Change	-0.67 ± 6.42	1.65 ± 4.08	0.180
	P[**]	0.640	0.090	
Disability	Before	1.33 ± 1.32	1.20 ± 1.32	0.610[#]
	After	1.24 ± 1.18	1.30 ± 1.38	0.930[#]
	Change	-0.10 ± 0.30	0.10 ± 0.45	0.110[#]
	P[***]	0.160	0.320	

BMI: Body mass index; SD: Standard deviation

[¥]Mean ± SD, [*]Independent sample t-test, [**]Paired sample t-test, [***]Wilcoxon signed-rank test, [#]Mann-Whitney U test; [€]Kilocalorie/day

Discussion

Despite the high prevalence of fatigue and its detrimental impact on the QOL of patients with MS, few studies have been conducted to address this issue. The effects of complementary treatment for fatigue improvement in patients with MS have remained uncertain.[36,37] One strong hypothesis expresses that the severity of fatigue is dependent on the inflammation situation in patients with MS.[38-41] In this way, supplementation with vitamins, minerals, and MVM could be a safe complementary therapeutic option that reduces the severity of fatigue by improving the inflammatory status.[20,21,23]

Table 4. Comparison of Inflammatory indexes between the two groups of study and between before and after supplementation in each group

Inflammatory indexes	Time	Groups[¥]		P[#]
		Supplementation	Placebo	
IL-4	Before	58.77 ± 71.05	54.31 ± 44.87	0.120
	After	84.23 ± 111.99	43.94 ± 36.87	0.970
	Change	25.46 ± 52.34	-10.37 ± 32.82	0.030
	P[***]	0.090	0.110	
IL-17	Before	33.10 ± 38.50	33.57 ± 37.73	0.270
	After	32.88 ± 37.69	35.58 ± 36.42	0.220
	Change	-0.22 ± 3.06	2.01 ± 6.30	0.160[*]
	P[**]	0.740	0.170	
TNF	Before	336.28 ± 170.40	315.03 ± 150.12	0.710
	After	318.21 ± 164.24	312.41 ± 155.38	0.970
	Change	-18.07 ± 40.93	-2.63 ± 23.42	0.150[*]
	P[**]	0.060	0.620	
INF-γ	Before	35.24 ± 54.48	24.28 ± 32.45	0.520
	After	25.16 ± 42.18	24.95 ± 32.33	0.880
	Change	-10.08 ± 16.18	0.67 ± 3.46	0.070
	P[***]	0.040	0.850	

SD: Standard deviation; IL: Interleukin; TNF: Tumor Necrosis Factor; INF-γ: Interferon Gama

[¥]Mean ± SD, [*]Independent sample t-test, [**]Paired sample t-test, [***]Wilcoxon signed-rank test, [#]Mann-Whitney U test

On the other hand, evidence has shown the adverse cumulative effects of the continuous intake of some minerals such as Iron, Copper, and Zinc through MVM supplements in patients.[26-28,42-44] Therefore, we proposed a specifically formulated MVM without Iron, Copper, and Zinc and aimed to investigate its effect on fatigue and cytokines in patients with MS. Finally, we showed the reductive trend for fatigue and inflammatory markers including CRP and INF-γ as well as a growing trend for IL-4 that is an anti-inflammatory cytokine.

However, our findings are supported by similar studies that have tested common vitamin or mineral supplements as a complementary treatment for MS. For example, improvement in fatigue scale in patients with chronic fatigue syndrome (CFS) as well as level of superoxide dismutase activity was reported after 2 months of MVM supplements.[45,46] Another study reported that short-term exposure to MVM intervention in older adults resulted in reduced circulating level of CRP and of oxidative stress.[47] In addition, Johnson et al. found that one supplement containing magnesium, zinc, selenium, and vitamins B6, D, A, and E could reduce MS incidence.[22]

Moreover, our interpretation was consistent with the finding of a meta-analysis in which the significant benefits of MVM supplementation were reported on fatigue.[48] However, subjects inversely responded to MVM treatment and exhibited a higher inflammatory status. For instance, MVM supplementation led to an unexpected increased oxidative stress in healthy adults that is a contributing factor to inflammation.[49,50] Studies reported that this discrepancy is likely through Fenton reaction by Iron molecules existing in the supplement.[51]

Some studies have provided evidence in support of the findings of our study. In this regard, recent studies have shown that retinoid, derived from vitamin A, has been shown to slow down MS progression for even the progressive phase of the disease.[52] Retinoid directly promotes the pro-inflammatory/anti-inflammatory balance and is associated with transcription of major anti-inflammatory mediators, followed by decreased Th1 and Th17 proliferation.[52,53] Furthermore, vitamin A has been shown to have a synergistic effect with INF-β.[54]

In our previous study on vitamin A, we provided the long term beneficial consequences on fatigue severity in patients with MS.[16] Another fat-soluble vitamin, calcitriol, present in our supplement has the same performance on the immune system as vitamin A.[55] There has been report of a reduced fatigue state after treatment with a single dose of vitamin D in healthy people.[55] It is thought that imbalances in the dopamine level in the CNS may motivate fatigue centers in the brain and vitamin D is able to modify this imbalance.[54] Further mechanisms for the involvement of vitamin D in reducing fatigue rate come from its ability in stimulating the production of the serotonin in the brain,[56] which has been shown to be inversely related to tiredness.[57] Moreover, the oral administration of calcium with or without vitamin D acts through various mechanisms, such as reducing inflammatory marker levels (TNF-α, IL-6), through which regulates the immune system.[58,59] As such, the role that calcium may have on fatigue by reducing these inflammatory markers could present insight into the benefit of its supplementation in MS.

The rationale for using antioxidants in our MVM is based on the knowledge that oxidative stress is one of the most critical components of the MS disease.[60] Unrestricted reactive oxygen species (ROS) production under chronic inflammatory conditions in MS is responsible for depleting the body's antioxidant reserves, including vitamins C and E and selenium.[61]

In this regard, studies showed the protective role of vitamins E and C and selenium in maintaining blood concentrations of glutathione peroxidase and decreasing prostaglandin E2 secretion from macrophages in patients with MS.[62-64] B vitamins, including B1, B2, B3, B5, B6, and B12 have been shown to play inter-related functions in reducing fatigue severity after physical activity in people living in hot climates.[65] Some observational studies have also reported that fatigue in patients with MS may be associated with mild intracellular vitamin B1 deficiency and subsequent impairment of thiamine-dependent cell reactions.[66] Another example of fatigue reducing properties of B vitamins is the role of vitamin B12 in synthesizing and maintaining myelin in patients with MS.[67] Furthermore, a significant relationship between folic acid deficiency and increased fatigue has been observed in these patients.[68]

Vitamin B6 and magnesium have a related role in regulating nitric oxide (NO) concentration within vascular endothelial cells and act as coenzymes in regulating intracellular NO production and the secretion of NO from cells. Therefore, deficiency of vitamin B6 or magnesium

leads to NO entrapment inside cells and its subsequent reaction with superoxide which produces nitrogen peroxide, leading to its accumulation and many adverse cellular consequences including myelin destruction.[22]

Magnesium homeostasis is physiologically linked to other minerals such as zinc, calcium, and aluminum that maintaining their concentration within optimal ranges is crucial for the desired function of both the immune system and CNS.[69] Adjunctive supplemented carnitine as well as ubiquinone (Q10) with vitamins and minerals may have benefits in reducing fatigue in patients with MS.[70,71]

Theoretically, the effect of carnitine is because of its role in energy production in mammalian cells and high excretion of this molecule has been observed in this disease because of stable inflammatory state.

In terms of Q10, the results of a study showed the anti-oxidative feature of this component in subjects with MS followed by reduced inflammation in these patients.[72,73]

A significant difference was seen between groups in BMI. However, malnutrition often occurs in patients with MS, so reduced BMI may not be desirable in patients with MS with normal weight.[74] The probable reason for this finding could be the effect of carnitine presence in the supplement, because of its decreasing effect on weight and BMI that has been proven in various studies.[75]

Furthermore, the remarkable reduction of ferritin levels was observed in both groups in our study. This could be attributed to the lack of Iron in the supplement used in this study in intervention group. Additionally, it was because of our inclusion criteria to cut the supplementation during 3 months before study in the placebo group. Liver enzymes were not affected in the present study. This outcome could be the desired result due to the oxidative characteristics of Iron.

The main strength of our study was being a triple-blind and randomized clinical trial study with a novel MVM formulation. Besides, the confounding effects of depression and disability on fatigue were measured and controlled. There were some limitations in the study: because of the restrictive inclusion criteria, we could not achieve enough sample size in the time specified for the study. One of the limitations was unwanted hair loss in the placebo group that was speculated to be a result of their previous supplementation discontinuation before the study initiation. On the other hand, more trials with larger sample sizes and longer supplementation periods are recommended to provide more certain results.

Conclusion

The present study indicated that the specially formulated MVM for patients with MS could probably improve fatigue and inflammatory state.

Conflict of Interests

The authors declare no conflict of interest in this study.

Acknowledgments

The authors would like to appreciate NanoHayat Darou Company for financial supports and MVM production. The authors are grateful to Tehran University of Medical Sciences and Health Services (Project registration number: 94-02-188-27795) for supervision and confirmation of the study.

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Journal of Integrative Neuroscience

J. Integr. Neurosci. **2023**; 22(1): 7
https://doi.org/10.31083/j.jin2201007

Commentary

Multiple Sclerosis Pathogenesis: Possible Interplay between Vitamin D Status and Epstein Barr Virus Infection

Giulia Bivona[1,2], Concetta Scazzone[1,2], Bruna Lo Sasso[1,2], Rosaria Vincenza Giglio[1,2], Caterina Maria Gambino[1,2], Luisa Agnello[1], Marcello Ciaccio[1,2,*]

[1]Department of Biomedicine, Neurosciences and Advanced Diagnostics, Institute of Clinical Biochemistry, Clinical Molecular Medicine and Clinical Laboratory Medicine, University of Palermo, 90127 Palermo, Italy
[2]Department of Laboratory Medicine, University Hospital "P. Giaccone", 90127 Palermo, Italy
*Correspondence: marcello.ciaccio@unipa.it (Marcello Ciaccio)
Academic Editor: Gernot Riedel
Submitted: 3 August 2022 Revised: 12 August 2022 Accepted: 17 August 2022 Published: 4 January 2023

Keywords: Vitamin D; Multiple Sclerosis; Immune response; Inflammation; Epstein Barr Virus

The role of vitamin D, Epstein Barr Virus (EBV), and endogenous retrovirus (ERV) in the pathogenesis of multiple sclerosis (MS) has been addressed in recent literature. Some authors hypothesized that a synergistic interplay between these factors could favor the onset and progression of the disease [1]. However, it seems that some considerations to better define this relevant topic should be expressed.

1. Vitamin D

Vitamin D is a nutrient that can be taken by diet and an endogenous steroid hormone regulating hundreds of gene expressions [2]. Beyond the regulation of calcium and phosphate metabolism and immune response, the active form of vitamin D can modulate brain function during development and in adulthood [3,4]. Hence, vitamin D deficiency has been broadly investigated in autoimmune, neurological, and neuropsychiatric diseases, both as a risk factor for developing some disorders or as a biomarker to identify specific features, including severity.

Before specifying the biological activities of vitamin D, it is worth mentioning the steps of its metabolism, since many roles of the hormone have been revealed after the discovery that many tissues and cells are capable of synthesizing and receiving vitamin D. Ultraviolet B rays (295–310 nM) transform the cutaneous precursor 7-dehydrocholesterol into cholecalciferol, which requires two sequential hydroxylations to form the active vitamin D3. The first hydroxylation produces 25-hydroxyvitamin D (25(OH)D) in the liver through the action of 25-hydroxylase. The 1-α-hydroxylase enzyme carries out the second hydroxylation, forming vitamin D3 (1,25(OH2)D) in the kidney, prostate, placenta, lung, brain, and immune cells. The enzymes involved in vitamin D metabolism mostly belong to the cytochrome P450 (CYP450) family. CYP2R1, CYP3A4, and CYP27A1 enzymes have 25-hydroxylase activities, whereas CYP27B1 is responsible for 1,25 hydroxylation [5]. Although the renal hormone drives calcium metabolism in an endocrine fashion, the extra-renal one acts in an autocrine/paracrine fashion and is responsible for the regulation of immune response and brain function.

The nuclear receptor of active vitamin D (VDR) binds to the membrane-associated rapid-response steroid-binding proteins (MARRS) or protein-disulfide-isomers family A members 3 (PDIA3), although a surface receptor is known as well [6]. After the binding of active vitamin D, VDR and MARRS carry on, respectively, the genomic and non-genomic actions of vitamin D [7].

As Brutting *et al*., found [8], low blood 25(OH)D levels have been widely reported in the healthy population, but vitamin D reference values are still a controversial topic. Vitamin D sufficiency is defined as a serum 25(OH)D level equal to or higher than 30 ng/mL, whereas vitamin D insufficiency is described as a serum 25(OH)D level from 20 to 30 ng/mL, and deficiency is described as lower than 20 ng/mL. Note that the optimum level to maintain skeletal health is defined as sufficiency, although no optimum for vitamin D status has been identified when considering the immunomodulation activities of the hormone [9]. In other terms, the vitamin D blood levels that are required to maintain proper immune or brain function are still debated. Indeed, as for some other analytes, no consensus on serum 25(OH)D reference intervals exists due to the lack of a standardization process [10].

Active vitamin D influences the immune system in different ways, but generally it can be said that the hormone drives the balance between anti-inflammatory and pro-inflammatory immune responses. Briefly, the immunomodulating activities of vitamin D can be described as follows: vitamin D can modulate the expression of CD14 and TLR4 co-receptor in macrophages and keratinocytes. VDR and 1,25(OH)2D are expressed by macrophages, dendritic cells, and activated B and T lymphocytes. The role of active vitamin D in the differentiation of dendritic cells is open to discussion, although it is known that it can lower their antigen-presenting capacity and survival [7,11].

The 1,25(OH)2D plays a key role in T-helper (Th) Th1, Th2 and Th17 lymphocyte balance by inhibiting the production of Th1 and Th17 cells and their cytokines, and enhancing the differentiation of Th2. Further, 1,25(OH)2D fosters the development of T- regulatory (T-reg) cells and the production of their cytokines [12].

Due to the immunomodulatory activity of vitamin D, it acts against pathogens in many ways: (a) it induces the expression of cathelicidin and defensins, which are antimicrobial peptides involved in the defense against microorganisms [6,12]; (b) it increases the antimicrobial activity of the innate response; and (c) it decreases adaptive pro-inflammatory effects.

However, the main impact of 1,25(OH)2D on the defense against pathogens is modulating Th lymphocyte subset balance, which is particularly important since Th balance and T-reg cells function both affect the efficacy and safety of immune responses against pathogens.

2. Vitamin D and MS

MS is a chronic autoimmune disease of the central nervous system and represents the most common cause of non-traumatic disabling in young adults [13]. Demyelination and axonal degeneration are characteristics of the disease. As for other neurological diseases, robust data from large genome-wide association studies support the role of genetics as one of the most significant risk factors for MS [14,15]. However, the impact of environmental factors on the etiology and pathogenesis of the disease is well-established, and many efforts have been made in past decades to identify possible environmental risk factors for MS. One of the most studied environmental risk factors for MS is vitamin-D-poor status.

It is interesting that among gene variants associated with MS risk, some are involved in the metabolic pathway influencing vitamin D status. An extensive dissertation on all vitamin D-related gene variants that have been evaluated in MS patients goes beyond of the scope of this commentary. However, the single-nucleotide polymorphisms of VDR have been largely investigated with some positive association to the risk of disease development, whereas research on others, including CYP27A1, CYP3A4, CYP27B1, CYP24A1, produced more controversial finding [16,17].

Beyond genetic investigations, several studies have addressed the question of whether low vitamin D levels could be a risk factor for the onset of the diseases or a biomarker for disease severity in MS patients [17].

Vitamin D levels have been reported in patients affected by MS in both neonatal and adult cohorts [18–20]. A large multi-center, randomized trial showed that serum 25(OH)D level is a strong risk factor for long-term MS activity and progression in the early disease course, and seems to predict new active lesions and relapse rate [21]. Munger *et al.* [18] found that low 25(OH)D levels ($<$30 nmol/L) were associated with a higher MS risk than were normal levels ($\geq$50 nmol/L). Although these findings could seem encouraging, it should be noted that 25(OH)D measurement was performed by chemiluminescence assay, whereas liquid chromatography-tandem mass spectrometry methods should be used to measure 25(OH)D, according to the National Institute for Standard and Technology recommendations [22]. The standardization issue for 25(OH)D measurement is the main reason there is no evidence-based consensus by which 25(OH)D values define vitamin D insufficiency, deficiency, and sufficiency. The lack of standardized data has hampered reaching univocal findings on the role of vitamin D in disease, and MS is not an exception.

3. Vitamin D, a Putative Link Bridging EBV, ERV and MS

Vitamin D levels have been studied in patients affected by respiratory tract infections, tuberculosis, virus infections (Human Immunodeficiency Virus, EBV), parasitic, and fungal infections in both adults and children, both as a risk factor and as a supplementation adjuvant therapy [11].

The relationship between vitamin D and EBV infection has been evaluated mainly in patients affected by MS, because EBV infection is one of the most studied environmental factors involved in the pathogenesis of the disease [23]. Several direct and indirect mechanisms have been hypothesized to explain the susceptibility of patients with mononucleosis, which is caused by EBV, to MS onset. Many studies reported high levels and frequencies of EBV antibodies in MS, but the presence of EBV is almost ubiquitous in adults, which weakens the hypothesis [24]. Further, assay methods used to detect antibodies in MS patients can influence the strength of the results to some extent. It is important to note that the presence of low vitamin D in healthy people, together with the lack of standardized data from affected ones, represent two main pitfalls weakening the findings available on this topic. Also, it should be remembered that, during chronic inflammatory disease, as MS is, many analytes can change without an established causal link between the disease and the molecule variation, due to the reverse causation issue [25,26].

Finally, ERV expression has also been related to the risk of MS onset; MS patients have been found to overexpress RNA from the HERV-W ERV family relative to healthy controls [14]. Molecular mechanisms underlying the relationship between ERV expression and MS onset include, among others, the expression of proteins from ERVs that induce pro-inflammatory cytokines (such as TNFα) [27]. Also, EBV infection has been reported to trigger the expression of HERV-W loci [27]. However, it seems that no conclusions can be drawn on this issue. Indeed, from a strictly theoretical point of view, the interplay among low vitamin D status, MS, EBV, and ERV, could be referred to as a pro-inflammatory *milieu* that could facilitate the etiopathogenesis mechanisms involved in the onset of

the disease. However, this scenario makes more likely the hypothesis that vitamin D, EBV, and ERV are pieces of a larger and composite mosaic that seems yet to be understood.

Author Contributions

GB—extraction and drafting of the manuscript; CS, BLS—analysis of data; LA, CMG, RVG—manuscript revision; GB, MC—design and revision.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest. GB and MC are serving as the Editorial Board members of this journal. GB served as Guest Editor of Special issue "Vitamin D and the Nervous System". We declare that GB and MC had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GR.

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Original article

Modified Mediterranean Diet VS. Traditional Iranian Diet: Efficacy of Dietary Interventions on Dietary Inflammatory Index Score, Fatigue Severity and Disability in Multiple Sclerosis Patients

Jalal Bohlouli [1], Iman Namjoo [2], Mohammad Borzoo-Isfahani [3], Fariborz Poorbaferani [4], Amir Reza Moravejolahkami [5*], Cain C. T. Clark [6], and Mohammad Ali Hojjati Kermani [7]

[1]Department of Nutrition, Nutrition and Food Security Research Centre, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Email: Bohlooljalal@gmail.com. ORCID ID: 0000-0003-0266-2537.

[2]Department of Community Nutrition, School of Nutrition & Food Science, Isfahan University of Medical Sciences, Isfahan, Iran. Email: inamjoo91@gmail.com; i.namjoo@nutr.mui.ac.ir. ORCID ID: 0000-0002-1889-2866.

[3] Department of Community Nutrition, School of Nutrition & Food Science, Isfahan University of Medical Sciences, Isfahan, Iran. Email: nutr.borzoo@gmail.com . ORCID ID: 0000-0003-2020-9920.

[4]Food Security Research Center, Department of clinical Nutrition, School of Nutrition & Food Science, Isfahan University of Medical Sciences, Isfahan, Iran. Email: fariborzporbafrani1371@gmail.com. ORCID ID: 0000-0001-8273-4005.

This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI
10.1017/S000711452100307X
The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

[5]Department of Clinical Nutrition, School of Nutrition & Food Science, Isfahan University of Medical Sciences, Isfahan, Iran. Emails: a.moravej@mail.mui.ac.ir; amimohs@gmail.com. ORCID ID: 0000-0001-9707-0352.

[6]Centre for Intelligent Healthcare, Coventry University, Coventry, CV1 5FB, U.K. Email: ad0183@coventry.ac.uk. ORCID ID: 0000-0002-6610-4617.

[7]Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. Email: Imhojjati@gmail.com. ORCID ID: 0000-0002-2364-3696.

***Corresponding author:** Dr. Amir Reza Moravejolahkami, Department of Clinical Nutrition, School of Nutrition and Food Sciences, Isfahan University of Medical Sciences, Hezar-Jerib Ave, Isfahan, IR Iran. P.O. Box 81746-73461. Tel: +98(31)33354453, Cell phone: +989136448771, Fax: +98(31)36681378, E-mails: a.moravej@mail.mui.ac.ir, amimohs@gmail.com, ORCID ID: 0000-0001-9707-0352.

Running head: Mediterranean diet and multiple sclerosis

This study has not been duplicate publication or submission elsewhere. The authors received no financial support for the research and/or authorship of this article. The authors declare that they have no conflict of interest to the publication of this article.

CRediT author statement

Jalal Bohlouli: Investigation, Funding acquisition

Iman Namjoo: Visualization, Project administration

Mohammad Borzoo-Isfahani: Data Curation, Resources

Fariborz Poorbaferani: Formal analysis

Amir Reza Moravejolahkami: Conceptualization, Methodology, Software, Validation, Formal analysis, Writing - Review & Editing, Supervision

Cain C. T. Clark: Writing - Review & Editing

Mohammad Ali Hojjati Kermani: Writing - Original Draft

Acknowledgement:

The authors thank all the participants of the study for their enthusiastic of involvement and to the personnel of the clinic. This research was supported by Student Research Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Grant NO. 197140).

Trial Registration: The trial was registered at the University of Medical Science "IR.MUI.RESEARCH.REC.1397.287" and WHO approving system "IRCT20181113041641N1".

The protocol is also visible at CCHRANE LIBRARY (https://www.cochranelibrary.com/central/doi/10.1002/central/CN-01949901/full)

Funding: The authors declare no support from any commercial organization for the submitted study.

Abstract

Background

Current evidence suggests that adherence to the Mediterranean Diet (MeD) can reduce inflammation in chronic diseases; however, studies pertaining to Relapsing-Remitting Multiple Sclerosis (RRMS) are limited. Therefore, the aim of this study was to investigate the potential of the modified MeD (mMeD) in improving Dietary Inflammatory Index (DII) scores, disability, and fatigue severity, compared to Traditional Iranian Diet (TID), in RRMS patients.

Methods

After initial screening (n=261), 180 RRMS patients were randomized to receive mMeD or TID (as control) for six months. DII score, Expanded Disability Status Scale (EDSS) and 21-item Modified Fatigue Impact Scale (MFIS) were evaluated at baseline and trial cessation. Multivariate analysis of covariance was conducted and adjusted for age, gender, body weight, body mass index, education level, supplement use, family history and duration of MS.

Results

Of the 180 patients enrolled, 147 participants were included in the final analysis (n of mMeD=68; n of TID=79). Self-reported adherence was good (~81%). Dietary intakes of 45 food parameters were assessed through the food frequency questionnaire. The mMeD significantly reduced DII scores after six months (2.38±0.21 to -1.87±0.86, P<0.001), but TID did not elicit any changes (2.21±0.44 to 2.14±1.01, P=0.771). Additionally, MFIS total score decreased significantly (72.4±17.2 to 63.9±14.2, P<0.001), whereas there was no considerable improvement for EDSS in the mMeD group.

Conclusion

Adherence to mMeD, for six months, improved dietary inflammatory status and fatigue severity in RRMS patients, however, the traditional Iranian diet did not positively impact dietary inflammation and MFIS score.

Keywords: Dietary inflammatory index; Mediterranean diet; fatigue; multiple sclerosis; randomized controlled trial

1. Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system, with unknown etiology, characterized by chronic inflammation, demyelination, and neuronal loss [1]. Around 2.5 million individuals, worldwide, are affected by this disease [2], although young adults and females are more susceptible [3]. Relapsing-Remitting MS (RRMS), the most common type of MS, is indicated in, roughly, 85% of patients [4].

Contentions in the literature regarding the relationship between some dietary components and MS progression are evident. For example, dietary polyphenols have been reported to mitigate demyelination [5], whereas, resveratrol - a polyphenol compound found in a variety of foods and beverages - reportedly exacerbated both autoimmune and viral models of MS [6]. Milk proteins and gluten may worsen the clinical manifestations in MS patients [7], however, milk consumption more than once per week was found to decrease the risk of developing MS [8]. Furthermore, high doses of vitamin C have been shown to worsen MS conditions [9], while some authors have reported that vitamin C promotes oligodendrocytes generation and remyelination [10]. Indeed, more nutrition-based research is required to clarify these conflicting findings.

Among the most advocated healthy diets, the Mediterranean Diet (MeD) has the strongest evidence for improvement in inflammatory status [11]. This diet is characterized by high intake of vegetables, legumes, fruits, whole grains, and unsaturated fatty acids (mostly in the form of olive oil), a moderately high intake of fish, and low to moderate intake of dairy products, meat, and poultry [12]. Indeed, previous studies have shown the potential effects of anti-inflammatory diets, such as Mediterranean-style diets, in reducing fatigue severity in MS patients [12-14].

Dietary Inflammatory Index (DII), a literature-based scoring system, is a tool used to classify forty-five pro or anti-inflammatory dietary items into an overall score [15]. Previous studies have reported that several foods and nutrients used in the DII calculation, such as whole grains, fruits, vegetables, fish, onion, and ginger possess anti-inflammatory effects [16, 17]. In contrast, refined grains, red meat, high-fat dairy products, and sweats have been routinely related to systemic inflammation [18]. In previous studies, MeD reportedly yielded a strong anti-inflammatory DII score [19] and greater MeD adherence has been negatively associated with DII scores [20, 21]. On the other hand, some findings suggest that higher DII scores during adolescence might be an important risk factor for MS onset [22].

Therefore, given the equivocality present within the literature, we sought to determine the effect of mMeD vs. TID, on DII, disease disability, and fatigue severity in RRMS patients. We hypothesized that the modified form of MeD (mMeD; mainly by elimination of alcohol-containing foods and beverages) would yield a lower DII score (i.e., greater dietary anti-inflammatory potential) in comparison with the Traditional Iranian Diet (TID).

2. Materials & Methods

2.1. Study design and sample size determination

In this single-center, two parallel arms, single-blind, randomized clinical trial, 180 RRMS patients were recruited, according to the Extended Disability Status Scale (EDSS 0-3, mild to moderate disability as diagnostic criteria) [23]. Intervention delivery was performed from July 2018 to February 2019.

The study protocol was approved by ethics committee located in the University Medical Sciences, and WHO-related Registry of Clinical Trials (IRCT20181113041641N1). The Helsinki ethical principles [24] were well observed throughout the trial. Study objectives were explained, and voluntary informed consent was taken prior to data collection.

Fatigue Severity Scale (FSS), a tool for measuring fatigue in MS, was used to calculate the sample size based on previous reports [25].

$$n = \frac{(z_1 + z_2)^2(s_1^2 + s_2^2)}{(\bar{x}_1 - \bar{x}_2)^2} \cong \frac{594/80}{9} \cong 66/08$$

By the use of sample size determination formula (S_1, SEM for FSS in control group=4.73; S_2, SEM for FSS in intervention group=4.85; $\bar{x}_1 - \bar{x}_2$, mean changes for FSS=3), with a confidence level of 95% (z_1=1.96), power of 80% (z_2=1.64), and drop-out rate of 35 % in the number of participants, the total sample size was estimated to be 180.

2.2. Inclusion and exclusion criteria

Eligible patients had mild to moderate RRMS (defined as EDSS up to 3, and who received dimethyl fumarate 240 mg twice daily in the last year), aged between 20-60 years old, and ability to write or recall dietary history. Subjects were excluded if they had any of the following: other forms of MS and disease duration of less than one year with active relapses, viral infections such as Epstein Barr, major medical illnesses (such as cancer, allergy, other autoimmune diseases,

anticoagulant or antiplatelet use, and psychiatric disorders), and current smokers (one or more cigarette per day). Subjects were also excluded if they left more than 40% blank items on the Food Frequency Questionnaire (FFQ) at baseline or were prescribed high dose corticosteroid therapy (greater than 30 mg/day methylprednisolone).

2.3. Interventions and control groups

The main composition of each diet has been described briefly in ***Table 1***. The intervention group followed a modified version of MeD (mMeD; 17 % protein, 51 % carbohydrate and 32 % fat [26]), based on higher intake of fresh fruits and vegetables, whole grains, monounsaturated fatty acids, fish, and low to moderate consumption of dairy products, meat, and poultry. In practice, the prescribed mMeD was individualized based on cultural and personal preferences, and the elimination of any alcohol-containing foods and beverages. The control group followed the TID (*low in low-fat dairy products, whole grains; high in red meats, solid oils, refined grains, and moderate intakes of legumes, fruits and vegetables*); based on prior investigations, this diet consisted of 13 % protein, 58 % carbohydrate and 29 % fat [27]. It must be noted that the TID group (as control) did not continue their normal eating pattern, i.e., the original dietary principles in the control group were maintained, however, the TID plan was adjusted for energy intake to avoid unexpected body weight changes.

Ideal body weight and the Harris-Benedict equation [28] were utilized to calculate the Basal Energy Expenditure (BEE) for each participant in both diets (mMeD and TID). Next, the above percentages were used to discern the macronutrient requirements in both diets. All the participants received an individualized diet plan, which had been designed according to the above principles. Dietary adherence was also measured with weekly with phone calls, and face-to-face interviewing every month.

2.4. Recruitment and randomization methods

Participants were recruited using advertisements in local media outlets and clinicians' invitation. Participants were randomly assigned into either the modified Mediterranean Diet (mMeD; intervention) or Traditional Iranian Diet (TID; control) group, with a computerized random sequence generator. Randomization was performed by a research assistant who did not participate in either the follow-up assessments or analysis.

2.5. Blinding

In this trial, blinding of participants and dietitians is not possible because of obvious differences between the intervention and control diets; however, where appropriate, trial personnel (research assistant who enrolled participants, outcome assessors and data analysts) remained blind to group allocation throughout the study period.

2.6. Outcome measurements

The primary outcome was the diet-induced change in DII. The secondary outcomes were change in disease disability (measured by EDSS) and fatigue severity (measured by MFIS). Sociodemographic and clinical characteristics were collected through a self-report survey completed at baseline, which included details on participants' age, body weight and height, Body Mass Index (BMI), education level, family history of MS, and supplement use. Baseline DII scores were also assessed in two states: dietary only and dietary plus supplements. However, the statistical analysis was conducted based on dietary DII scores.

2.6.1. Dietary assessment

Food intake of individuals during the previous year was assessed using a validated 168-item semi-quantitative FFQ [29], which included a list of foods with standard serving sizes commonly consumed [30-32]. Nutritionist IV software (N-squared Computing, Salam, OR, USA) was used to analyze the composition of consumed foods. Some DII parameters such as ginger, saffron, turmeric, thyme/oregano, and rosemary were additionally added to the FFQ. For calculation of flavonoids (flavan-3-ol, flavones, flavonols, flavonones, anthocyanidins and isoflavones), the USDA Databases for the Flavonoid Content of Selected Foods (Release 3.3, March 2018) [33] and Isoflavone Content of Selected Foods (Release 2.0, September 2008) [34] were used. Dietary intake of eugenol was estimated according to Phenol-Explorer database (latest version 3.6; released on December 2016) [35]. There were two timepoints for dietary assessment: one before the dietary intervention and one 6 months after the start of the study.

2.6.2. DII calculation

Shivappa et al. [36], after evaluation of 1943 articles (were published between 1950 and 2010), examined the association between inflammation and 45 food and nutrient parameters; this resulted in the development and validation of DII, where the score ranged from 7.98 (i.e.,

strongly pro-inflammatory) to -8.87 (i.e. strongly anti-inflammatory). In the present study, we calculated the DII scores at baseline and after 6 months of intervention. Estimated dietary intake data were adjusted against a reference global daily mean and standard deviation intake (from 11 countries) [36] for each parameter to obtain a Z-score; each Z-score was converted to percentile, and this value was multiplied by 2 and then subtracted from 1. This number for each intake parameter was multiplied by its respective parameter-specific inflammatory effect score to obtain the parameter-specific DII score. Each of these 45 scores were then summed to obtain an overall DII score.

2.6.3. Fatigue Severity Assessment

The Modified Fatigue Impact Scale (MFIS) was used to determine the MS-related fatigue [37] at baseline and 6 months after the intervention. This standard 21-item questionnaire has three subscales (Physical, ranges from 0-36; Cognitive, 0-40; and Psychosocial, 0-8). The total score is computed by summing scores from the three subscales and ranges from 0-84, where higher scores represent greater fatigue severity. In the present study, the validated Persian version of MFIS [38], with excellent test-retest reliability [39], was utilized.

2.6.4. Disability Assessment

A trained neurologist measured EDSS to assess MS-related disability [23, 40] at baseline and 6 months after the intervention. Scales for the total EDSS in the current study ranged from 0 (no disability at all) to 3 (mild to moderate disability).

2.7. Statistical analysis

Data were presented as means ± Standard Deviation (SD) for continuous variables and number (percent) for categorical variables. The Kolmogorov–Smirnov test was used to assess the normality of continuous variables. In addition, independent student t and paired t tests (or non-parametric Mann–Whitney U and Wilcoxon tests) were used to compare the continuous variables. Categorical variables were compared using the $\chi 2$ or Fisher's Exact test. Multivariate Analysis of Covariance (MANCOVA) was performed to evaluate the differences for change in DII scores, where the related values were adjusted for age, gender, body weight, BMI, education level, supplement use, family history, and duration of MS. The mean changes (Δ) were

calculated by subtracting the baseline and 6 months (end) values. To identify the relationship between DII (and other covariates) and fatigue severity/ disease activity scores at end of trial, multiple regression analysis was performed. All statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS), version 24 (SPSS Inc.). $P<0.05$ was considered to represent statistical significance.

3. Results

3.1. Enrollment and adherence

Between July 2018 and February 2019, we screened 261 RRMS patients, however, sixty-seven subjects were excluded as they did not meet the inclusion criteria, and fourteen patients declined to participate (***Figure 1***). 180 patients were dichotomized to the mMeD or TID group. Thirty subjects dropped out during the study follow-up: twenty due to lack of compliance, two due to lack of willingness to continue the study, one due to a driving accident, and ten subjects due to incomplete questionnaires. Overall, 147 participant-related data (intervention=68; control=79) were analyzed (based on per-protocol analysis). No side effects (diarrhea, abdomen pain, constipation, and appetite changes) were reported during the study period.

3.2. Baseline characteristics

Sociodemographic and medical characteristics, between the groups at baseline, are reported in ***Table 2***. Overall, the participants were middle-aged adults (with mean age 39.3 ± 9.2 years old; ~83% female). More than 40 % were overweight and obese, 15 % had family history of MS, and the majority had already completed a degree to diploma level. More than 80 % of the study population were taking at least one type of nutritional supplement, of which vitamin D (~83 %) and omega-3 (~33%) were the most common. Additionally, ~20 % of subjects had consumed L-carnitine or caffeine-containing supplements during the past six months. A small number of male participants (13 %) were irregular smokers (average 1-2 cigarettes in a week). Mean EDSS score was slightly higher in the control group (2.0 vs. 1.7), although there were no significant differences between the study groups for EDSS and DII scores.

3.3. Clinical outcomes

3.3.1. Impact of diet interventions on DII

Table 3 details the mean daily intake for 45 DII parameters and overall DII score for each diet. Within the mMeD group, there was a significant decrease from 2.38 ± 0.21 to -1.87 ± 0.86 at 6 months for overall DII score (P< 0.001). Compared with control group (TID), the mean changes for overall DII score were also statistically significant (-4.25 ± 1.54 vs. -0.07 ± 0.62; P< 0.001).

For mean daily intake of DII food/nutrient parameters after 6 months (***Table 3***), there was a significantly higher intake of protein, n-6 Fatty acids, Monounsaturated Fatty Acids (MUFA), Polyunsaturated Fatty Acids (PUFA), selenium, beta carotene, vitamin E, riboflavin, garlic, onion, ginger, turmeric, pepper, thyme/oregano, rosemary, flavan-3-ol, anthocyanidins, isoflavones; in addition to lower intake of energy, carbohydrate, total fat, saturated fat, trans fat, iron, and caffeine in the mMeD group compared to TID group (P< 0.05).

3.3.2. Impact of diet interventions on fatigue

Table 4 details the results for fatigue severity. At the end of the study period, MANCOVA revealed a significant difference between the groups for MFIS total score (Δ for mMeD= -8.5 ± 2.74 vs. Δ for TID= 6.4 ± 1.62, P< 0.001). These findings were adjusted for age, gender, body weight, BMI, education level, supplement use, family history, and duration of MS.

Participants who received mMeD had a statistically significant improvement in physical and cognitive MFIS subscales. After six months, there was a 2.7 and 5.6 points reduction in physical (P< 0.001) and cognitive (P= 0.027) MFIS subscales, respectively. However, no significant change in the psychosocial subscale of MFIS was evident.

3.3.3. Impact of diet interventions on disability

There was a non-significant reduction in EDSS at the end of the study period in the mMeD group (Δ= -0.02 ± 0.07, P= 0.334). Contrastingly, a non-significant rising trend in EDSS was seen in the TID group. MANCOVA, adjusted for age, gender, body weight, BMI, education level, supplement use, family history, and duration of MS, did not indicate any significant change for EDSS, in mMeD vs. TID (P= 0.065) (***Table 4***).

3.3.4. Relationship between DII and the fatigue severity/disease disability

Significant predictors and covariates for MFIS total score and EDSS are presented in ***Table 5***. DII score significantly predicted fatigue severity in the intervention group (*B*= 1.701, P= 0.041; adjusted R^2= 0.098), however, age, gender, body weight, BMI, education level, supplement use, family history, and duration of MS had no significant association with MFIS total score.

In addition, regression analysis revealed that DII score does not predict disease disability in mMeD group (*B*= 3.809, P= 0.067; adjusted R^2= 0.157). Other covariates (age, gender, body weight, BMI, education level, supplement use, family history, and duration of MS) did not show any significant association with EDSS.

4. Discussion

This study assessed the effects of dietary intervention on DII score in Iranian RRMS patients. Our findings showed that the mMeD possesses significant anti-inflammatory properties, whereas the Traditional Iranian Diet (TID) had no significant effect on overall DII score. The key components of DII i.e., MUFA and polyphenols, increased significantly after six months adherence to the mMeD group. Moreover, mMeD also reduced fatigue severity (MFIS score); however, the effect on disease disability (measured by EDSS) was not significant.

Iranian dietary intervention studies typically use TID as the control diet [11, 41, 42]. TID (low in low-fat dairy products, whole grains; high in red meats, solid oils, refined grains, and moderate legumes, fruits and vegetables) is the most prevalent diet in Iran, and has both positive and negative health-related aspects [43]. In a case-control study, conducted by Jahromi et al. [44], the traditional dietary pattern was inversely related to the risk of RRMS; however, high amounts of red/organ meat in the TID can lead to both neurodegeneration and autoimmune disorders [45]. Indeed, it must be noted that nutritional transition in Iran has resulted in a change in TID, which must be considered in further research [43, 46]; however, the control group adhered to the prescribed TID, as defined in the present study.

In this research, DII was considered as an index that effectively represents dietary inflammatory status, based on several previous studies that have verified by the significant association of DII with inflammatory markers [47]. This index presents an alternative assessment tool for inflammation, as opposed to the laboratory-based techniques which are obtained through invasive methods and economically prohibitive [48]. Moreover, some previous studies have

indicated that the DII may also be correlated with other dietary indices i.e., Healthy Eating Index-2010 (HEI-2010), the Alternative Healthy Eating Index (AHEI), and the Dietary Approaches to Stop Hypertension Index (DASH) [49].

Regardless of the pro-inflammatory DII score for the TID, the mMeD elicited a reduction in the total DII score in the present study. Indeed, the association between MeD and DII score has been evaluated in previous research; for instance, in a Spanish cross-sectional study, adherence to the MeD was higher in the lowest quintile of DII scores [20]. Moreover, an inverse correlation was also observed between the DII and Mediterranean Diet Score [21], whilst Mayr et al. [19] showed that six months MeD adherence, compared with a low-fat diet, elicited an improvement in DII scores in patients with coronary heart disease. Interestingly, Mayr et al. evaluated 45 parameters of DII and reported an anti-inflammatory DII score of -1.74, which is comparable to the total score of -1.87 in the present study. Although we removed the alcohol-containing foods and beverages from our intervention, the current findings were comparable to the intact versions of the MeD reported in the literature.

Recently, it was reported that DII was not significantly associated with the clinical condition of individuals with MS [50]. Moreover, Silva et al. [51], in a cross-sectional study, reported that DII does not correlate to waist circumference, waist-hip ratio, body roundness index, body shape index, body shape z score index, and percentage of body fat among MS patients. In contrast, Shivappa et al. [52] observed that a pro-inflammatory diet (with a higher DII score) may be associated with an increased risk of MS in an Iranian population. In the current study, an anti-inflammatory diet was prescribed to assess the possible effects on DII in RRMS patients. The link between diet and chronic inflammation has been well established [53, 54], and the association between inflammation and neurodegeneration in MS is generally well-supported [55]. According to previous work, the Mediterranean diet is inversely associated with biomarkers of inflammation [56, 57].

The MUFA and PUFA content of mMeD appears to be responsible for the anti-inflammatory DII score in the current study. Omega-3 PUFAs inhibit NF-kB signaling through activation of SIRT1-mediated pathway [58] and the reduction of pro-inflammatory cytokines (e.g., IL-12, IL-23) [59]. Olive oil polyphenols, which are a major part of the mMeD, also have an inhibitory effect on endothelial Nitric Oxide Synthase (eNOS) and Brain-Derived Neurotrophic Factor

(BDNF) expression [60]. However, two systematic reviews in 2012 and 2020, respectively, reported that PUFAs do not elicit any significant effect on MS-related outcomes [61, 62].

In the present study, flavonoids intake increased after six months adherence to the mMeD; moreover, flavan-3-ol, anthocyanidins, and isoflavones levels were significantly greater in comparison with the TID group. Flavan-3-ols, mainly extracted from green tea, have previously been advocated as neuroprotective compounds [63]. Furthermore, anthocyanidins possess anti-inflammatory and anti-proliferative effects through inhibition of the cyclooxygenase-2 expression in LPS-evoked macrophages [64]. Recently, Freedman et al. [65] found that a high-isoflavone diet ameliorates Experimental Autoimmune Encephalomyelitis (EAE) through modulation of gut microbiota in MS patients.

In the present study, fatigue severity was reduced by 12 percent (measured by MFIS total score). In a 12-week randomized trial, Mousavi-Shirazi-Fard et al. [13] observed the fatigue-modulatory effect of an anti-inflammatory diet among 100 RRMS patients. Another study, conducted by Yadav et al. [66], reported that a plant-based, low-fat diet, can reduce the MFIS by ~0.2 points per month in RRMS patients. Indeed, it seems that the bioactive components of mMeD are responsible for fatigue improvement.

The mMeD administration in the present study did not elicit any improvement in disease-related disability in RRMS participants. We hypothesized that the mMeD may have improved the level of disability from moderate (~3) to mild disability (≤2); however, in this study, and our previous work, there was no association between a Mediterranean-like dietary pattern and disability (measured by EDSS) [14]. EDSS is the most important secondary endpoint in MS trials addressing RRMS patients; this instrument is suitable for detecting the efficacy of clinical interventions, to monitor disease progression, and is internationally utilized [67].

While our study provides initial insights into understanding the potential role of dietary interventions in the management of MS, it has some limitations that should be considered. The current study is representative of patients with RRMS undergoing intensive pharmacotherapy, and who are potentially motivated and health-conscious. Therefore, the current findings are not necessarily pertinent to healthy subjects, or other disease populations. The sample size for the present study was calculated based on a secondary variable, i.e., FSS. Furthermore, incumbent findings could have been affected by insufficient statistical power, relative to DII score, small sample size, short follow-up period, and high drop-out rate (>18%). Moreover, there was an

imbalance between groups at follow-up in the drop-out rate (more than 22% in the intervention group versus 3.3 % in the control group). The nature of this study was single-blind and was vulnerable to selection and recall biases. The lack of neuroimaging data, which may be useful in evaluating the effect of diet on neurodegeneration, was the most important clinical limitation. The calculation of trans fat intake was predominantly based on high-fat dairy and meat products; thus, underestimation was possible. Finally, EDSS was used to measure disease disability in the current research; however, this tool may not be sensitive to clinical change, especially in short-term studies (≤ 6 months) and milder levels of disability [68].

However, despite the aforementioned limitations, the present study has several strengths worth mentioning. The MS participants recruited were relatively homogenous, allowing pertinent inferences to be drawn. Furthermore, adjustment in the final analysis allowed detailed consideration of potential confounding variables. In this trial, all 45 parameters of the DII were measured and a non-invasive method was used for evaluating inflammatory condition; these strategies helped to improve the accuracy and precision of our findings. Finally, although current evidence suggests that adherence to a Mediterranean-style diet can reduce inflammation in chronic diseases, studies pertaining to Relapsing-Remitting Multiple Sclerosis (RRMS) are limited; therefore, the present study provides a novel and important addition to the literature.

5. Conclusion

Our results demonstrated that adherence to the mMeD for 6-months can reduce DII score in RRMS participants. Indeed, the mMeD improved fatigue severity, without any significant change on disability. Comparatively, adherence to the Traditional Iranian Diet did not impact DII scores. Additional studies are required to evaluate the long-term safety and immunomodulatory properties of the MeD, and TID in progressive forms of MS, as well as in patients with other autoimmune diseases.

Acknowledgements

The authors thank all the participants of the study for their enthusiastic of involvement and to the personnel of the clinic.

Disclaimer statements

Funding

The authors declare no support from any commercial organization for the submitted study.

Conflicts of interest

The Authors declare that there is no conflict of interest.

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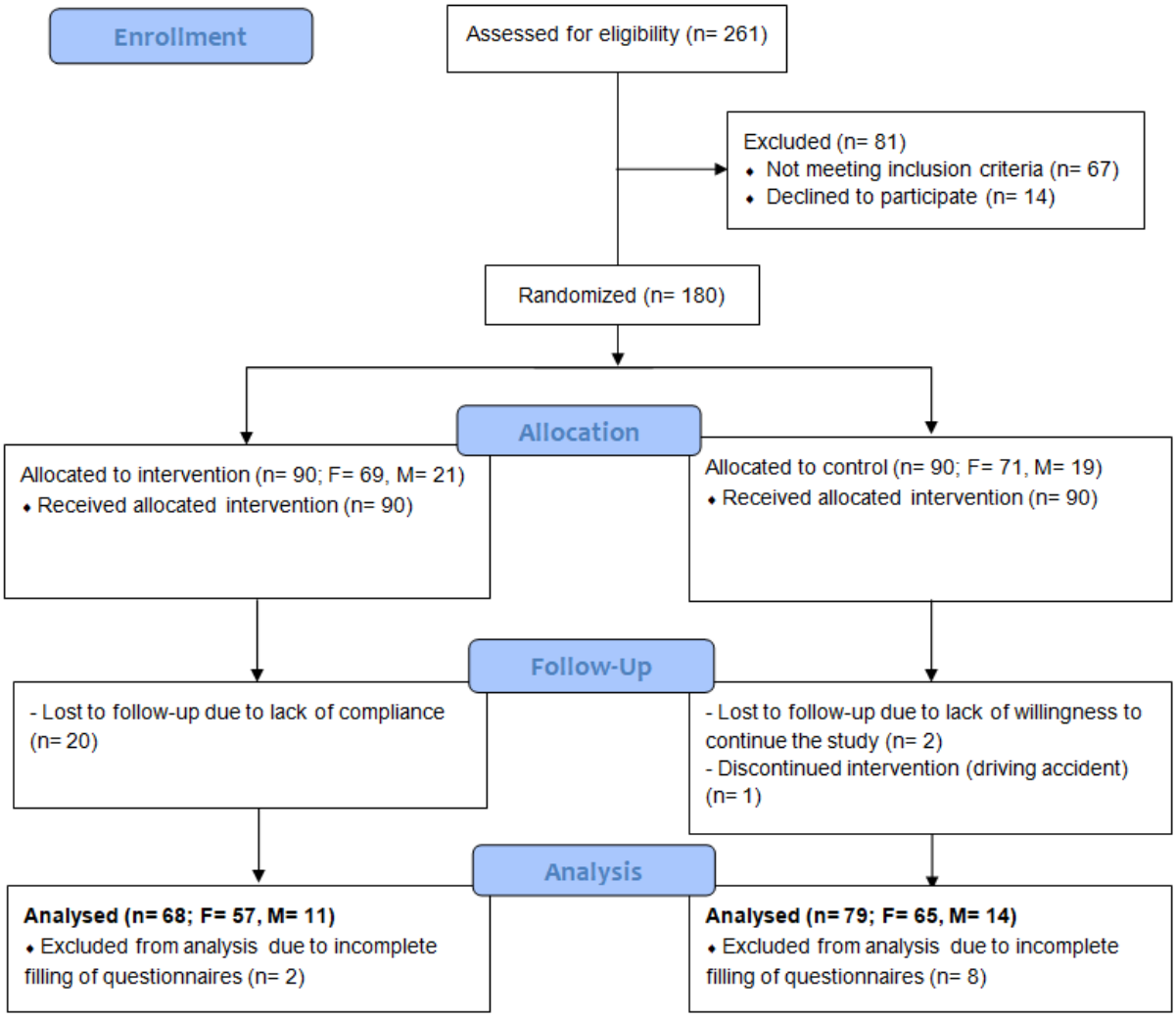

Figure 1. Flow diagram representing study plan.

Table 1. The main composition of modified Mediterranean (mMeD) and Traditional Iranian (control) diets.

Major nutrients	**mMeD**	**Control†**
	% of calories	% of calories
Protein	17[1]*	13
Carbohydrate	51[1]	58
Fat	32[1]	29
	% of total fat	
Saturated	21[1]	32
Monounsaturated	56[1]	33
polyunsaturated	15[1]	14
ω6/ω3 Fatty Acids	2.1-3/1[2]	3.8/1
Cholesterol mg/Cal	0.16[1]	0.12
Fiber g/Cal	0.03[3]	0.005
Sodium mg/Cal	1.3[1,3]	1.6

*Reference Number:

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† Values were calculated based on average usual intakes of the participants in Traditional Iranian Diet.

‡modified Mediterranean Diet; adopted from 1999 Greek Dietary Guidelines (1999): Ministry of health and welfare, supreme scientific health council: Dietary guidelines for adults in Greece.Arch. Hell. Med. 1999, 16, 516–524. Serving sizes specified as: 25 g bread, 100 g potato, 50–60 g cooked pasta, 100 g vegetables, 80 g apple, 60 g banana, 100 g orange, 200 g melon, 30 g grapes, 1 cup milk or yoghurt, 1 egg, 60 g meat, 100 g cooked dry beans.

Table 2. Characteristics of participants between diet study groups.

group / **continuous variables**		**mMeD (n=68) M ± SD**	**Control (n=79) M ± SD**	**P-value**[†]
Age (y)		38.6 ± 8.6	40.0 ± 9.6	0.309
Body Weight (kg)		71.2 ± 10.1	68.5 ± 10.5	0.556
Height (cm)		165.4 ± 7.0	163.0 ± 6.8	0.473
BMI (kg/m^2)		26.1 ± 4.1	25.9 ± 4.5	0.757
Duration of the disease (y)		8.1 ± 5.7	9.3 ± 6.9	0.395
EDSS		1.7 ± 0.7	2.0 ± 0.9	0.059
DII[‡]		2.38 ± 0.21	2.38 ± 0.21	0.687
DII (plus supplements)		1.87 ± 1.25	2.01 ± 0.93	0.069
Categorical variables		**mMeD (n=68) n (%)**	**Control (n=79) n (%)**	**P-value**[§]
gender	Male (17%)	11 (16.2)	14 (17.7)	0.830
	Female (83%)	57 (83.8)	65 (82.3)	
Education	Illiterate	3 (4.4)	3 (3.8)	0.332
	Elementary	7 (10.3)	5 (6.3)	
	Junior school	7 (10.3)	9 (11.4)	
	Diploma	29 (42.6)	46 (58.2)	
	University	22 (32.4)	16 (20.3)	
Family history	Yes	10 (14.7)	12 (15.2)	0.999
	No	58 (85.3)	67 (84.8)	
BMI classification	Normal	28 (41.2)	34 (43.0)	0.948
	Underweight	2 (2.9)	3 (3.8)	
	Overweight	18 (26.5)	22 (27.8)	
	Obese	20 (29.4)	20 (25.3)	
Nutritional supplement	Vitamin D	60 (88.2)	62 (78.5)	0.764
	Omega-3	26 (38.2)	23 (29.1)	

intakes	Multivitamin & minerals	18 (26.5)	17 (21.5)	
	L-carnitine or caffeine	11 (16.1)	18 (22.8)	

Abbreviations: mMeD, modified Mediterranean Diet; BMI, Body Mass Index; yr, year; SD, Standard Deviation; EDSS, Extended Disability Status Scale; %, within group percent; DII, dietary inflammatory index; control, Traditional Iranian Diet.

[†]obtained from independent t-test, EXCEPT for Duration of the disease, BMI, and EDSS, that was analyzed by Man-Whitney U test; $P<0.05$ considered significant

[‡]determined with Chi Square, EXCEPT for gender, and Family history, that was analyzed by Fisher's Exact test, $P<0.05$ considered significant

[§]Negative number represents an anti-inflammatory score, while positive number reflects a proinflammatory score.

Table 3. Dietary inflammatory index (DII) parameters and scores in patients with relapsing-remitting multiple sclerosis that received either modified Mediterranean Diet (mMeD) or Traditional Iranian Diet (control).

Dietary group	DII food parameters	Group	Baseline	End of trial	P-value[1]	Δ^2	P-value[3]
energy	Energy (kcal/day)	mMeD	2670.6 ± 468.6	2193.6 ± 317.3	**<0.001***	-477.0 ± 300.1	**<0.001***
		Control	2575.7 ± 437.0	2588.7 ± 419.5	0.828	13.0 ± 531.5	
Macro nutrients	Carbohydrate (g/day)	mMeD	405.2 ± 86.6	317.5 ± 57.7	**<0.001***	-87.7 ± 54.2	**<0.001***
		Control	391.4 ± 78.3	394.8 ± 75.1	0.083	3.4 ± 97.3	
	Protein (g/day)	mMeD	92.0 ± 7.5	87.0 ± 8.4	**<0.001***	-5.0 ± 8.8	0.327
		Control	90.5 ± 6.9	90.1 ± 7.1	0.642	-0.36 ± 10.0	
	Total fat (g/day)	mMeD	77.3 ± 13.1	66.9 ± 7.5	**0.001**	-10.4 ± 12.4	0.052
		Control	74.0 ± 13.0	74.0 ± 12.7	0.057	0.10 ± 15.8	
	Cholesterol (mg/day)	mMeD	307.1 ± 105.3	266.0 ± 37.2	0.055	-81.1 ± 103.2	0.134
		Control	283.8 ± 104.6	284.8 ± 105.2	0.072	1.0 ± 132.4	
	Saturated fat (g/day)	mMeD	26.5 ± 10.4	18.7 ± 5.2	**0.002**	-7.8 ± 9.7	0.061
		Control	24.3 ± 10.4	24.4 ± 10.5	**0.044**	0.07 ± 13.0	
	Trans fat (g/day)	mMeD	1.2 ± 0.98	0.6 ± 0.87	**0.012**	-0.6 ± 0.3	0.671
		Control	1.3 ± 0.85	1.2 ± 0.60	0.741	-0.1 ± 0.7	
	n-6 Fatty acids (g/day)	mMeD	11.3 ± 1.8	12.8 ± 0.8	**0.002**	1.5 ± 1.7	**0.036**
		Control	11.5 ± 2.0	11.6 ± 1.8	0.292	0.1 ± 2.5	
	n-3 Fatty acids	mMeD	0.18 ± 0.07	0.2 ± 0.06	0.429	0.02 ± 0.09	0.711

	(g/day)	Control	0.18 ± 0.07	0.18 ± 0.06	0.207	-0.00 ± 0.08	
	MUFA (g/day)	mMeD	22.9 ± 2.6	26.0 ± 4.0	**0.013**	3.1 ± 4.1	**0.019**
		Control	25.1 ± 3.7	25.1 ± 3.7	0.091	-0.01 ± 4.6	
	PUFA (g/day)	mMeD	15.2 ± 3.5	17.0 ± 1.5	**0.022**	1.9 ± 4.1	0.172
		Control	15.3 ± 3.4	15.3 ± 3.3	0.333	0.00 ± 4.5	
	Fiber (g/day)	mMeD	27.7 ± 8.0	32.0 ± 5.1	0.407	4.2 ± 9.0	0.431
		Control	29.0 ± 8.0	28.9 ± 8.4	0.431	-0.1 ± 11.1	
minerals	Iron (mg/day)	mMeD	29.6 ± 6.5	24.6 ± 3.6	**<0.001***	-5.0 ± 5.34	**0.029**
		Control	28.5 ± 6.0	28.4 ± 6.0	0.139	-0.1 ± 7.8	
	Magnesium (mg/day)	mMeD	253.4 ± 50.5	292.7 ± 36.8	0.348	39.2 ± 58.9	**0.038**
		Control	259.0 ± 51.0	257.0 ± 52.2	0.611	-1.9 ± 70.9	
	Zinc (mg/day)	mMeD	9.0 ± 1.1	9.0 ± 0.9	0.650	0.0 ± 1.5	0.168
		Control	8.9 ± 1.0	8.9 ± 1.1	0.543	-0.05 ± 1.6	
	Selenium (µg/day)	mMeD	118.3 ± 25.2	93.0 ± 13.1	**<0.001***	-25.3 ± 20.5	**0.004**
		Control	112.2 ± 24.9	114.0 ± 24.4	0.091	1.8 ± 31.4	
Fat-soluble vitamins	Beta Carotene (µg/day)	mMeD	710.7 ± 895.8	1746.2 ± 973.6	**0.006**	1035.5 ± 1084.4	**0.032**
		Control	856.2 ± 962.3	801.8 ± 924.4	0.214	-54.3 ± 1236.6	
	Vitamin E (mg/day)	mMeD	3.6 ± 0.6	3.8 ± 0.6	**0.001**	0.2 ± 1.0	0.650
		Control	3.6 ± 0.6	3.5 ± 0.6	0.226	-0.1 ± 0.8	
	Vitamin D (µg/day)	mMeD	1.6 ± 1.2	2.3 ± 1.2	0.319	0.6 ± 1.8	0.061
		Control	1.6 ± 1.2	1.6 ± 1.2	0.657	0.0 ± 1.7	

	Vitamin A (RE/day)	mMeD	1139.9 ± 545.5	1663.2 ± 648.4	0.085	523.3 ± 754.3	0.152
		Control	1203.8 ± 600.0	1172.1 ± 569.1	0.757	31.6 ± 779.6	
Water-soluble vitamins	Vitamin C (mg/day)	mMeD	94.4 ± 53.2	141.5 ± 37.3	0.**109**	47.0 ± 58.7	0.069
		Control	104.4 ± 54.6	101.9 ± 54.3	0.215	-2.5 ± 71.4	
	Vitamin B12 (ųg/day)	mMeD	5.1 ± 2.0	3.7 ± 0.8	0.289	-1.3 ± 2.2	0.401
		Control	4.6 ± 2.1	4.6 ± 2.1	0.298	0.06 ± 2.8	
	Vitamin B6 (mg/day)	mMeD	1.7 ± 0.3	1.6 ± 0.4	0.581	-0.08 ± 0.5	0.478
		Control	1.7 ± 0.4	1.7 ± 0.4	0.708	0.00 ± 0.5	
	Folic acid (ųg/day)	mMeD	334.2 ± 138.8	395.7 ± 102.9	0.828	61.4 ± 170.5	0.374
		Control	357.6 ± 144.0	355.9 ± 148.0	0.310	-1.6 ± 194.2	
	Niacin (mg/day)	mMeD	29.8 ± 6.3	24.0 ± 3.6	**<0.001***	-5.7 ± 4.2	**0.018**
		Control	28.7 ± 5.5	28.7 ± 5.3	0.326	0.01 ± 7.2	
	Riboflavin (mg/day)	mMeD	2.1 ± 0.4	2.2 ± 0.4	**0.006**	0.02 ± 0.7	0.444
		Control	2.1 ± 0.4	2.1 ± 0.4	0.935	0.00 ± 0.6	
	Thiamin (mg/day)	mMeD	2.9 ± 0.5	2.4 ± 0.4	**<0.001***	-0.5 ± 0.3	**<0.001***
		Control	2.8 ± 0.4	2.8 ± 0.4	0.200	0.01 ± 0.6	
Specific foods	Alcohol[5] (g/day)	mMeD	0	0	-	0	-
		Control	0	0	-	0	
	Garlic (g/day)	mMeD	0.68 ± 1.87	1.24 ± 3.47	**<0.001***	0.56 ± 1.22	**<0.001***
		Control	0.70 ± 2.00	0.59 ± 1.55	0.803	-0.11 ± 2.52	
	Onion (g/day)	mMeD	8.9 ± 2.78	10.23 ± 5.74	0.142	1.23 ± 4.03	**0.049**

		Control	8.0 ± 1.89	7.4 ± 3.22	0.241	-0.6 ± 1.20	
	Ginger (g/day)	mMeD	0.32 ± 0.87	1.0 ± 0.9	**0.023**	0.7 ± 1.5	**0.015**
		Control	0.26 ± 0.80	0.20 ± 0.91	0.441	-0.06 ± 0.51	
	Saffron (g/day)	mMeD	0.02 ± 0.11	0.03 ± 0.27	0.095	0.01 ± 0.47	0.314
		Control	0.02 ± 0.28	0.02 ± 0.54	0.176	-0.00 ± 0.44	
	Turmeric (mg/day)	mMeD	8.4 ± 6.5	10.7 ± 3.8	**0.047**	2.3 ± 1.3	**0.041**
		Control	7.9 ± 4.4	8.0 ± 4.5	0.341	0.1 ± 1.4	
	Green/black tea (g/day)	mMeD	4.5 ± 0.4	5.0 ± 1.1	0.057	0.5 ± 1.7	0.274
		Control	4.1 ± 0.6	4.1 ± 0.6	0.748	0.00 ± 0.8	
	Pepper (g/day)	mMeD	0.7 ± 1.5	5.3 ± 2.3	**<0.001***	4.6 ± 3.1	**0.031**
		Control	0.8 ± 1.1	0.9 ± 1.8	0.188	0.1 ± 1.1	
	Thyme/oregano (mg/day)	mMeD	0.04 ± 0.27	29.8 ± 8.1	**<0.001***	29.7 ± 9.9	**<0.001***
		Control	0.16 ± 0.44	0.13 ± 0.79	0.552	-0.03 ± 0.20	
	Rosemary (mg/day)	mMeD	0.00 ± 0.05	33.4 ± 6.6	**<0.001***	33.4 ± 8.2	**<0.001***
		Control	0.00 ± 0.02	0.00 ± 0.06	0.656	0.00 ± 0.02	
Flavonoids[6]	Flavan-3-ol (mg/day)	mMeD	74.2 ± 24.2	160.3 ± 69.8	**0.046**	86.1 ± 12.4	0.068
		Control	68.9 ± 51.1	77.1 ± 44.4	0.121	8.2 ± 10.7	
	Flavones (mg/day)	mMeD	4.2 ± 1.6	5.5 ± 4.8	0.180	1.3 ± 0.9	0.059
		Control	3.3 ± 5.1	3.1 ± 2.2	0.470	-0.2 ± 0.5	
	Flavonols (mg/day)	mMeD	30.8 ± 8.4	44.5 ± 10.3	0.065	13.7 ± 4.8	0.214
		Control	22.7 ± 4.5	20.3 ± 6.6	0.113	-2.4 ± 1.1	

	Flavonones (mg/day)	mMeD	11.6 ± 6.1	17.3 ± 9.9	0.080	5.7 ± 3.3	0.247
		Control	11.0 ± 2.5	11.0 ± 1.9	0.914	0.00 ± 0.47	
	Anthocyanidins (mg/day)	mMeD	10.6 ± 5.5	59.3 ± 11.2	**<0.001***	48.7 ± 10.0	**<0.001***
		Control	10.2 ± 4.4	11.0 ± 3.8	0.252	0.8 ± 1.8	
	Isoflavones[7] (mg/day)	mMeD	4.2 ± 3.1	13.7 ± 0.9	**0.044**	9.5 ± 1.6	**0.049**
		Control	4.1 ± 3.0	4.5 ± 2.8	0.230	0.4 ± 0.6	
miscellaneous	Eugenol[8] (mg/day)	mMeD	0.01 ± 0.2	0.2 ± 0.1	0.074	0.19 ± 0.2	**0.033**
		Control	0.00 ± 0.1	0.00 ± 0.1	0.234	0.00 ± 0.1	
	Caffeine (g/day)	mMeD	0.004 ± 0.003	0.003 ± 0.001	**<0.001***	-0.001 ± 0.000	0.618
		Control	0.004 ± 0.003	0.004 ± 0.003	0.164	-0.000 ± 0.000	
Overall DII score[4]		**mMeD**	**2.38 ± 0.21**	**-1.87 ± 0.86**	**<0.001***	**-4.25 ± 1.54**	**<0.001***
		Control	**2.21 ± 0.44**	**2.14 ± 1.01**	**0.771**	**-0.07 ± 0.62**	

DII, Dietary inflammatory index; mMeD, modified Mediterranean Diet; control, Traditional Iranian Diet; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Data has been presented as mean ± SD.
[1]obtained from paired t test; except for protein and Vitamin A, that were analyzed via nonparametric Wilcoxon test. P<0.05 considered as significant.
[2]mean changes between end of trial and baseline values.
[3]obtained from MANCOVA test; adjusted for age, gender, body weight, body mass index, education level, supplement use, family history and duration of MS, and P<0.05 considered as significant.
Flavonoid intake was estimated based on "USDA Database for the Flavonoid Content of Selected Foods; Release 3.2". Other micro &macronutrients were calculated with N4 software.
[4] obtained by dietary intakes only. Negative number indicates an anti-inflammatory score, while positive number reflects a proinflammatory score.
[5]due to the cultural features of Iranian, the intake of alcohol-contained products was close to zero; thus, we modified the standard version of MeD by eliminating any alcohol beverages e.g. red wine.
[6] calculated from USDA Database for the Flavonoid Content of Selected Foods Release 3.3 (March 2018)
[7] calculated from USDA Database for the Isoflavone Content of Selected Foods Release 2.0 (September 2008)
[8] Eugenol (mg) intake was measured based on Phenol-Explorer database (latest version 3.6; released on December 2016)

Table 4. Comparison of fatigue & disability-related variables in patients with relapsing-remitting multiple sclerosis that received either modified Mediterranean Diet (mMeD) or Traditional Iranian Diet (control).

Variables	Subscale	Group	Baseline	End of trial	P-value[1]	Δ^2	P-value[3]
MFIS	Physical	mMeD	31.2 ± 10.4	28.5 ± 8.8	**<0.001****	-2.7 ± 0.7	**<0.001***
		Control	32.9 ± 9.2	33.7 ± 10.2	0.124	0.8 ± 0.8	
	Cognitive	mMeD	35.8 ± 11.1	30.2 ± 8.5	**<0.01***	-5.6 ± 1.8	**0.027**
		Control	36.6 ± 9.9	36.1 ± 7.1	0.092	-0.5 ± 0.1	
	Psychosocial	mMeD	5.4 ± 3.1	5.2 ± 2.6	0.244	-0.2 ± 0.2	0.088
		Control	6.0 ± 2.9	6.1 ± 3.4	0.157	0.1 ± 0.4	
	Total score	mMeD	72.4 ± 17.2	63.9 ± 14.2	**<0.001****	-8.5 ± 2.74	**<0.001***
		Control	69.5 ± 13.2	75.9 ± 15.3	0.771	6.4 ± 1.62	
EDSS		mMeD	1.7 ± 0.7	1.7 ± 0.6	0.334	-0.02 ± 0.07	0.065
		Control	2.0 ± 0.9	2.1 ± 0.8	0.112	0.1 ± 0.02	

MFIS, Modified Fatigue Impact Scale; EDSS, Extended Disability Status Scale; mMeD, modified Mediterranean Diet; control, Traditional Iranian Diet; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Data has been presented as mean ± SD.

[1]obtained from paired t test. P<0.05 considered as significant.

[2]mean changes between end of trial and baseline values.

[3]obtained from MANCOVA test; adjusted for age, gender, body weight, body mass index, education level, supplement use, family history and duration of MS, and P<0.05 considered as significant.

Table 5. Predictors and covariates for MFIS total score and EDSS in mMeD group.

Dependent variables	Covariates	*B*	95% CI		P value	Adjusted *R*
			Lower	Upper		
MFIS total score	Age	0.035	-0.006	0.068	0.122	0.098
	Gender	-0.362	-0.388	-0.340	0.950	
	body weight	0.045	0.033	0.057	0.069	
	BMI	0.277	0.127	0.428	0.212	
	education level	0.022	0.018	0.026	0.199	
	supplement use	0.860	0.510	1.176	0.320	
	family history	0.232	0.047	0.417	0.478	
	duration of MS	1.007	0.946	1.069	0.321	
	DII	1.701	1.329	2.073	**0.041***	
EDSS	Age	0.018	-0.024	0.060	0.625	0.157
	Gender	0.112	-0.089	0.313	0.164	
	body weight	1.022	0.142	1.902	0.311	
	BMI	0.987	0.975	0.998	0.584	
	education level	-0.221	-0.381	-0.061	0.358	
	supplement use	0.675	0.504	0.846	0.166	
	family history	1.328	0.297	2.359	0.254	
	duration of MS	2.547	2.113	2.981	0.323	
	DII	3.809	2.505	5.114	0.067	

B, unstandardized regression coefficient; 95% CI, 95% confidence interval of the unstandardized regression coefficient. MFIS, modified fatigue impact scale; DII, dietary inflammatory index; EDSS, extended disability status scale; BMI, body mass index; MS, multiple sclerosis

doi:10.1093/brain/awy131 BRAIN 2018: 141; 1900–1916 | 1900

BRAIN
A JOURNAL OF NEUROLOGY

REVIEW ARTICLE

The intestinal barrier in multiple sclerosis: implications for pathophysiology and therapeutics

Carlos R. Camara-Lemarroy,[1,2] Luanne Metz,[1,2] Jonathan B. Meddings,[3] Keith A. Sharkey[2,4] and V. Wee Yong[1,2]

Biological barriers are essential for the maintenance of homeostasis in health and disease. Breakdown of the intestinal barrier is an essential aspect of the pathophysiology of gastrointestinal inflammatory diseases, such as inflammatory bowel disease. A wealth of recent studies has shown that the intestinal microbiome, part of the brain-gut axis, could play a role in the pathophysiology of multiple sclerosis. However, an essential component of this axis, the intestinal barrier, has received much less attention. In this review, we describe the intestinal barrier as the physical and functional zone of interaction between the luminal microbiome and the host. Besides its essential role in the regulation of homeostatic processes, the intestinal barrier contains the gut mucosal immune system, a guardian of the integrity of the intestinal tract and the whole organism. Gastrointestinal disorders with intestinal barrier breakdown show evidence of CNS demyelination, and content of the intestinal microbiome entering into the circulation can impact the functions of CNS microglia. We highlight currently available studies suggesting that there is intestinal barrier dysfunction in multiple sclerosis. Finally, we address the mechanisms by which commonly used disease-modifying drugs in multiple sclerosis could alter the intestinal barrier and the microbiome, and we discuss the potential of barrier-stabilizing strategies, including probiotics and stabilization of tight junctions, as novel therapeutic avenues in multiple sclerosis.

1 Department of Clinical Neurosciences, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
2 Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
3 Department of Medicine, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
4 Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada

Correspondence to: Carlos R. Camara-Lemarroy
MS Clinic, FMC and University of Calgary
1403 29 Street NW, T2N 2T9
Calgary, Alberta, Canada
E-mail: Carlos.Camara-Lemarroy@albertahealthservices.ca

Keywords: multiple sclerosis; intestinal barrier; microbiota; disease-modifying therapies; inflammatory bowel disease

Abbreviations: EAE = experimental autoimmune encephalomyelitis; IBD = inflammatory bowel disease; LPS = lipopolysaccharide; SCFA = short chain fatty acid

Introduction

Biological barriers separate the internal milieu from the external environment and are essential components of maintaining homeostasis. A compromised intestinal barrier function is a prominent feature of many diseases, such as inflammatory bowel disease (Choi *et al.*, 2017; Martini *et al.*, 2017; Mu *et al.*, 2017), graft versus host disease (Nalle and Turner, 2015) and coeliac disease (Schumann *et al.*, 2017), but other biological barriers also fail in a

Received December 1, 2017. Revised February 28, 2018. Accepted March 24, 2018. Advance Access publication May 30, 2018

myriad of pathological conditions (e.g. renal tubules in glomerulonephritis and lung alveoli in acute respiratory distress syndrome). The CNS is highly sensitive to homeostatic changes, and as such requires its own specialized barrier, the blood–brain barrier, for appropriate functioning. Breakdown of the blood–brain barrier is an essential hallmark of multiple sclerosis pathophysiology. Immune mediated dysregulation of the blood–brain barrier allows for migration of activated inflammatory cells into the brain, which in turn induces demyelination, axonal loss and other tissue damage (Ortiz *et al.*, 2014; Kamphuis *et al.*, 2015). Interestingly, many of the tight junction molecules in endothelial cells of the brain–blood barrier are identical to those in intestinal tissues, such as occludin, claudins and zona occludens-1 (Reinhold and Rittner, 2017). In this review, we examine the multiple lines of evidence, albeit mostly indirect, linking the intestinal barrier function and multiple sclerosis pathophysiology. We also discuss the possible effect of multiple sclerosis disease-modifying therapies and their association with the gut microbiome.

The intestinal barrier

The intestinal barrier maintains homeostasis by preventing the unwanted movement of antigenic molecules and microbes from the lumen of the gastrointestinal tract, while allowing the products of digestion and water to enter the body. The intestinal barrier consists of a physical barrier provided by the inter-epithelial tight junctions, a secretory barrier that includes antimicrobial peptides, mucus and fluid and an immunological barrier, including cells and molecules of the innate and adaptive immune system. The secretory component of the epithelial barrier is regulated by neural mechanisms that integrate this component of barrier function with digestive processes in the gut. Intestinal barrier function refers to ability of the intestinal mucosa and extracellular barrier components (e.g. mucus, antimicrobial peptides) to modulate epithelial permeability and act as a physical and functional limiting step for organism-luminal interactions.

The intestinal lumen and its contents are separated from the rest of the gastrointestinal tissue (and the body) by a single layer of epithelial cells along the length of the gastrointestinal tract. These cells are being constantly renewed and thus require constant proliferation (Delgado *et al.*, 2016). Intestinal stem cells, present in the crypts of the intestinal mucosa, differentiate into both enterocytes, and specialized secretory (Paneth cells and goblet cells) and sensory cells (enteroendocrine cells and tuft cells), a process regulated by complex transcriptional and epigenetic mechanisms (Smith *et al.*, 2017). The intestinal barrier is permeable to water and other small molecules, a property modulated by tight junctions, located around the apical surface of adjacent epithelial cells. Tight junctions consist of a heterogeneous group of transmembrane proteins such as occludins, claudins, junctional adhesion molecules and zona occludens-1, each with specific roles (Gasbarrini and Montalto, 1999; Sturgeon and Fasano, 2016; Volynets *et al.*, 2016; Capaldo *et al.*, 2017; France and Turner, 2017).

The intestinal barrier (Fig. 1) is continuously exposed to a number of immunological and microbiological factors. When the permeability of the intestinal barrier is breached, undesired large molecules, and commensal bacteria, may enter the lamina propria with pathological consequences (Odenwald and Turner, 2017). One of the main causes of increased permeability of the intestinal barrier is inflammation, an event thought to be essential in the pathophysiology of inflammatory bowel disease (IBD) (de Souza *et al.*, 2017; Martini *et al.*, 2017), coeliac disease and sepsis (Yoseph *et al.*, 2016; Schumann *et al.*, 2017). Inflammatory cytokines including interferons, interleukin (IL)-17 and tumour necrosis factor alpha (TNFα), as well as calcium-dependent oxidative stress, have been shown to alter the expression of tight junction proteins and lead to increased intestinal permeability (Reynolds *et al.*, 2012; Yang *et al.*, 2014; Al-Sadi *et al.*, 2016; Gangwar *et al.*, 2017).

Together with intestinal epithelial cells as the first layer of the intestinal barrier are Paneth cells (Fig. 1), which are specialized secretory cells derived from intestinal stem cells. Paneth cells produce antimicrobial peptides, the defensins, which are secreted into the mucus layer (Dupont *et al.*, 2014; Yu *et al.*, 2016; Capaldo *et al.*, 2017). Mucus, secreted from goblet cells, is composed of heavily glycosylated oligomeric mucin proteins, water, ions and secretory IgA. This layer modulates bacterial growth in the intestinal lumen adjacent to the intestinal barrier, prevents bacterial adherence and acts as part of the innate immune response of the organism against microbial pathogens (Dupont *et al.*, 2014).

After the mucus and the epithelial lining of the gastrointestinal tract, the next layer of the intestinal barrier is mostly immunological. Innate lymphoid cells, located in the epithelial layer, can be activated to produce a variety of inflammatory mediators, which play a defensive or a pathogenic role in mammal gut homeostasis (Bostick and Zhou, 2016). Found in close proximity to the single layer of enterocytes, intraepithelial lymphocytes are a heterogeneous population of cells that provide immune protection against pathogens and also regulate immune responses that, if unchecked, could jeopardize the integrity of the barrier (Cheroutre *et al.*, 2011; Olivares-Villagómez and Van Kaer, 2018). The lamina propria (Fig. 1) is populated by B, T and dendritic cells that can initiate and modulate a host of immunological responses (Persson *et al.*, 2013; Gronke *et al.*, 2017). Peyer's patches are secondary lymphoid tissues present in the intestinal mucosa. They are continuously exposed to a variety of antigens, presented to Peyer's patches by microfold epithelial cells and resident dendritic cells (Rochereau *et al.*, 2011; Hashiguchi *et al.*, 2015).

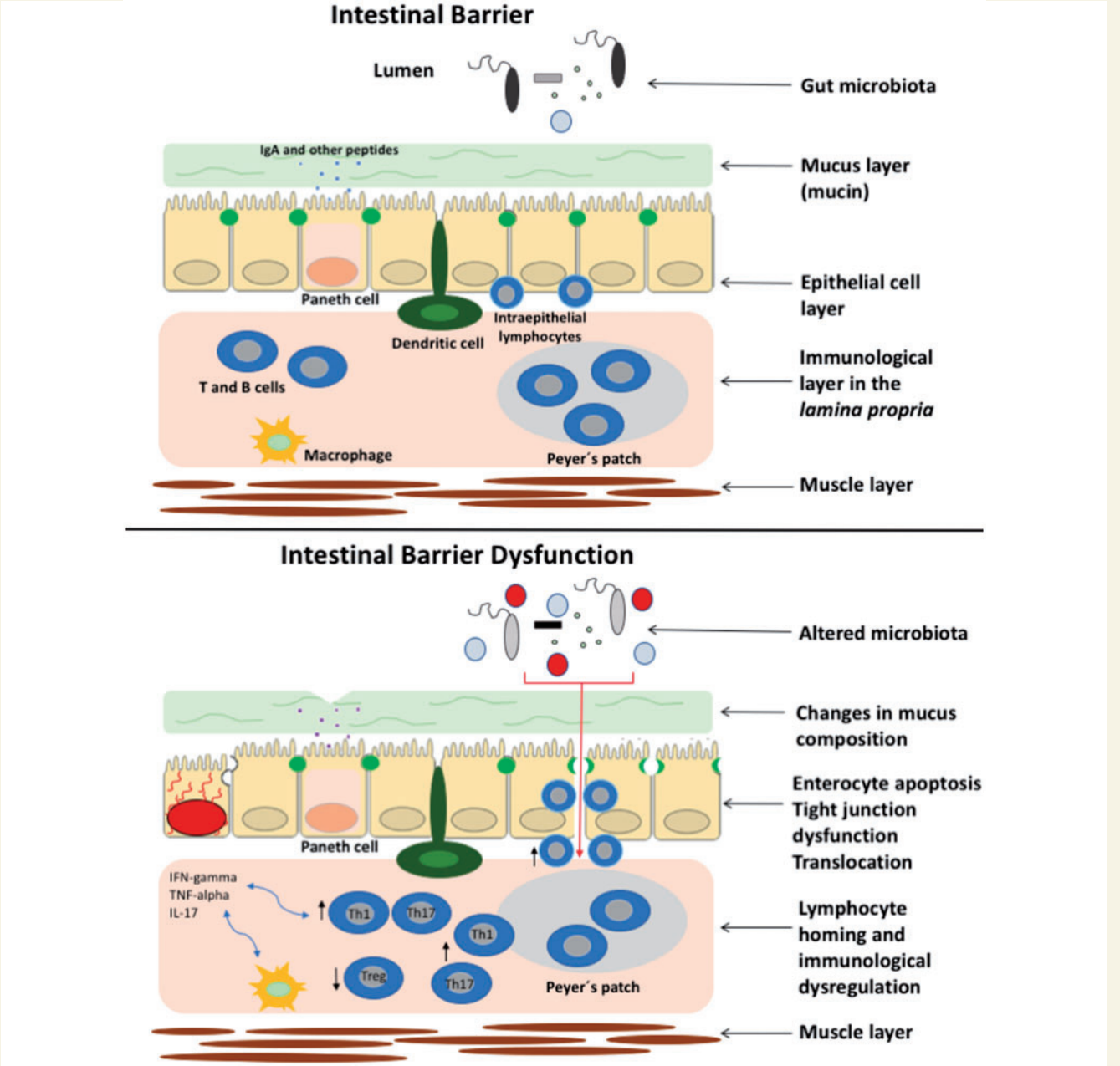

Figure 1 The intestinal barrier and possible mechanisms of barrier dysfunction in multiple sclerosis. The normal intestinal barrier is composed of multiple layers (*top*). From the luminal side outwards, there is a mucus layer in close contact with the commensal microbiota, the single cell epithelial layer (woven together by tight junction proteins depicted here as green closed circles), the lamina propria and submucosa containing the immunological barrier, and finally the muscle and connective tissue layer. Changes in microbiota, mucus composition, epithelial cell death, tight junction function and immunological dysregulation could all lead to breakdown of the intestinal barrier and increased permeability (*bottom*).

CNS demyelination and intestinal barrier breakdown in gastrointestinal disorders: an important link?

An association between multiple sclerosis and IBD has been suggested because of common epidemiological, immunological and genetic patterns (Barcellos *et al.*, 2006). IBD patients have an increased risk for cerebrovascular disease, peripheral neuropathy and demyelinating disease (Casella *et al.*, 2014; Ferro *et al.*, 2014; Morís, 2014), and anti-TNF therapies that are widely used in IBD have also been associated with CNS demyelination (Katsanos and Katsanos, 2014). Indeed, a recent meta-analysis of 10 case-control studies including over 1 million patients found a risk ratio of 1.54 for multiple sclerosis/IBD comorbidity, with no difference between Crohn's disease

and ulcerative colitis (Kosmidou *et al.*, 2017). Certain authors propose that IBD can be conceived as a disorder of the intestinal epithelial barrier, and barrier breakdown is known to be an essential step in the pathophysiology of both Crohn's and ulcerative colitis (for reviews see Jäger *et al.*, 2013; Antoni *et al.*, 2014; Goll and van Beelen Granlund, 2015). Evidence showing white matter involvement in IBD could also provide a link between intestinal barrier breakdown and CNS demyelination.

In an early study, investigators found a 3-fold increase of white matter hyperintensities in the MRIs of patients with IBD (Geissler *et al.*, 1995). A recent estimate suggested that over half of all IBD patients will have white matter hyperintensities in a routine MRI (Ferro *et al.*, 2014). Other findings in IBD include decreased grey matter volume and decreased axial diffusivity in major white matter tracts (Zikou *et al.*, 2014). The aetiology of the white matter lesions found in patients with IBD is uncertain, and some authors suggest that ischaemia and vasculitis might be responsible (Zikou *et al.*, 2014). However, in a report of five cases of patients with Crohn's disease with symptomatic acute white matter lesions suggestive of demyelination, systemic infection, coagulation disorders, or vasculitis were ruled out (de Lau *et al.*, 2009). Additionally, other studies have attempted to describe white matter lesions in patients with Crohn's disease with more detail. White matter lesions suggestive of demyelination were found in 72% of 54 patients compared to 34% in age- and sex-matched controls (Chen *et al.*, 2012). The role of anti-TNF therapy is also debated, and some observational studies have not found an association between therapy and presence of white matter hyperintensities (Chen *et al.*, 2012). In a retrospective analysis of 9095 patients with IBD, anti-TNF therapy was not found to increase the risk of confirmed inflammatory demyelinating CNS lesions (de Felice *et al.*, 2015).

Other gastrointestinal diseases where the intestinal barrier is impaired have also been associated with CNS demyelination. In patients with multiple sclerosis, serological and histological markers of coeliac disease are more frequent than in healthy controls (Rodrigo *et al.*, 2011), although other studies have found inconsistent results (Salvatore *et al.*, 2004). Cases of comorbid coeliac disease and multiple sclerosis are abundant in the literature (Batur-Caglayan *et al.*, 2013; Casella *et al.*, 2016), as are cases of coeliac disease with white matter lesions mimicking multiple sclerosis or other CNS demyelinating diseases (Mirabella *et al.*, 2006; Finsterer and Leutmezer, 2014; Krom *et al.*, 2017). MRI studies in patients with coeliac disease have also shown higher proportion of white matter lesions and grey matter atrophy (Bilgic *et al.*, 2013).

Although a causal link between intestinal barrier breakdown and CNS demyelination cannot be concluded with certainty in these cases, there appears to be an association not solely explained by their shared epidemiological and immunological characteristics. The association between these entities is certainly complex and in need of further study.

Intestinal barrier homeostasis, the microbiome and neuroinflammation: possible mechanisms linking these entities

The interactions between the microbiome and the intestinal barrier, particularly the contribution of the microbiome in maintaining barrier homeostasis, could be central in accounting for its regulation of neuroinflammation (Fig. 2). Several studies have established that there are alterations in the gut microbiome of patients with multiple sclerosis, which has further fuelled the interest in the brain-gut-microbiome connection in multiple sclerosis research.

Early studies showed that, when compared to controls, patients with relapsing-remitting multiple sclerosis have an abundance of *Anaerostipes*, *Faecalibacterium*, *Pseudomonas*, *Mycoplasma*, *Haemophilus*, *Blautia*, and *Dorea* and a relative decrease of *Bacteroides*, *Prevotella*, *Parabacteroides* and *Adlercreutzia* (Cantarel *et al.*, 2015; Miyake *et al.*, 2015; Chen *et al.*, 2016). In paediatric multiple sclerosis, patients have higher levels of members of *Desulfovibrionaceae* and depletion in *Lachnospiraceae* and *Ruminococcaceae* (Tremlett *et al.*, 2016*a*). However, a clear and consistent 'multiple sclerosis microbiome phenotype' has not been described, and a myriad of different species have been implicated. For example, studies have found a significant depletion in *Clostridial* species (Rumah *et al.*, 2013; Miyake *et al.*, 2015), *Butyricimonas* (Jangi *et al.*, 2016), *Roseburia* (Swidsinski *et al.*, 2017) and increases in *Streptococcus* (Cosorich *et al.*, 2017), *Methanobrevibacter*, *Akkermansia* and *Coprococcus* (Cantarel *et al.*, 2015; Jangi *et al.*, 2016). Multicentre studies aiming at defining a 'core microbiome' are underway (Pröbstel and Baranzini, 2018). Furthermore, some of these changes in the microbiome have been associated with immunological derangements, such as differences in the expression of genes involved in interferon and nuclear factor kappa-B (NF-κB) signalling (Jangi *et al.*, 2016), and numbers of pro-inflammatory T helper 17 (Th17) cells in the intestine (Cosorich *et al.*, 2017). At least one study found that differences in the microbiota could predict relapse risk in paediatric multiple sclerosis patients (Tremlett *et al.*, 2016*b*).

Insights into how the microbiome could alter neuroinflammatory responses (reviewed in Colpitts and Kasper, 2017; Wekerle, 2017) have been illuminated by studies in germ-free mice where the microbiome regulates the shift back-and-forth of immune cells from pro- to anti-inflammatory phenotypes (Berer *et al.*, 2011). Mice maintained under germ-free conditions have an attenuated form of experimental autoimmune encephalomyelitis (EAE), an inflammatory model of multiple sclerosis, and show lower levels of IL-17 in both the gut and the CNS, while also

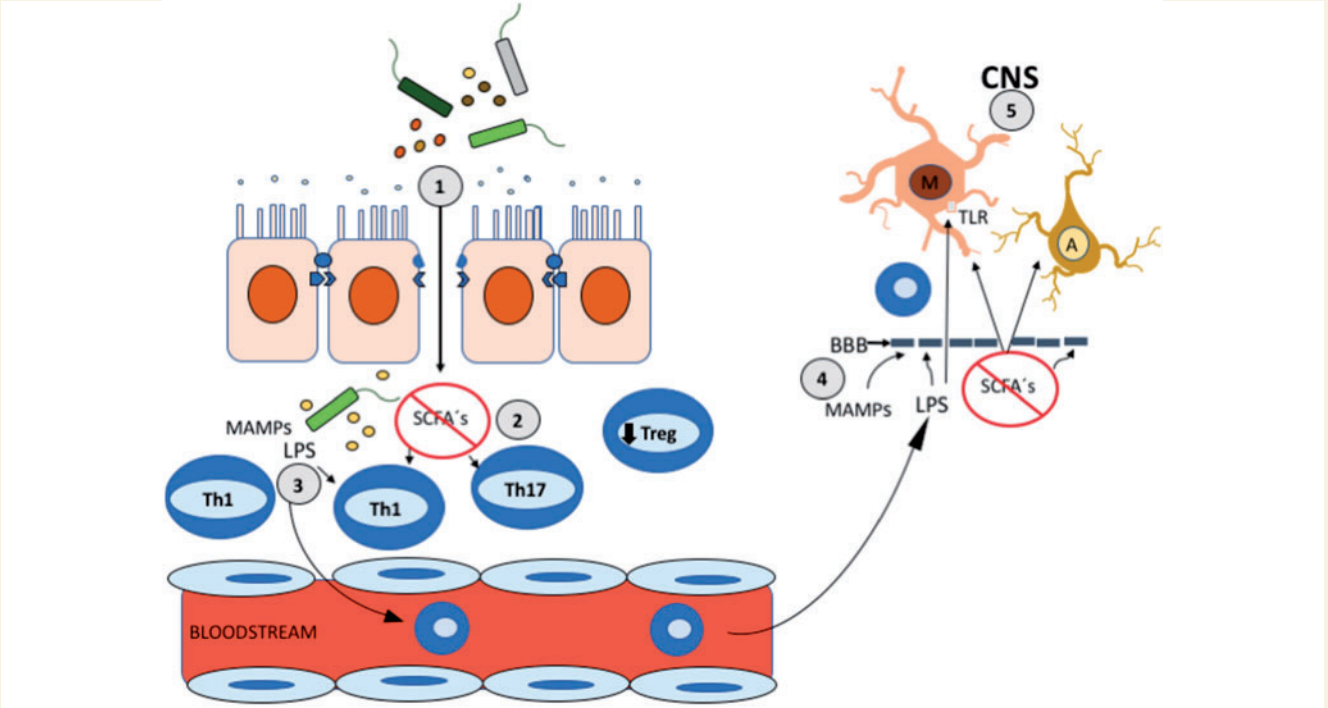

Figure 2 An altered intestinal barrier leads to immune changes in the gut and the CNS. (1) Multiple sclerosis-associated microbiota and immune derangements lead to an altered barrier and increased permeability. (2) Microbiota diversity is reduced, as is production of SCFA's, and some bacteria translocate to the lamina propria. (3) LPS produced by bacteria cause low-grade inflammation and endotoxaemia, and loss of SCFA signalling alters lymphocyte phenotypes. (4) LPS, microbial-associated molecular patterns (MAMPs) and reduced SCFAs alter the blood–brain barrier. (5) LPS and activated lymphocytes reach the CNS, where in absence of normal SCFA concentrations, microglia and astrocyte neuroimmune responses are affected. A = astrocytes; BBB = blood–brain barrier; M = microglia; TLR = Toll-like receptors.

showing an increase in regulatory T cells (Tregs) peripherally (Lee *et al.*, 2011). Colonization with segmented filamentous bacteria in germ-free mice leads to increased production of IL-17 and development of severe EAE. In contrast, other gut commensals such as *P. histicola* are able to suppress EAE severity, by decreasing pro-inflammatory Th1 and Th17 cells, and increasing Tregs and suppressive macrophages (Mangalam *et al.*, 2017). *B. fragilis*, another common commensal strain, can also suppress EAE by expanding Tregs expressing the ectonucleotidase CD39, allowing for increased migration of this regulatory cell type into the CNS (Wang *et al.*, 2014). Microbiota abundant in patients with multiple sclerosis induce the differentiation *in vitro* of human peripheral blood mononuclear cells into Th1 cells while reducing Treg numbers; conversely, microbiota that are decreased in patients with multiple sclerosis stimulate anti-inflammatory IL-10-expressing T cells and FoxP3+ Tregs (Cekanaviciute *et al.*, 2017). Microbiota from patients with multiple sclerosis transplanted to mice prone to develop spontaneous EAE increases their susceptibility to EAE (Berer *et al.*, 2017). Interestingly, multiple sclerosis patient-derived microbiota transplantation did not lead to changes in tight junction protein expression in the mouse recipient gut, but splenic lymphocytes had impaired IL-10 production (Berer *et al.*, 2017).

An altered microbiome also leads to changes in some bacteria-associated products known to influence neuroimmune responses. Short chain fatty acids (SCFAs) such as butyrate, propionate and acetate are produced by bacterial fermentation of dietary carbohydrate and fibre. They play important roles in maintaining intestinal homeostasis, such as mediating sodium transport, serving as the principal energy source of intestinal epithelial cells and modulating gene transcription via inhibition of histone deacetylase activity (Kiela and Ghishan, 2016). Although not focusing on the concentration of SCFAs, CSF metabolomics studies from patients with multiple sclerosis have shown significant differences when compared to controls. SCFAs such as acetate are reduced (Simone *et al.*, 1996; Kim *et al.*, 2017), while others such as formate (Kim *et al.*, 2017) have been found to be elevated in patients CSF. In studies evaluating metabolites in urine, propionate metabolism has also been found to be altered in patients with multiple sclerosis (Gebregiworgis *et al.*, 2016).

In experimental models, eradication of the gut microbiota, or even just limiting the intestinal microbiome diversity, leads to impaired microglia structure and immune function, a process regulated by SCFAs (Erny *et al.*, 2015, 2017). Astrocytes may also be influenced by SCFAs and the microbiome. Dietary tryptophan is metabolized by the gut microbiota into aryl hydrocarbon receptor agonists

such as indoxyl-3-sulfate and indole-3-propionic acid, which can modulate astrocyte inflammatory function trough limiting NF-κB activation in a suppressor of cytokine signalling 2-dependent manner (Rothhammer *et al.*, 2016). SCFAs also reduce T cell proliferation and cytokine production in the gut (D'Souza *et al.*, 2017; Wan Saudi and Sjöblom, 2017). In EAE models, the administration of SCFAs led to amelioration of disease severity in association with a reduction of Th1 cells and an increase in Tregs (Mizuno *et al.*, 2017). Interestingly, an altered microbiota may also alter innate immune responses in the gut favourable for systemic autoimmunity. For example, some types of intraepithelial lymphocytes may act as Tregs that suppress the pathogenic response to the immunizing antigen in EAE (Tang *et al.*, 2007). CD4(+) intraepithelial lymphocytes obtained from transgenic mice prone to develop spontaneous EAE can infiltrate the CNS and ameliorate EAE severity in wild-type mice on transfer, showing regulatory properties (Kadowaki *et al.*, 2016). These same cells proliferate in response to gut-derived antigens, aryl hydrocarbon receptor ligands and microbiota.

SCFAs could also modulate blood–brain barrier permeability. It is well known that SCFAs enhance intestinal epithelial cell barrier function by increasing the expression of tight junction proteins (D'Souza *et al.*, 2017; Wan Saudi and Sjöblom, 2017). Butyrate has also been shown to increase the expression of occludin and zona occludens-1, thus restoring blood–brain barrier permeability in models of traumatic brain injury (Li *et al.*, 2016). In germ-free mice exhibiting an altered blood–brain barrier, butyrate administration led to increased occludin expression and preserved blood–brain barrier permeability (Braniste *et al.*, 2014). Overall, changes in SCFA-producing bacteria in the gut, and the influx of SCFAs into the blood stream, could thus have a distal effect in microglia and astrocyte functions, as well as in modifying blood–brain barrier permeability and the entrance of immune cells into the CNS (Fig. 2).

Besides the above-discussed mechanisms suggesting bystander activation, another possible immunopathogenic link between multiple sclerosis and the gut microbiota is that of molecular mimicry. CNS-specific, self-reactive lymphocytes might be cross-activated by both gut microbiota antigens and myelin (Berer and Krishnamoorthy, 2014). Although there is no conclusive evidence for these mechanisms, commonly found pathogenic and non-pathogenic gut bacteria such as *Bacteroides* spp. and *Enterococcus faecalis* possess potential myelin basic protein encephalitogenic mimics (Westall, 2006).

The intestinal barrier in multiple sclerosis: consequences of a leaky gut

Recent attention in the brain-gut connection in multiple sclerosis research has been focused on the role of the commensal gut microbiome while largely ignoring the interface of the microbiome with the organism, i.e. the intestinal barrier. Therefore, actual evidence for an alteration of the intestinal barrier in multiple sclerosis is limited. In a study of 12 jejunal biopsies from multiple sclerosis patients, Lange and Shiner (1976) found subtle histological changes, such as two cases of villous atrophy, as well as some cases of intestinal inflammatory cell infiltration. A later study found similar infiltrates, and also evidence of intestinal malabsorption in close to 20 of 52 patients with multiple sclerosis (Gupta *et al.*, 1977).

In 1996, Yacyshyn *et al.* (1996) showed that 5 of 20 patients with multiple sclerosis had an altered lactulose/mannitol permeability test, suggesting increased intestinal permeability, a finding also associated with peripheral expression of CD45RO on CD20+ B cells. In the most recent study to date, the lactulose/mannitol permeability test was again used to evaluate intestinal permeability in 22 patients with multiple sclerosis and compared with age- and sex-matched controls (Buscarinu *et al.*, 2017). Investigators found abnormal permeability in 73% of cases versus 28% in controls, but no association between permeability and brain MRI lesion load.

Similar findings have been recently described in the EAE model, the prototypic inflammatory animal model of multiple sclerosis. Investigators have found altered intestinal permeability, reduced submucosa thickness and altered tight junction expression in intestinal epithelial cells (Nouri *et al.*, 2014). These alterations could also be induced in mice by adoptive transfer of pathogenic T cells. Furthermore, a recent study showed that the degree of intestinal permeability disturbance is closely associated with EAE severity (Secher *et al.*, 2017). Treatment with *Escherichia coli* strain Nissle 1917, a probiotic known to improve intestinal barrier function, preserved tight junction expression and decreased intestinal permeability, leading to reduced EAE severity and decreased secretion of pro-inflammatory cytokines and an increased production of the anti-inflammatory cytokine IL-10 (Secher *et al.*, 2017). This reduction of intestinal permeability led to a reduction of the migration of inflammatory T cells to the CNS, suggesting an impact on blood–brain barrier permeability as well (Secher *et al.*, 2017).

The above studies suggest that there is indeed an alteration in the intestinal barrier in patients with multiple sclerosis and that these changes are at least partly due to an altered intestinal immune response (Buscarinu *et al.*, 2017). The clinical relevance of these findings is unclear, but several possibilities arise. Intestinal barrier dysfunction has been associated with susceptibility to systemic infections (König *et al.*, 2016), and both CNS and systemic infections are a common complication in patients with multiple sclerosis (Venkatesan, 2015). Another possibility is that the intestinal barrier's interplay with commensal microbiota could modulate the immune response pathologically. Finally, alterations in intestinal permeability may modulate or perpetuate neuroimmune dysregulation by increased

transmucosal passage of injurious or immunogenic antigens.

The essential role of the commensal microbiome in the regulation of intestinal immunity is beginning to be recognized, and several recent reviews have been published on this subject (Haak and Wiersinga, 2017; Shi *et al.*, 2017). Commensal bacteria are able to strengthen the gut barrier and regulate intestinal permeability (Lin and Zhang, 2017). A healthy microbiota also preserves intestinal epithelial cell integrity through the production of SCFAs that increase tight junction expression and through toll-like receptor activation (Wells *et al.*, 2017). Intestinal commensal bacteria are recognized by toll-like receptors, a process leading to protection of intestinal epithelium against injury and barrier disruption (Rakoff-Nahoum *et al.*, 2004). Toll-like receptor signalling also promotes epithelial cell proliferation, IgA secretion and expression of antimicrobial peptides in Paneth cells (Abreu, 2010; Wells *et al.*, 2011).

Alterations in the gut homeostatic mechanisms in multiple sclerosis could have as one of its consequences increased bacterial translocation through an impaired intestinal barrier. One recent study found elevated levels of endotoxin [lipopolysaccharide (LPS)] in plasma of patients with multiple sclerosis, and endotoxin concentrations were related to *in vivo* IL-6 production and increased *in vitro* T-helper 17 (Th17)-like responses (Teixeira *et al.*, 2013). Circulating endotoxin was also correlated with the Expanded Disability Status Scale, a measure of clinical disability in multiple sclerosis. In another study, LPS and LPS-binding protein were found to be elevated in the serum of EAE-induced mice; investigators also found increased LPS-binding protein levels in the serum of multiple sclerosis patients compared to healthy controls (Escribano *et al.*, 2017). These studies are evidence of a low-grade endotoxaemia that could be present in patients with multiple sclerosis, possibly due to bacterial translocation in the setting of an altered intestinal barrier.

Besides LPS, enteric bacteria also produce microbial-associated molecular patterns (MAMPs) such as bacterial lipoproteins and double-stranded RNA that can enter the systemic circulation and act through toll-like receptors to modulate the immune system (Patten and Collett, 2013). Toll-like receptors are known to be expressed in microglia and to modulate initiation and severity of EAE in experimental models (Miranda-Hernandez and Baxter, 2013). LPS is a well-known stimulant of microglial responses and is able to disrupt the blood–brain barrier by increasing microglial production of matrix metalloproteinases (Frister *et al.*, 2014). LPS and other MAMPs could constitute another pathway by which an altered intestinal barrier could affect neuroimmune responses in multiple sclerosis.

Finally, the use of oral disease-modifying therapies and/or symptomatic drugs in multiple sclerosis also constitute a concern, as the intestinal barrier is essential in drug absorption (Sánchez-Navarro *et al.*, 2016). On the other hand, there are no currently marketed therapies to improve intestinal barrier function; nutritional, microbial-derived and probiotic agents are being investigated. In the next section, we will discuss the possible effects of currently used disease-modifying therapies on the intestinal barrier as well as other pathophysiological considerations.

Disease-modifying therapies and the intestinal barrier

An interesting aspect of the above mentioned findings is that the microbiome can also be altered by whatever immunomodulatory therapy the multiple sclerosis patient is receiving (Cantarel *et al.*, 2015; Tremlett *et al.*, 2016*b*). The question of whether gut dysbiosis precedes the development of multiple sclerosis or follows the immune alterations (innate, acquired or drug-induced) is also a matter of debate (Ochoa-Repáraz *et al.*, 2017). Disease-modifying therapies are medications that have improved the clinical course of relapsing-remitting multiple sclerosis. While their principal mechanisms are thought to be immune-modulating, their possible effects over the intestinal barrier that may contribute to therapeutic efficacy have not been explicitly evaluated. Below we summarize evidence suggesting that disease-modifying therapies could modulate the intestinal barrier, the gut microbiome and the interaction between the two (Fig. 3). However, the evidence is indirect, and whether this actually plays a meaningful role in clinical response remains to be established.

Interferons

There is evidence suggesting that endogenous interferons could affect the intestinal barrier. Type I interferons, including IFNα and IFNβ, are an integral part of the innate host immune response to gut microbiota, and they modulate bilateral interactions between epithelial cells and commensal flora (Giles and Stagg, 2017). For example, IFNβ has shown stabilizing properties in biological barriers (such as the intestinal, blood–brain and blood–lung barriers), partly through the upregulation of tight junction proteins in endothelial cell layers (Kraus *et al.*, 2004; LeMessurier *et al.*, 2013; Long *et al.*, 2014). The commensal microbiota also stimulates dendritic cell IFNβ production, which increases the proliferation of Tregs in the intestine, a process itself inhibited by intestinal epithelial cell apoptosis (Nakahashi-Oda *et al.*, 2016). Type I interferons also inhibit the continuous proliferation of the intestinal epithelium by activating the p53 pathway and inducing epithelial cell apoptosis (Katlinskaya *et al.*, 2016), and mice lacking type I interferon receptor on Paneth cells show an altered microbiota (Tschurtschenthaler *et al.*, 2014).

Glatiramer acetate

Various studies have shown that glatiramer acetate reduces colonic injury in animal models of colitis, through reduction of TNFα signalling, elevation of regulatory T cells and

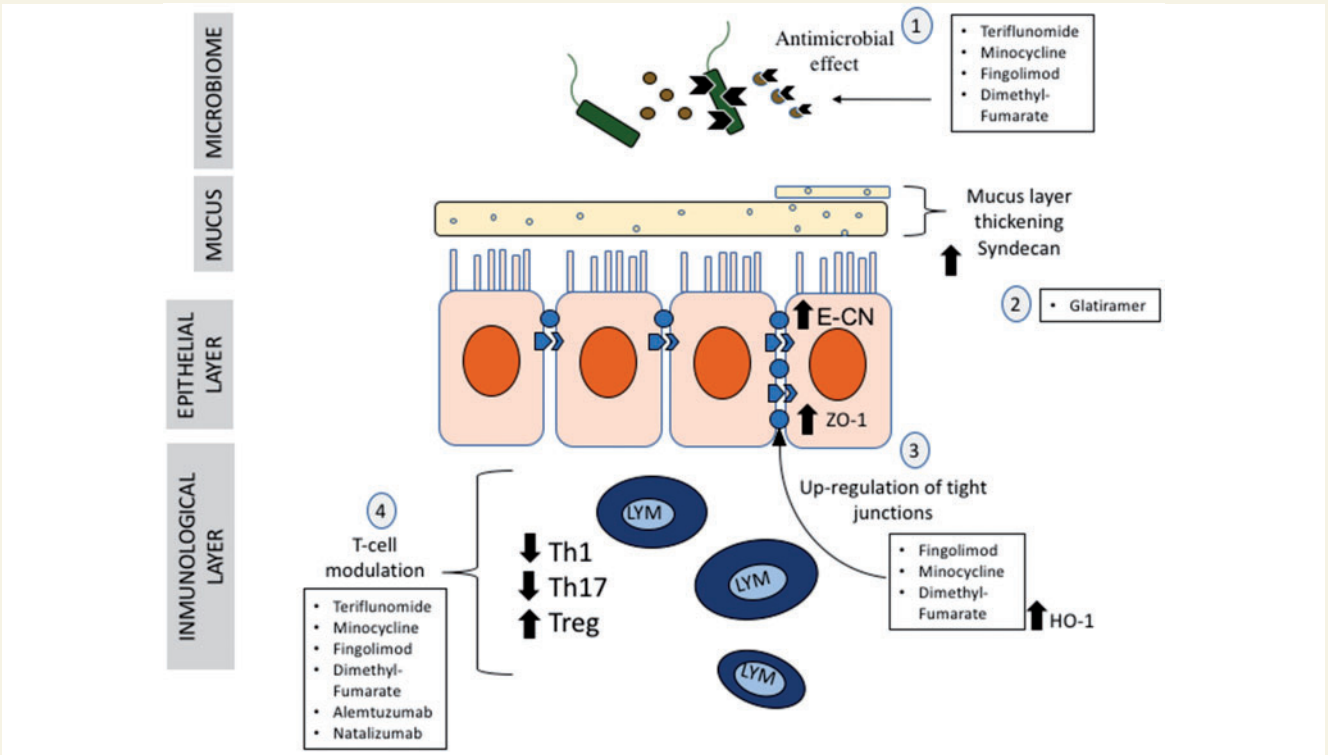

Figure 3 Disease-modifying therapies can modulate the intestinal barrier. Different disease-modifying therapies in clinical use may beneficially modulate intestinal barrier function through a variety of mechanisms. (1) Oral disease-modifying therapies have antimicrobial properties, while minocycline is a tetracycline antibiotic. Dimethyl fumarate acts as a Michael acceptor and can deplete bacterial nucleophilic thiols. (2) Glatiramer acetate has been shown to increase syndecan, the most abundant heparan sulphate proteoglycan in the gastrointestinal tract. (3) Fingolimod, dimethyl fumarate and minocycline increase tight junction expression. Dimethyl fumarate increases zona occludens-1 (ZO-1) in a heme-oxygenase-1 (HO-1) dependent pathway, while S1P signalling increases E-cadherin (E-CN). (4) Most disease-modifying therapies modulate lymphocyte (LYM) populations and functions in non-neurological tissues, such as in the lamina propria. Whether any of these effects have a mechanistic relevance for their therapeutic action is unknown.

increase in anti-inflammatory mediators such as IL-10 and TGFβ (Aharoni *et al.*, 2005, 2007). In one such study, glatiramer acetate attenuated colitis severity and prevented the destabilization of the intestinal epithelial barrier (Yablecovitch *et al.*, 2011). There is also evidence suggesting that patients with multiple sclerosis treated with glatiramer acetate have different microbiota composition. In a small study, glatiramer-treated patients had stool taxonomic units (evaluated by hybridization of 16S rRNA to a DNA microarray) of *Bacteroidaceae*, *Faecalibacterium*, *Ruminococcus*, *Lactobacillaceae*, *Clostridium*, and other *Clostridiales* that were significantly different than those of untreated patients (Cantarel *et al.*, 2015).

Natalizumab

Dysregulated recruitment of leucocytes into the intestine is one of the components of the immune response responsible for barrier breakdown in IBD (Danese *et al.*, 2005; Fiorino *et al.*, 2010). Integrins are expressed on intestinal lymphocytes and are essential in their homing to intestinal lymphoid tissues and trafficking through the intestinal mucosa (Hamann *et al.*, 1994; Tanaka *et al.*, 1995; Miura *et al.*, 1996; Farstad *et al.*, 1997; Bradley *et al.*, 1998; Fujimori *et al.*, 2002). Natalizumab, which blocks the activity of integrins (both α4β1 and α4β7), has shown effectiveness in reducing the severity of IBD (Fiorino *et al.*, 2010; Bamias *et al.*, 2013). However, its association with JC virus-related CNS complications has led to the development of specific α4β7-antibodies such as vedolizumab, now routinely used in the treatment of IBD (Zundler *et al.*, 2017).

Nonetheless, the effects of natalizumab on integrins and lymphocyte trafficking in the gut suggests it could modulate the inflammatory response in this site in multiple sclerosis. A potential role for intestinal lymphocytes and integrins in multiple sclerosis pathophysiology has been suggested by results from mouse EAE models. Th17 cells, prominent drivers of EAE, are controlled and redirected in the small intestine. Th17 cells, which are normally pro-inflammatory, acquire a regulatory phenotype in the intestine and are ultimately eliminated through the intestinal lumen (Esplugues *et al.*, 2011). In EAE, there is infiltration of proinflammatory Th1/Th17 cells and reduction of Tregs in the gut, in association with functional and morphological changes (Nouri *et al.*, 2014). Furthermore, mice lacking integrin α show a loss of Th17 cells in the intestine and resistance

against EAE (Acharya *et al*., 2010; Melton *et al*., 2010). In spontaneously EAE resistant B10.S mice, blocking $\alpha 4\beta 7$ integrin leads to peripheral availability of Th17 cells and increased severity of EAE (Berer *et al*., 2014). In patients with multiple sclerosis, natalizumab treatment reduces the populations of integrin α-4-positive Th1, Th17 and Tregs differentially, while affecting the immune function of residual integrin α-4-positive T cells (Kimura *et al*., 2016). The gut might act as a checking point, a reservoir and an activation site for Th17 and other T cells, a process regulated in part by intestinal integrins. Natalizumab and its non-selective integrin blockade could lead to changes in the way lymphocytes interact with the intestinal tissue. Considering the abovementioned findings, it is possible that natalizumab's therapeutic properties in multiple sclerosis could depend, at least in part, on these intestinal effects, besides those seen in blood–brain barrier, integrins and lymphocyte trafficking.

Fingolimod

Another drug that acts through the regulation of leucocyte trafficking is fingolimod, a functional antagonist of the sphingosine 1-phosphate receptor (S1P). S1P1 receptors are highly expressed on lymphocyte membranes and are critical for T and B cell egress from secondary lymphoid organs. S1P can affect the intestinal barrier by modulating tight junction proteins (Greenspon *et al*., 2011; Pászti-Gere *et al*., 2016), particularly under inflammatory conditions (Dong *et al*., 2015). For instance, fingolimod reduces endothelial barrier dysfunction in blood vessels and lung epithelium in experimental models of sepsis and haemorrhagic shock (Lundblad *et al*., 2013; Bonitz *et al*., 2014). Fingolimod also sequesters and alters the activation of lymphocytes in intestinal tissues (Chiba *et al*., 1998; Yanagawa *et al*., 1998; Henning *et al*., 2001; Halin *et al*., 2005; Sugito *et al*., 2005; Daniel *et al*., 2007), an effect thought to be mechanistically relevant in multiple sclerosis therapeutics. In the mouse EAE model, development of EAE was associated with increased accumulation of T cells in Peyer's patches, a process increased by fingolimod (Spirin *et al*., 2014). Fingolimod can also directly affect the microbiota. Both sphingosine and fingolimod inhibit *C. perfringens* growth and endotoxin production *in vitro*, suggesting an intrinsic antibacterial property (Rumah *et al*., 2017).

Dimethyl fumarate

Dimethyl fumarate (DMF) is derived from the simple organic acid fumaric acid, and it acts as an immunomodulator by promoting T cell apoptosis, shifting to a Th2 response and acting as an antioxidant. There is limited but interesting evidence suggesting DMF could beneficially affect both the intestinal barrier and the gut microbiota. DMF alleviates experimentally induced colitis and reduces the Th1 response in mouse models and protects human intestinal epithelial cells against oxidative barrier dysfunction by preserving zona occludens-1 and occludin expression *in vitro* (Casili *et al*., 2016). DMF also preserves intestinal mucosa morphology after mycotoxin exposure and decreases intestinal permeability by strengthening tight junctions (Ma *et al*., 2017). In this model, DMF also led to increased microbiome diversity, with more abundance of bacteria producing SCFAs, such as *Gemella*, *Roseburia*, *Bacillus* and *Bacteroides*. DMF can also directly reduce *C. perfringens* growth and exhibits anti-mildew and antibacterial properties (Ma *et al*., 2017; Rumah *et al*., 2017).

Alemtuzumab

Alemtuzumab is an anti-CD52 antibody that causes depletion of mainly lymphocytes and is highly effective in the clinical management of multiple sclerosis (Hartung *et al*., 2015). Despite its specific mechanism of action, there is evidence suggesting it has detrimental effects over the integrity of the intestinal barrier and might alter the gut microbiome.

In mice, anti-CD52 antibodies induce increased intestinal barrier permeability (Qu *et al*., 2009) and lead to reductions in epithelial cell populations and to altered tight junction ultrastructure (Shen *et al*., 2013, 2015). In macaques, alemtuzumab-induced intestinal barrier disruption is associated with epithelial cell apoptosis as well as with increased circulating levels of D-lactate and endotoxin, indirect markers of intestinal barrier breakdown and bacterial translocation (Li *et al*., 2011; Qu *et al*., 2015). Lymphocyte depletion with alemtuzumab treatment in macaque models also resulted in dramatic changes in the gut microbiota (Li *et al*., 2010). *Lactobacillales*, *Enterobacteriales*, *Clostridiales*, and the genus *Prevotella* and *Faecalibacterium* were primarily responsible for the variations of the gut microbiota after lymphocyte depletion (Li *et al*., 2013). The diversity of fungal microbiota was similarly affected (Li *et al*., 2014). Despite this preclinical evidence, alemtuzumab-induced intestinal barrier disruption is infrequent in clinical practice. However, a case of spontaneous pancolitis was described in a patient with multiple sclerosis treated with alemtuzumab recently (Vijiaratnam *et al*., 2016), and historically, the use of alemtuzumab in haematological malignancies has been associated with the development of diarrhoea and opportunistic intestinal infections (Goteri *et al*., 2006; Ronchetti *et al*., 2014).

Teriflunomide

Teriflunomide selectively and reversibly inhibits dihydroorotate dehydrogenase, leading to a reduction in the number of activated lymphocytes that enter the CNS (Miller, 2015). Teriflunomide could alter the microbiome and the host response to enteral pathogens. Treatment of porcine intestinal epithelial cells with teriflunomide led to reduced capacity to fight bacterial infection through suppression of STAT-6

signalling (Yi *et al.*, 2016). Teriflunomide could also directly inhibit *C. perfringens* growth *in vitro* (Rumah *et al.*, 2017). Animals treated with teriflunomide in a mouse model of EAE had fewer antigen-presenting cells in Peyer's patches as well as an increase in gut-specific CD39(+) Treg cells that could protect against EAE when used in an adoptive transfer regimen (Ochoa-Repáraz *et al.*, 2016).

Minocycline

Minocycline is a second-generation tetracycline that was first introduced over half a century ago. Besides its antibiotic effects, it also has anti-inflammatory, immune-modulating and anti-apoptotic properties, all of which have been proposed as possible pathways towards neuroprotection (Yong *et al.*, 2004; Giuliani *et al.*, 2005). A recent randomized, double-blind, placebo controlled trial showed that oral minocycline could delay the appearance of a new demyelinating events in patients with clinically isolated syndrome, as well as reduce the appearance of T_2 lesions in the brain (Metz *et al.*, 2017).

Minocycline's immune-modulating and anti-inflammatory properties have also been observed in intestinal tissues. In a chemically-induced colitis model in mice, minocycline reduced intestinal inflammation, mucosal injury, restored microbiota and preserved tight junction protein expression (Huang *et al.*, 2009; Garrido-Mesa *et al.*, 2011*a*, *b*). As an antibiotic, minocycline also alters the gut microbiome. A recent study evaluated the effects of various commonly used antibiotics, including minocycline, on the salivary and gut microbiome in 66 healthy adults. Antibiotic exposure led to reductions in health-associated butyrate-producing species as well as proliferation of potentially resistant strains in the gut microbiome, although the changes were more robust after amoxicillin and ciprofloxacin administration (Zaura *et al.*, 2015). Other studies have shown that some gut commensals such as *Bifidobacteria* and *E. coli* are susceptible to minocycline (Moubareck *et al.*, 2005; Kirchner *et al.*, 2014). Minocycline thus presents an intriguing option in dual modulation of the intestinal barrier function. It could have protective anti-inflammatory properties while also altering the composition of the gut microbiome.

Treating the diseased intestinal barrier

Current treatments for a diseased intestinal barrier are limited, but there are various interesting avenues of research. One of the main therapeutic targets are tight junctions. Larazotide acetate, also known as AT-1001, is a synthetic octapeptide related to the zonula occludens toxin produced by *Vibrio cholera*, developed as a treatment for coeliac disease. It acts locally to decrease tight junction permeability by blocking zonulin receptors and thus preventing actin rearrangement in response to stimuli, and *in vitro* it can stabilize tight junctions and decrease intestinal permeability (Paterson *et al.*, 2007; Gopalakrishnan *et al.*, 2012; Khaleghi *et al.*, 2016). However, clinical trials in coeliac disease have yielded conflicting results, despite showing a beneficial effect over intestinal permeability (Kelly *et al.*, 2013; Leffler *et al.*, 2012, 2015).

Another approach in improving intestinal barrier function is enrichment of the mucus layer, a strategy being explored in IBD (Stange, 2017). Lecithin, or phosphatidylcholine, accounts for the majority of the phospholipids in the intestinal mucus layer, and is available as a delayed release oral formulation. In randomized phase II controlled studies, delayed-release lecithin was proven to be clinically and endoscopically effective in ulcerative colitis, and phase III studies are underway (Stremmel and Gauss, 2013; Stange, 2017). Recent interest has also been placed on stem cell-based therapies to regenerate the intestinal epithelium, through luminal transplantation (Holmberg *et al.*, 2018), but these approaches are still in an experimental phase.

There has also been recent interest in the effects of vitamin D over intestinal barrier function and immune homeostasis (Dimitrov and White, 2017). In a model of experimental colitis, mice overexpressing vitamin D receptor in the intestinal epithelium show preserved intestinal permeability, reduced caspase expression and less induction of apoptosis (Liu *et al.*, 2013). Vitamin D also attenuates TNFα-induced apoptosis in human colonic cells through reduction of NF-κB activation and mucosal IKK kinase activity, thereby preserving barrier function (see Li *et al.*, 2015 for a review). Vitamin D signalling also preserves the mucosal barrier integrity by abrogating myosin light chain kinase dependent tight junction dysregulation during colonic inflammation through suppression of NF-κB *in vitro* (Du *et al.*, 2015). Cultured colonic samples from patients with ulcerative colitis have altered expression of the tight junction claudin as well as increased pro-inflammatory cytokine expression; these changes were reversed by incubation with vitamin D (Stio *et al.*, 2016). A recent small, randomized and placebo controlled study reported improvements in intestinal permeability [assessed by excretion of oral sugars (lactulose and mannitol were used as markers of small intestine permeability, sucrose as a marker of gastro-duodenal permeability, and sucralose as marker of combined small- and large-bowel permeability)] as well as serum immune markers in patients with IBD after vitamin D treatment (Raftery *et al.*, 2015). Vitamin D appears to be important in the regulation of the intestinal barrier function, a mechanism not yet thoroughly evaluated in multiple sclerosis research.

Probiotics have emerged as an interesting option in regulating intestinal barrier function, fuelled by research in both *in vitro* and *in vivo* models that show that some microbiota can stabilize the intestinal barrier (Bron *et al.*, 2017). However, small clinical studies in necrotizing

Table 1 Possible therapeutic interventions to improve barrier function

Intervention	Target
AT-1001 (larazotide)	Tight junction proteins
Lecithin	Mucus layer composition
Probiotics/faecal transplantation	Pleiotropic
Vitamin D	Epithelial and immunological homeostasis
Dietary/nutritional	Pleiotropic. 'High' short chain fatty-acid diet?

enterocolitis, irritable bowel syndrome and IBD have shown only modest effects. There are no large randomized, placebo controlled studies and there is no obvious standardization of the quantities and composition of a given therapeutic probiotic 'agent', making trials difficult (Bron *et al*., 2017). There has been growing interest in the use of faecal microbiota transplantation (the ultimate microbiome modification) for the treatment of patients with chronic gastrointestinal infections and IBD (Smits *et al*., 2013), with excellent results observed in *C. difficile* colitis. It is also a safe procedure. Its effectiveness in autoimmune diseases and multiple sclerosis is unknown at this time.

There are other sources of interest in probiotics in multiple sclerosis. Probiotic administration is known to modulate the immune response in the mouse EAE model. Different formulations have been shown to reduce EAE duration (Ezendam *et al*., 2008), inhibit the pro-inflammatory Th1/Th17 polarization (Kwon *et al*., 2013), induce IL-10 producing Treg cells (Ochoa-Repáraz *et al*., 2010*a*, *b*; Takata *et al*., 2011) and enhance CD103 expression in dendritic cells (Ochoa-Repáraz *et al*., 2010*b*), all while preventing, delaying or attenuating EAE. *E. coli* strain Nissle 1917 has been shown to reduce EAE-induced intestinal barrier dysfunction, while also reducing disease severity and beneficially modifying T cell functions (Secher *et al*., 2017).

Despite these encouraging studies, few clinical trials have been performed using probiotics in multiple sclerosis. In one early trial, investigators used the non-pathogenic helminth *Trichuris suis* (Fleming *et al*., 2011). Five newly diagnosed patients with relapsing-remitting multiple sclerosis were given *T. suis* orally for 3 months, and favourable trends were seen in MRI outcomes (reduction in enhancing lesions from baseline) and immunological assessments (increased IL-10). A recent double-blind, placebo-controlled trial randomized 60 multiple sclerosis patients to receive a probiotic capsule or placebo for 12 weeks (Kouchaki *et al*., 2017). Probiotic treatment mildly improved Expanded Disability Status Scale (an absolute 0.4-point difference) and depression and anxiety symptoms, reduced high-sensitivity C-reactive protein and improved other metabolic measures such as insulin sensitivity and high-density lipoprotein-cholesterol levels. Probiotics also downregulated the gene expression of some pro-inflammatory cytokines in patients' peripheral blood-derived mononuclear cells (Tamtaji *et al*., 2017). In these studies, the treatment was safe and tolerable, but follow-up was too short to show any meaningful benefit in radiological or clinical outcome measures. Nonetheless, the encouraging results seen in the EAE model will surely promote further clinical trial development.

SCFAs are bacterial fermentation products from indigestible diet components. The most common SCFAs are acetate, propionate and butyrate. SCFAs could have a beneficial effect over the intestinal barrier. Butyrate was shown to be able to accelerate tight junction protein assembly and preserve permeability in a single enterocyte layer *in vitro* model, a process mediated by AMP-activated protein kinase activity (Peng *et al*., 2009). SCFAs could also increase prostaglandin-dependent mucin expression in intestinal epithelial cells, enhancing their mucoprotective properties (Willemsen *et al*., 2003). In an EAE model, dietary SCFA ameliorated the course of EAE through expanded Treg cell populations in the lamina propria, through suppression of the JNK1 and p38 pathway (Haghikia *et al*., 2015). CD44 knockout mice that show attenuated EAE also have increased microbiota diversity and SCFA production in the gut (Chitrala *et al*., 2017).

Dietary interventions that increase the availability of SCFAs and reduce other types of fatty acids could be an interesting therapy in improving the intestinal barrier function in multiple sclerosis, with the additional possibility of beneficial immunological effects. However, evidence showing a benefit for any kind of dietary interventions in multiple sclerosis is scarce, despite widespread acceptance that a 'healthy' diet is probably best (Altowaijri *et al*., 2017; Esposito *et al*., 2017). Some probiotic species are also rich sources of SCFAs, suggesting the possibility of a combination approach.

Concluding remarks

The recent interest in the role of the gut microbiota in multiple sclerosis has not been accompanied by a similar interest in the intestinal barrier. The intestinal barrier is the physical and functional zone of interaction between the luminal microbiome and the organism, and it is also responsible for modulating multiple biochemical processes and immune modulation of the mucosa. It appears that besides dysbiotic changes in the gut microbiome, the intestinal barrier function is also altered both in EAE models and in patients with multiple sclerosis, but the precise consequences of this alteration are unclear. Evidence of CNS demyelination in gastrointestinal disorders where there is barrier breakdown and basic studies showing how the intestinal barrier homeostasis can directly influence microglia and neuroinflammation provide some insights. Furthermore, most disease-modifying therapies appear to also impact on the intestinal barrier and the gut

microbiome. To advance the understanding of this complex interaction, future studies will have to take into consideration the microbiome, the intestinal barrier and the downstream neuroimmunological changes to accommodate for them in a single integrative model. Both the precise mechanisms involved in the breakdown of the intestinal barrier, and the value, if any, of therapeutic modulation of the intestinal barrier in multiple sclerosis, also require further study.

Funding

C.R.C.L. holds the Lejoie-Lake Fellowship awarded by the Hotchkiss Brain Institute, K.A.S. is the Crohn's and Colitis Canada Chair in IBD Research at the University of Calgary, while V.W.Y. is a Canada Research Chair (Tier 1). The authors acknowledge operating grant support from the Canadian Institutes of Health Research, the Multiple Sclerosis Society of Canada, and the Alberta Innovates – Health Solutions CRIO Team program.

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NUTRITIONAL NEUROSCIENCE
2022, VOL. 25, NO. 8, 1633–1640
https://doi.org/10.1080/1028415X.2021.1885240

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Adherence to dietary guidelines is associated with better physical and mental quality of life: results from a cross-sectional survey among 728 Dutch MS patients

I. Evers [a*], M. Heerings[b*], N.M. de Roos[a], P.J. Jongen[c,d] and L.H. Visser [e,f]

[a]Division of Human Nutrition and Health, Wageningen University, Wageningen, Netherlands; [b]Department of Human Genetics, Radboud University Medical Center, Nijmegen, Netherlands; [c]MS4 Research Institute, Nijmegen, Netherlands; [d]Department of Community & Occupational Medicine, University of Groningen & University Medical Centre Groningen, Groningen, Netherlands; [e]Department of Neurology, St. Elisabeth-Tweesteden Hospital, Tilburg, Netherlands; [f]Department Ethics of Care, University of Humanistic Studies, Utrecht, Netherlands

ABSTRACT

Background: A healthy diet has been associated with less symptoms or progression of disease in multiple sclerosis (MS). However, whether specific diets are needed, or general healthy diet recommendations are sufficient is unknown.

Objective: To investigate the association between diet quality, use of diets, and quality of life (QoL) in men and women with MS.

Methods: Diet quality was measured with the Dutch Healthy Diet-index, which measures adherence to the Dutch Guidelines for a Healthy Diet. QoL was assessed with the MSQoL-54 questionnaire. A total of 728 people were included (623 women, 105 men). Multiple linear regression, stratified for gender, was used to analyse the data.

Results: In women with MS, an association was found between diet quality and both physical and mental QoL after adjusting for several confounders (Physical Health Composite Score (β=0.410; P=0.001); Mental Health Composite Score (β=0.462; P=0.002)). Similar results were less pronounced in men. Subjects following a specific diet had higher diet quality and QoL than subjects not following a diet.

Conclusion: Adherence to the Dutch dietary guidelines is associated with better physical and mental QoL, especially in women. Following an MS-specific diet may help to adhere to these guidelines.

KEYWORDS
Multiple sclerosis; diet; quality of life; nutrition; dietary guidelines; MS-specific diet; mental health; lifestyle

Introduction

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system. With a prevalence of 100 per 100,000 in Europe [1], MS is the most common demyelinating disease in high-income countries and one of the leading causes of disability in young adults [2,3]. Onset of disease is on average around 30 years of age [4] and MS occurs mostly in women, with a female-to-male ratio of 1.4–2.3 [5].

MS has a major impact on quality of life (QoL) [6], due to various common physical symptoms such as vision problems, spasticity, weakness and fatigue and psychological symptoms, like cognitive impairment and mood disorders [6]. For now, the goal of disease management is to minimise these symptoms and, if possible, improve function [7].

Patients with MS are often proactive and ask for guidance from their physician on modifiable lifestyle-factors that may slow MS disease progression [8]. Modifying lifestyle-factors may be important since it is known that vascular comorbidity, whether present at symptom onset, diagnosis, or later in the disease course, is associated with a substantially increased risk of disability progression in MS [9]. Questions about exercise and supplements are common, but most questions from patients are about their diet [8]. Although there is limited scientific evidence supporting the use of specific diets in the management of MS, there is a wide variety of 'MS cookbooks' and Web-based dietary advice [8,10]. These advices are mostly based on individual experiences and have not been scientifically tested [10].

There is, however, some scientific evidence that certain dietary patterns may be beneficial for patients with MS. One of these diets is the Mediterranean diet, which has been shown to reduce inflammatory markers in other autoimmune diseases and cardiovascular diseases

CONTACT I. Evers ilse.evers@wur.nl
*Equal contribution.
This article has been republished with minor changes. These changes do not impact the academic content of the article.
Supplemental data for this article can be accessed at https://doi.org/10.1080/1028415X.2021.1885240

and might therefore be interesting for MS patients [11]. Various MS-specific diets have been proposed as well. These diets include the Swank diet, the Jelinek diet and the modified Palaeolithic Wahls diet [12]. All diets include a high intake of fruits, vegetables and whole grains [11]. Alcohol consumptions is restricted in these diets [11]. Some evidence for reduction in complaints when using these diets has been published [11]. For example, a pilot study showed that a Palaeolithic diet may reduce perceived fatigue and increased mental and physical quality of life [13]. In a recent trial, a ketogenic diet and a Palaeolithic diet were compared [14]. This trial showed that participants consuming a ketogenic diet achieved nutritional ketosis, however, it was not associated with significant clinical improvements in fatigue and QoL, whereas the modified Palaeolithic diet was associated with significant clinical improvements [14]. Although some studies are available, the results are still inconclusive, and no diet has been shown to be superior to others [11]. One of the main limitations of the studies is a small sample size.

Next to this, information about the differences between patients following a diet and not following a diet and their diet quality and QoL is scarce. Moreover, most of the dietary studies in MS have been performed in the United States of America [12], and it would be interesting to see if similar results would be found in a European population. This cross-sectional study aims to investigate the association between diet quality and QoL in Dutch patients with MS and furthermore looks at the role of MS-specific diet use in this association.

Methods

Participants recruitment

Subjects were recruited from the volunteer database of the National Multiple Sclerosis Foundation, Rotterdam, The Netherlands and via social media, posters/flyers, MS nurses, and MS centres. An online survey was conducted between May 2017 and February 2018 and filled out by 745 respondents. For this survey, the online programme LimeSurvey™ was used. Eligibility was checked with the first three screening questions about age (18 years or older), diagnosis (by a neurologist) and for women pregnancy or lactation (these were excluded). Respondents who did not meet the criteria were automatically sent to the end of the questionnaire.

Participant characteristics

Via an online survey, the following sociodemographic characteristics were asked: age, sex, country of birth, education level, and living situation (number of persons in household). Questions about clinical characteristics were included, involving information about height, weight, comorbidities, smoking status, years of complaints of MS, age at MS diagnosis, type of MS, exacerbations, and progression of MS. Questions about treatment including current use of medication, exercise supervised by a professional, exercise not supervised, diet supervised by a professional, diet not supervised, and use of complementary and alternative medication.

Body mass index (BMI, kg/m2) was calculated as weight (in kg) divided by body height (in m) squared. Underweight, normal weight, overweight and obesity were classified using BMI cut-off points classified by the WHO: < 18.5 kg/m2 is underweight, 18.5–24.9 kg/m2 is normal weight, ≥25 kg/m2 is overweight and ≥ 30 kg/m2 is obesity [15].

Diet quality

Diet quality was measured with the Dutch Healthy Diet index (DHD-Index), a dietary quality score which includes eight diet components representing the Dutch Guidelines for a Healthy Diet [16]. For each component, the score ranges between zero and ten, resulting in a total score between zero (no adherence) and 80 (complete adherence). The eight components are vegetable, fruit, fibre, fish, saturated fat, trans fat, salt and alcohol. Threshold values (minimum score) and cut-off values (maximum score) of each diet component are shown in Table 1. Scores were calculated according to the method described by van Lee et al. [16]. The DHD-Index is a short version of an FFQ and takes around 10 min to complete [16]. The DHD-index is a validated questionnaire and shown to be capable of ranking participants according to their adherence to the Dutch Guidelines for a Healthy Diet [16]. The score reflects variation in diet components, and was originally based on two 24-hour recalls [16].

Quality of life

QoL was measured using an MS-specific questionnaire, called the Multiple Sclerosis Quality of Life-54 (MSQoL-54) [17]. It is based on the 36-items Short Form Health Survey with eighteen additional MS-specific items. The MSQoL-54 provides two scores: the Physical Health Composite Score (PHCS) and the Mental Health Composite Score (MHCS), both ranging from 0 to 100. The PHCS is a score based on the categories physical function, health perceptions, energy/fatigue, physical limitation, pain, sexual function, social function and health distress. The MHCS includes the categories health

Table 1. Diet components and Dutch dietary guidelines of the Dutch Healthy Diet index and their threshold values (minimum score) and cut-off values (maximum score).

Diet component (per day)	Dutch Guidelines for a Healthy Diet	Minimum score (=0)	Maximum score (=10)
1. Vegetables	Eat 150–200 grams of vegetables.	0 g	≥ 200 g
2. Fruit + fruit juices[1]	Eat 200 grams of fruit a day.	0 g	≥ 200 g
3. Fibre	Eat 30–40 grams a day of dietary fibre, especially from sources such as fruit, vegetables and whole-grain cereal products.	0 g/4.2 MJ	≥ 14 g/4.2 MJ
4. Fish[2]	Eat two portions of fish a week, at least one of which should be oily fish.	0 mg EPA = DHA	≥ 450 mg EPA + DHA
5. Saturated fatty acids	Limit saturated fatty acid consumption to less than 10 percent of energy intake.	≥ 15 en %	< 10 en %
6. Trans fatty acids	Limit mono trans-fatty acid consumption to less than 1 percent of energy intake.	≥1 en %	< 1 en %
7. Sodium	Limit consumption of table salt to 6 grams a day.	≥ 2520 mg	< 1680 mg
8. Alcohol	If alcohol is consumed at all, male intake should be limited to two Dutch units (20 gram ethanol) a day and female intake to one.	Male: ≥ 6 drinks Female: ≥ 4 drinks	Male: ≤ 2 drinks Female: ≤1 drink

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; en %: energy %; [1]A maximum of 100 g of fruit juice containing vitamin C and folate could be included. [2]Fish intake was estimated based on dietary fish fatty acids (EPA + DHA) and fish oil capsules.

distress, overall QoL, emotional well-being, role of limitations and cognitive function [17]. Scores were calculated based on the method described by Vickrey et al. [17]. Overall wellbeing was based on one of the questions within the MSQoL-54 questionnaire. 'Overall, how would you rate your own quality-of-life?'. This question could be rated by a score between 1 and 10.

Following a diet

Patients were asked whether they were following a diet or had made dietary changes for disease management. If patients were following a diet they were asked to choose that diet from the list, that was most closely to the diet they were following. Possible diet options were a 'low carbohydrate diet', 'high carbohydrate diet', 'high fibre diet', 'gluten free diet', 'sugar free diet', 'vegan diet', 'vegetarian diet', 'Atkins diet', 'Jelinek diet' and 'Paleo diet'. They also had the option to fill in 'others'. Only diets that were reported by a minimum of 20 patients are reported.

Data analysis

Data were analysed by using IBM SPSS Statistics version [22]. Normally distributed variables are presented as mean ± standard deviation (SD) and skewed variables as median with interquartile range (IQR). For categorical variables cases (n) and percentages (%) are presented. For continuous data an independent samples t-test was used. If data were nominal, a Chi-square test was performed. Data that were not normally distributed or measured on an ordinal scale were analysed using the Mann–Whitney U test. Significance was set to $p<0.05$. Based on experience and literature, differences in baseline characteristics between men and women were expected in diet quality scores but also in disease characteristics: men are more often diagnosed with progressive types of MS than women [18]. For these reasons, data were stratified for gender.

Sex-specific tertiles of the DHD-Index score were made, based on previous research [16]. These tertiles were used to check if all components of the DHD-Index contributed equally across the DHD-Index. Next to this, possible trends in lifestyle factors across the DHD-Index could be checked by using tertiles. Differences between groups were tested using a one-way ANOVA when normally distributed, not normally distributed variables were analysed using the Kruskal Wallis test. For the categorical variables the chi-square test was used to analyse the trend.

Differences between diet quality, mental QoL and physical QoL between patients following a diet or one of the specific diets was tested with an independent sample t-test. Following a diet was tested against not following a diet. Following one of the specific diets was tested against not following that specific diet. For example people following a 'High in fibre diet' were tested against all patients not following a 'High in fibre diet'.

Multiple linear regression was used to analyse the possible association between diet quality and QoL. Covariates which were considered to be confounders were age, gender, type of MS, duration of disease, body mass index (BMI), past and current smoking, level of education and physical activity. In the final model, only covariates that changed the crude model with more than 10% were included.

Ethical aspects

The study protocol was presented to the Medical Research Ethics Committee Brabant, Tilburg, the Netherlands, this committee declared that this study did not come within the scope of the Medical Research Involving Human Subjects Act (WMO) and therefore needed

no approval. The study was performed in agreement with the Declaration of Helsinki and the WMO. After having completed informed consent online, the patients received a personal code and logged on to the website.

Results

Characteristics of study population

A total of 745 subjects started the screening questionnaire. Seventeen were excluded: fifteen because they did not fulfil the inclusion criteria, two based on implausible values for height or weight. Baseline characteristics of the remaining 728 subjects, 623 women and 105 men, are shown in Table 2. Men had an average age of 53 years, women 45 years. In both genders, almost half of the subjects had a high education level and the majority of subjects had a healthy BMI. Over 80% of the subjects were not current smokers. Only one out of five subjects met the physical activity guideline of 30 min per day for 5 days per week. In many subjects, MS-related complaints started more than ten years ago followed by a diagnosis some years later. Women were diagnosed more often with the relapsing-remitting type of MS, where men more often had a progressive type of MS.

Table 2. Sociodemographic and clinical characteristics of 728 research subjects diagnosed with MS, stratified by gender.

Characteristics	Men	Women	*P*-values difference men and women
N	105	623	
Sociodemographic			
Age (years), mean ± SD	52.6 ± 9.9	45.4 ± 11.2	0.000
Country of birth,[1] n (%)			0.464
The Netherlands	99 (94.3)	591 (94.9)	
Western country	3 (2.9)	25 (4.0)	
Non-Western country	3 (2.9)	7 (1.1)	
Living situation			0.464
Alone	15 (14.3)	107 (17.2)	
Together	90 (85.7)	516 (82.8)	
Level of education[2], n (%)			
Low	22 (21.0)	114 (18.3)	0.519
Medium	33 (31.4)	201 (32.3)	
High	50 (47.6)	308 (49.4)	
BMI, n (%)			0.781
Underweight	1 (1.0)	9 (1.4)	
Normal weight	56 (53.3)	329 (52.8)	
Overweight	31 (29.5)	156 (25.0)	
Obese	15 (14.3)	126 (20.2)	
Smoking status, n (%)			
Current	10 (9.5)	73 (11.7)	0.514
Past	58 (55.2)	241 (38.7)	0.002
Never	37 (35.2)	309 (49.6)	0.001
Physical activity (≥30 min/day)[3], n (%)			0.136
<5 times per week	88 (83.8)	485 (77.8)	
≥5 times per week	17 (16.2)	138 (22.2)	
Clinical			
Start of complaints			0.054
<1 year ago	2 (1.9)	13 (2.1)	
1-3 years ago	3 (2.9)	62 (10.0)	
3-5 years ago	15 (14.3)	85 (13.6)	
5-10 years ago	27 (25.7)	167 (26.8)	
>10 years ago	25 (55.0)	296 (47.5)	
MS type, n (%)			0.038
CIS	2 (1.9)	9 (1.4)	
Benign	10 (9.5)	60 (9.6)	
Relapsing-remitting	35 (33.3)	380 (61.0)	
Primary progressive	30 (28.6)	46 (7.4)	
Secondary progressive	24 (22.9)	112 (18.0)	
Unknown	4 (3.8)	16 (2.6)	
Current use drugs, n (%)			0.087
Yes	47 (44.8)	335 (53.8)	
No	58 (55.2)	288 (46.2)	

MS: Multiple Sclerosis; SD: Standard Deviation; BMI: Body Mass Index; CIS: Clinically Isolated Syndrome; [1]Country of birth: based on definitions made by Statistic Netherlands (CBS: Centraal Bureau Statistiek) [2]Level of education: based on the standard classification of education in the Netherlands made by Statistic Netherlands [3]Physical activity: groups based on the guidelines for physical activity in the Netherlands made by the Dutch Health Organisation (Gezondheidsraad).

Diet quality

Women had a DHD-Index of 57.9, which was 2.9 points higher than the DHD-Index of men (p=0.005) (Table 3). Their higher score was mainly due to a higher score for the diet components vegetable, fibre and salt, meaning a higher or more frequent intake of vegetables and fibre and a lower intake of salt. In both men and women, significant trends from lowest to highest tertile of the DHD-Index were seen for all diet components ($p \leq 0.002$) (Supplemental Tables I and II), but especially for the components vegetables, fruits, fish and saturated fat. High adherence to trans-fat and alcohol guidelines was seen in all tertiles. In women, smaller differences between the tertiles were seen for salt than for men.

Women in the highest tertiles of the DHD-Index were more highly educated (p=0.002), had a normal

Table 3. Nutritional characteristics of 728 research subjects diagnosed with MS, stratified by gender.

Characteristics	men	women	*P*-values difference men and women
N	105	623	
Diet quality			
DHD-Index Total[1], mean ± SD	55.0 ± 11.7	57.9 ± 9.6	0.005
Vegetable	6.0 ± 3.0	7.0 ± 2.9	0.001
Fruit	6.8 ± 3.4	7.4 ± 3.0	0.094
Fibre	6.2 ± 2.1	6.6 ± 2.2	0.035
Fish	2.7 ± 3.7	2.1 ± 3.2	0.768
Saturated fat	8.0 ± 3.5	7.7 ± 3.4	0.560
Trans fat	9.3 ± 0.2	9.6 ± 0.8	0.336
Salt	7.0 ± 2.5	8.5 ± 1.4	0.000
Alcohol	9.0 ± 2.7	9.0 ± 2.5	0.941
Currently on a diet, n (%)	27 (25.7)	233 (37.4)	0.014
Willing to change diet, n (%)	98 (93.3)	591 (94.9)	0.520

MS: Multiple Sclerosis; SD: Standard Deviation; DHD-Index: Dutch Healthy Diet-Index **1** Per diet component the score ranges between zero and ten, resulting in a total score between zero (no adherence) and 80 (complete adherence).

Table 4. Association between DHD-index and Physical Health Composite Score or Mental Health Composite Score.

	Sample size	Crude model (β [1]± SE)		Fully adjusted model (β[1] ± SE)	
Type of score					
PHCS					
Men	105	0.351 ± 0.18	$p = 0.056$	0.233 ± 0.19	$p = 0.256$
Women	623	0.498 ± 0.12	$p = 0.000$	0.410 ± 0.12	$p = 0.001$
MHCS					
Men	105	0.511 ± 0.26	$p = 0.051$	0.419 ± 0.27	$p = 0.126$
Women	623	0.548 ± 0.14	$p = 0.000$	0.462 ± 0.15	$p = 0.002$

Note: Adjusted for age, level of education, smoking, physical activity and BMI. Stratification for gender.

DHD-Index: Dutch Healthy Diet-Index; BMI: Body Mass Index; **1** ß indicates change in PHCS or MHCS for one point change in DHD-Index; PHCS: Physical Health Composite Score; MHCS: Mental Health Composite Score

BMI ($p<0.001$), were not smoking ($p<0.001$) and met the guideline for physical activity ($p=0.004$) more frequently compared to people in the lowest tertiles of the DHD-Index (Supplemental Table III). No differences were seen for age and type of MS. In men, no significant differences in characteristics were found across tertiles of the DHD-Index, except for age ($p=0.038$) (Supplemental Table IV).

Wellbeing

Overall wellbeing was graded with a 6.8 out of 10 in men and 7.0 out of 10 in women. Mental Health Composite Scores (MHCS) of the MSQoL-54 questionnaire were higher than the Physical Health Composite Scores (PHCS), 67.2 (men) and 69.0 (women) vs 53.8 (men) and 56.6 (women) respectively. Although women had slightly higher scores, these differences were not significant.

Association diet quality and QoL

The DHD-Index score significantly predicted physical and mental QoL in univariate analysis in women: PHCS ($\beta=0.498$; $p<0.001$); MHCS ($\beta=0.548$; $p<0.001$). After adjusting for the confounders age, level of education, smoking, physical activity and BMI the association remained significant (Table 4). In univariate analysis in men, positive associations were seen as well, however not significant: PHCS ($\beta=0.351$; $p=0.056$); MHCS ($\beta=0.511$; $p=0.051$). This trend was weakened after adjusting for the confounders.

Specific diets for disease management

More than a third of the respondents indicated to follow a diet, which was most often restricted in carbohydrates (Table 5). Remarkably, followers of a diet had a 7-point higher DHD-index compared to patients not following a diet ($p<0.001$). Patients following a diet were more often female, higher educated, non-smokers and more physically active. The Jelinek diet, which is specific for MS patients, was mentioned by less than 5% of respondents, but had the highest score on diet quality: on average more than 10 points higher than patients not following a diet. The highest mental QoL was found in patients following a 'high fibre diet', whereas people following a 'vegetarian diet' had the highest physical QoL.

Discussion

Better adherence to dietary guidelines, measured as a higher score on the DHD-Index, was associated with better physical and mental QoL in women with MS after adjusting for multiple confounders. These results were less pronounced in men. Vegetables, fruits, fish and saturated fat were the main discriminating factors in the DHD-index, whereas trans-fat and alcohol were quite similar across all levels of the index in both sexes. Patients following a specific diet for the management of MS had higher diet quality and higher QoL than patients not following a specific diet.

Our findings in this sample of the Dutch population of MS patients confirm those of an international cross-

Table 5. Specific diets for managing MS and their diet quality, Physical Health Composite Score and Mental Health Composite Score.

Type of diet	N	Mean DHD-Index	*p*-values	PHCS	*p*-values	MHCS	*p*-values
No diet	468	55.0 ± 9.8	-	54.6 ± 17.1	-	68.2 ± 17.2	-
Following a diet[1]	260	62.0 ± 8.5	**<0.001**	59.1 ± 16.4	**0.001**	69.8 ± 17.0	0.230
Sugar free diet[2]	86	63.1 ± 6.8	**<0.001**	60.5 ± 15.3	**0.014**	72.3 ± 15.0	**0.038**
Low carbohydrate diet[2]	85	61.5 ± 8.2	**<0.001**	58.2 ± 14.9	0.215	68.2 ± 16.6	0.769
High fibre diet[2]	48	65.0 ± 8.3	**<0.001**	54.0 ± 15.7	0.347	74.5 ± 14.2	**0.006**
Paleo diet[2]	40	60.1 ± 8.1	**0.050**	60.3 ± 15.7	0.117	71.3 ± 15.0	0.327
Jelinek diet[2]	32	65.3 ± 8.2	**<0.001**	62.1 ± 16.9	**0.045**	67.6 ± 20.6	0.708
Gluten free diet[2]	31	62.9 ± 8.6	**0.002**	59.9 ± 15.1	0.222	69.3 ± 13.5	0.850
Vegetarian diet[2]	23	62.8 ± 8.6	**0.009**	65.7 ± 16.7	**0.007**	68.8 ± 17.8	0.982

[1]Following a diet is tested against not following a diet.
[2]Specific diet is tested against not following that specific diet.
MS: Multiple Sclerosis; DHD-Index: Dutch Healthy Diet-Index; PHCS: Physical Health Composite Score; MHCS: Mental Health Composite Score.

sectional study, in which diets that were characterised by ample amounts of vegetables, fruits, fibre and healthy fats were associated with better physical and mental health [19]. Similar positive associations were found between intake of fruits and vegetables and disability and symptom severity in a survey including almost 7000 MS patients in the U.S.A. [12].

The large sample size is a strength of our study. In addition, our findings in Dutch patients are comparable to those of other studies and clinically relevant. In other studies using the MSQOL-54, a 5-point change was considered to be clinically relevant for patients with MS [20]. The association in our study implies that for a 5-point change in the MSQol-54, a change of 10–12 point on the DHD-Index is needed. Presuming a causal relationship, this change in DHD-Index is feasible, since it can be achieved by improving on at least two diet component (e.g. stop drinking alcohol and start eating fish or eat more fruit and vegetables).

Our study population is skewed towards a higher educated population with a healthy lifestyle, which makes our findings less generalisable. However, other studies in patients with MS showed similar characteristics for their patients group, when looking at education levels, smoking status and physical activity [20]. When comparing the participants to studies in the healthy Dutch population, comparable results are seen as well. This is reflected in the findings that diet quality scores in the population with MS resemble the diet quality scores of the healthy Dutch population (mean DHD-Index healthy population 59.2 ± 11.2; MS population 57.5 ± 9.9) [16].

In our study, different results for men and women were found. Only recently the importance of studying both sexes separately has gained attention in the field of disease susceptibility and progression, in MS as well as other diseases [18]. Women have an increased susceptibility for MS and an increased rate of disability progression than men, suggesting a gender effect in the mechanism of the disease [18]. Indeed, in our study women were diagnosed more often than men with the relapsing-remitting type of MS, where men more often had a progressive type of MS. In addition, although not significant, around 10% more women than men reported the use of medication in our study. A review on medication use in patients with MS found a positive association between medication use and quality of life [21]. Both types of MS and medication may explain the different findings between men and women in our study. Next to this, our findings showed that significantly more men than women had smoked in the past. Since smoking is an evidence-based risk factor for developing the disease, past smoking might also affect further progression in disease. Sex differences, as in any disease, may be related to sex hormones. However, different studies have shown that the worse disease progression of men compared to women, is probably related to other factors than testosterone [18]. Independent studies show that testosterone is protective in men with MS [18]. In developed countries, cooking (including skills, frequency and involvement in time) has been associated with dietary benefits, such as consumption of healthier food groups and better adherence to dietary guidelines [22]. When looking at cooking at home, positive associations have been shown with being female, married, older, greater time availability and more [22]. Therefore, this may be another factor why women have higher diet quality compared to men. Lastly, the smaller sample size for men decreased the chance of finding significant associations. Thus, although it seems plausible that the relation between diet and QoL is different in men and women, this needs more substantiation in studies that include more men.

About a third of the patients reported to follow a diet to manage their disease. Remarkably, these patients on average scored higher on diet quality than patients not following a specific diet. Patients following a 'high-fibre' diet scored high on mental QoL. Recently, the interest in how diet influences brain function via the gut microbiome and the role of a high fibre diet has gained attention [23]. Although more research is needed into these specific pathways, our results support this promising field of interest. Patients following the MS-specific Jelinek diet had the highest diet quality score, which was 10 points higher than patients not following a diet. This diet focusses on high intake of omega-3 fatty acids and low omega-6 fatty acids. Consequently, fish, fruit and vegetable intake are important in this diet, which are categories in line with the DHD-Index. Thus, although health professionals generally do not advocate diets for MS, following any of these popular diets might improve adherence to the Dutch dietary guidelines and should therefore not be discouraged.

Causal inference cannot be made based on cross-sectional studies. It is also possible that poor physical or mental health leads to food choices that result in a poorer diet quality. This reversed causality is considered to be the main limitation of our study. Likewise, it could be conceived that diet quality and QoL are both causally linked to a third factor, like personality traits or financial situation. E.g. financial means may impact access to foods or physical activity, which could have negative impact on QoL. In addition, patients with higher diet scores could be better in self-management. The idea of having control of your own life, could help in the perception of your QoL. This is in line with our observation

that patients who followed a specific diet, had higher QoL scores than patients not following a diet. Fatigue could also be a third factor since it is a frequently reported symptom of MS which may reduce QoL. People who experience more fatigue, could be less capable of preparing fresh meals and therefore have a lower diet quality.

Generalisability of our research results to the whole (Dutch) MS population might be another shortcoming in our study. Selection bias might be present; it is imaginable that people who are already interested in (and complying with) diet and a healthy lifestyle are more likely to fill out a questionnaire on this topic. In our study, patients who followed a diet were more often women, highly educated and physically active, all of which are attributes associated with better diet quality. Conversely, filling out a survey may be burdensome, especially for patients in a further state of the disease or with a lower QoL. It could therefore be possible that such patients decided to not fill in the questionnaire. Although patients were given the option to save the answers and resume at a later moment, this might still play a role in our results.

Conclusion

A statistically significant positive association was found between adherence to the Dutch dietary guidelines and physical and mental QoL in women with MS. These results were less pronounced in men. One third of the patients was following a diet to manage MS, which seemed to help them to better adhere to the dietary guidelines. As this was a cross-sectional study, longitudinal studies and randomised controlled trials are needed to test whether starting an MS-diet or taking measures to better adhere to the general dietary guidelines improves QoL and reduced disease activity and slows disease progression.

Acknowledgement

The authors would like to give special thanks to the National Multiple Sclerosis Fund for their support in this research.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

ORCID

I. Evers http://orcid.org/0000-0001-9422-8281
L.H. Visser http://orcid.org/0000-0001-6695-2432

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ORIGINAL RESEARCH
published: 16 June 2022
doi: 10.3389/fnut.2022.882426

Eating Hubs in Multiple Sclerosis: Exploring the Relationship Between Mediterranean Diet and Disability Status in Italy

Federica Felicetti[1], Silvia Tommasin[2,3], Maria Petracca[2], Laura De Giglio[2†], Flavia Gurreri[2], Antonio Ianniello[1,2], Riccardo Nistri[1], Carlo Pozzilli[1,2] and Serena Ruggieri[2,3]*

[1] MS Center, Sant'Andrea Hospital, Rome, Italy, [2] Department of Human Neurosciences, Sapienza University of Rome, Rome, Italy, [3] Neuroimmunology Unit, IRCSS Fondazione Santa Lucia, Rome, Italy

OPEN ACCESS

Edited by:
Christelle Guillet,
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United States

***Correspondence:**
Serena Ruggieri
serena.ruggieri@uniroma1.it

†Present address:
Laura De Giglio,
Neurology Unit, San Filippo Neri Hospital, Rome, Italy

Specialty section:
This article was submitted to Clinical Nutrition, a section of the journal Frontiers in Nutrition

Received: 23 February 2022
Accepted: 25 May 2022
Published: 16 June 2022

Citation:
Felicetti F, Tommasin S, Petracca M, De Giglio L, Gurreri F, Ianniello A, Nistri R, Pozzilli C and Ruggieri S (2022) Eating Hubs in Multiple Sclerosis: Exploring the Relationship Between Mediterranean Diet and Disability Status in Italy. Front. Nutr. 9:882426. doi: 10.3389/fnut.2022.882426

Background: Multiple Sclerosis (MS) is a complex disease in which multiple factors contribute to disability accrual. Mediterranean Diet (MeDi) has shown beneficial effects across neurodegenerative diseases. We hypothesize that specific food habits, rather than global adherence to MeDi, might impact on MS. We aimed to (i) evaluate differences in adherence to MeDi between people living with MS (PwMS) and healthy controls (HC); (ii) characterize eating patterns in PwMS and HC, identifying the most influential MeDi items for each group by the use of network analysis; (iii) explore the relationship between patients' eating habits and disability.

Materials and Methods: In this cross-sectional study, we consecutively recruited 424 PwMS and 165 matched HC. Data were obtained through the administration of self-reported questionnaires. Expanded Disability Status Scale (EDSS) and Fatigue Severity Scale (FSS) were evaluated in the MS population. We performed between-groups comparisons via unpaired two-sample *t*-test and X^2 test as appropriate. We calculated food networks in both MS cases and HC using and tested the association between hub nodes and disability. Finally, we conducted a *post-hoc* analysis, investigating the relationship between food items, lifestyle factors (smoking, exercise) and clinical outcomes.

Results: Most participants adhered sufficiently to MeDi. Exploring each group separately, fruit, vegetables, cereal, and fish were identified as hubs in PwMS, while meat and alcohol were identified as hubs in HC. Hubs were all inter-correlated, indicating that eating habits of PwMS include a large intake of all the foods identified as hubs. EDSS was predicted by the intake of vegetables (beta $= -0.36$, $p < 0.03$) and fish (beta $= -0.34$, $p < 0.02$). The model including smoking pack/year, International Physical Activity Questionnaire (IPAQ) score and intake of "negative foods" predicted 6% of the variance in EDSS ($p < 0.001$), while the model including smoking pack/year and IPAQ score predicted 4% of the variance in FSS ($p < 0.001$).

Conclusions: We identified a sufficient adherence to MeDi in our population. PwMS showed overall a healthier dietary pattern than HC. Vegetables and fish intake were associated with disability outcomes. Future longitudinal studies applying integrated approaches are needed to understand lifestyle added value to the use of standard pharmacological therapies.

Keywords: multiple sclerosis, Mediterranean Diet, lifestyle, disability, food network analysis

INTRODUCTION

Multiple Sclerosis (MS) is a complex disorder with genetic, immunological and environmental factors contributing to the disease onset and evolution. Among environmental factors, viral infections, vitamin D deficiency, childhood obesity, smoking, incorrect dietary habits, and vascular risk factors might play a role in the development of MS (1). Whether dietary habits and lifestyle affect the course of MS is still a matter of discussion and established MS therapies are not usually integrated by specific indications on diet. Nonetheless, the known effects on the disease evolution of migration, low vitamin D levels and obesity during adolescence make a strong case for the relevance of dietary habits in MS (2). A recent study based on web interviews provided data on dietary behavior of more than 7,000 MS patients, demonstrating a strong association between healthy dietary habits, better physical and mental outcomes and lower level of disability (3). On the other hand, unhealthy lifestyle habits and their consequences, such as being overweight or obese, smoking and sedentary life, are common in MS population with unfavorable sequelae (4). Among healthy dietary regimens, the traditional Mediterranean Diet (MeDi), has shown beneficial effects across neurodegenerative diseases (5). Dietary supplementation and education on food intake, with a particular focus on MeDi components, has shown a positive impact on quality of life and cognitive performance in patients with Alzheimer's disease (6), on motor symptoms in Parkinson's disease (7) and on global disability in amyotrophic lateral sclerosis (8). Indeed, a modified MeDi approach has recently been associated to improvements in fatigue and other MS symptoms, as well as disability (9). MeDi has shown promising results not only on health-related issues but also on cognitive performance sustained by brain volume preservation in MS. Particularly in a cross-sectional study of patients with early MS, higher hybrid MeDi score correlated with preserved thalamic volumes (10) a site known to be crucial for cognitive decline (11). Such beneficial effects are likely mediated by the antioxidant components of MeDi, counteracting the oxidative stress secondary to mitochondrial dysfunction, which represent a pathological hallmark in all neurodegenerative disorders (12, 13). MeDi is characterized by consumption of fruit, vegetables, whole grains, legumes, nuts, lean fish, dairy products, white meat, small quantities of red meat, moderate consumption of alcohol and extra virgin olive oil as a fat source. These foods not only exert an antioxidant action due to their composition but can positively influence the function of immune cells in MS, probably favoring a shift toward an anti-inflammatory profile (14).

Given these premises, MeDi seems a good candidate for a dietary intervention in People Living with MS (PwMS). However, before suggesting specific dietary indications, a deeper knowledge of the dietary patterns commonly followed by PwMS, should be acquired. This is not trivial as, even though Italy belongs to the Mediterranean basin, a region where high level of adherence to MeDi is expected, surveys on eating behavior of adult population revealed that only one third of the population had an adequate intake of vegetables and fish, and the energy intake from saturated fats and sugars was globally very high (15).

Additionally, even in populations showing an overall good adherence to MeDi, relative consumption of specific foods, as well as the relationship between different food categories within the diet (i.e., consumption of one food driven or associated to the consumption of other foods) might differ across subgroups.

We hypothesize that the consumption of specific food items, or their eating pattern, rather than the global adherence to MeDi (16), might characterize MS patients and potentially affect their disability outcomes. Indeed, when studying diet behaviors, it is crucial to evaluate the multifaceted interdependence of foods in the habitual diet. In this context a recent work has implemented network science tools to identify novel diet patterns in prodromal dementia (17), showing how network methods may progress our knowledge of associated risk factors for complex disease as dementia and MS. With network analysis we can recognize eating hubs (i.e., items that are consumed in association with many other items within the dietary regimen), thus highlighting complex relations, hidden in the eating behavior, that may be related to patients' clinical status. This analysis would be complementary to the wide investigations on dietary habits, that have been performed and are still ongoing, and that so far have elucidated the relation between incorrect dietary behavior and MS poor clinical status and progression (3).

Given these premises, we set out to explore dietary patterns and their relationship with disability outcomes in a sample of PwMS followed in a large tertiary center in Central Italy, implementing a novel network analysis approach. Additionally, as diet is only one of the lifestyle habits potentially affecting disability outcomes in MS, our analysis was complemented by the exploration of aspects such as smoking and physical exercise (9).

Specifically, the aims of this work were (i) to evaluate differences in adherence to MeDi, intake of specific food, lifestyle habits between PwMS and HC; (ii) to describe the dietary pattern of PwMS and HC identifying the most influential demographic or MeDi items that characterize each group's eating habits (i.e., eating hubs); (iii) to explore the relationships between those hubs and disability outcomes.

METHODS

Participants

Participants older than 18 at the time of screening were prospectively enrolled at the MS Center of the Sant'Andrea Hospital in Rome between March 2018 and August 2019. Participants with a diagnosis of MS according to most recent revised McDonald criteria 2017 (18) regardless of disease phenotype were recruited together with their family members and hospital staff to act as control group. The ethical committee board of Sapienza University of Rome at Sant'Andrea Hospital provided approval for the project. Informed, written consent has been obtained in all participants.

Exclusion criteria for both groups were: (1) diagnosis of metabolic diseases; (2) mental or psychiatric diseases; (3) pregnancy or breastfeeding; (4) food allergies and food intolerances; (5) ongoing vegetarian or vegan diet; (6) any condition preventing participants to provide adequate answers to the administered questionnaires; (7) ability to walk without support/aid. For latter exclusion criteria, PwMS with and Expanded Disability Status Scale (EDSS) (19) higher than 5.5 were not recruited.

Study Procedures

The following information were collected in PwMS and HC: demographic data, pharmacological therapies, smoking (number of cigarettes smoked per day and duration of habit, i.e., smoking pack/year), telephone and email contacts.

We also evaluated the following anthropometric indicators: weight and height to calculate Body Mass Index (BMI kg/m^2), waist (WC) and hip circumferences, waist-hip ratio (WHR).

For BMI, we used World Health Organization (WHO) cut-points to asses underweight ($<$18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obese ($>$30 kg/m^2). For men WC$\geqslant$94 cm and for women WC$\geqslant$80 cm are cut-points used to assess cardiovascular risk in the general population (20). Fat distribution is further defined as WHR above 0.90 for males and above 0.85 for females (21).

Subsequently, the following questionnaires were administered:

- MeDi adequacy questionnaire (22). This is a nine-question test, one for each food group (fruit, vegetables, legumes, cereals, fish, meat and cold cuts, milk and derivatives, olive oil, alcohol). For each question there are three different answers, regarding the frequency intake of those particular foods, daily or weekly, with a score from 0 to 2, begin 0 = no intake and 2 = more than once a week, referring to the last month. Adding all the results, we evaluated the adequacy to MeDi (0–4 not adequate; 5–9 poorly adequate; 10–15 sufficiently adequate; 16–18 completely adequate). Moreover, we computed the frequency of intake for each food item and grouped food categories as "positive" (fruit, vegetables, legumes, cereals, fish, olive oil) vs. "negative" (meat and cold cuts, milk and derivatives, alcohol): the higher the score in the "positive" group the better the diet of the participant, while the higher score in the "negative" group the worse the nutrition habits.
- The reduced form of the International Physical Activity Questionnaire (IPAQ) assessing the engagement in physical activity (23). The questionnaire is divided into 4 sections in which intense activities, moderate activities, walking and sitting are distinguished. For each of these categories the participant needs to specify how many days per week the various activities had been carried out and the total minutes of activity in one of those 7 days referring to the previous week. According to the scores obtained in the test, the person falls into three different groups that specify the levels of physical activity: inactive (lower score 700 Met); sufficiently active (score between 700 and 2,519 Met) and active or very active (score higher than 2,520 Met).

For each PwMS, the respective level of disability as measured by EDSS (and fatigue in terms of Fatigue Severity Scale (FSS) (24) were evaluated the same day of questionnaire completion.

PwMS were dichotomized into two subgroups according to their EDSS score: no disability (EDSS $\leq$1.5; i.e., no or minimal signs in more than one functional system) and disability (EDSS $\geq$2).

Network Analysis

To identify what demographic or MeDi items were most influential for the eating habits of PwMS and how they were linked to disability and fatigue, we performed a network analysis. We created a structure depicting the connections, namely edges, among all the variables of interest, namely nodes, such as age, sex, and MeDi items (fruit, vegetables, legumes, cereals, fish, meat, dairy, olive oil, alcohol). Thanks to this representation, we were able to identify the hubs, that are the most connected variables, thus the variables with the highest amount of connections with the other variables. First, to generate the edges connecting the nodes of the network, we built a mutual information matrix, representing the relation among age, sex, and MeDi items for both patients and HC (17). The mutual information matrix was selected to describe the relation among age, sex and MeDi items, as it provided information on the mutual dependence between couples of variables, without any a priori assumption of linear association among them. Since the investigated variables were all categorical except for age, the continuous variable age was grouped by ranges of years and devised in 5 classes (from class 0 if age $<$20 years to class 4 if age$>$49 years and the other classes covering 10 years each) to build the mutual information matrix.

Then, we investigated the difference of age, sex and MeDi items association between PwMS and HC We obtained 1,000 random matrices by reshuffling, 1,000 times, values taken from the information matrices of PwMS and HC, and placing them into two matrices, that had the same dimensionality of the mutual information matrices but casual entries, and that were subtracted one from the other. Then, we averaged these 1,000 random matrices and compared the result with the difference between the mutual information matrix of HC and that of PwMS, by means of a Z-score. Z-scores were considered significant at alpha = 0.05 if Z$>$|1.96|.

Moreover, on the mutual information matrices of both PwMS and HCs, we calculated the hubs. Analytically, in a network,

hubs are the variables with the degree larger than the average plus a standard deviation. The degree of an item is the number of its neighbors, e.g., of the number of links incident upon it. Network analysis was implemented in the open-source R environment (https://www.r-project.org/. For a flowchart of the network analysis see **Figure 1.**

Statistical Analysis

All values are presented as mean (standard deviation) or median [range], as appropriate. Normal distribution assumption was checked for continuous variables by using Shapiro-Wilk tests.

Unpaired two-sample *t*-test and X^2 test were used to compare demographic, clinical, anthropometric indicators, adherence to MeDi, frequency of food intake and IPAQ scores between PwMS MS and HC, as well as between PwMS with different levels of disability, as appropriate.

To test the association between hub nodes from network analysis and disability, a multivariate logistic regression was conducted. Specifically, we included hubs (obtained by network analysis) as independent variable and scores of either disability or fatigue as dependent variable. Classes of disability were devised as no disability (class 0, EDSS$<=$ 1.5), or disability (class 1, EDSS$>$1.5). Similarly, classes of fatigue were devised as no fatigue (class 0, FSS lower than the median value) or fatigue (class 1, FSS higher than the median value). Initially, we performed a sensitivity analysis to evaluate the minimum regression coefficient (beta) relative to sample size, considering alpha = 0.05 and power = 0.90. In multiple regression, collinearity exists if one variable is linearly predicted from the other and it may affect the reliability of the calculation of regression coefficients. Therefore, we checked for collinearity via Spearman correlation and removed correlated variables ($p < 0.05$ not corrected for multiple comparisons to be conservative at the most). However, for completeness, also association between dependent variables and removed hubs was calculated as bivariate logistic regression. Significance was reached if $p < 0.05$.

Finally, as the network analysis identified as hubs only items pertaining to the "positive foods," and did not include lifestyle factors other than diet, we conducted a *post-hoc* analysis, investigating the relationship between "negative foods," lifestyle factors (smoking, exercise) and clinical outcomes (EDSS and FSS) via hierarchical regression.

Analyses were conducted with the Statistical Package for Social Sciences 25.0 (SPSS, Chicago, IL, USA). Network statistical analyses were implemented in the open-source R environment (https://www.r-project.org/. Sensitivity analysis was performed with G-power (https://stats.idre.ucla.edu/other/gpower/.)

RESULTS

Participants in the Study

From an initial screening of 571 PwMS we enrolled 424 (74%) participants. Among the excluded patients there were 52 PwMS with metabolic diseases (9%), 47 with food allergies or food intolerances (8%), 19 people following a vegetarian or vegan diet (4%), 13 women in a gestational or breastfeeding state (2%), 12 (2%) have not been included due to incomplete data and 4 (1%) patients had psychiatric comorbidities. Two hundred and eighty four participants were screened as HC, of which 165 people (58%) were found to be suitable for study inclusion. Among the excluded participants there were 30 people with metabolic diseases (10%); 48 with food allergies or intolerances (17%); 18 people following a vegetarian or vegan diet (6%), 9 women in a gestational or breastfeeding state (3%), 7 have not been included due to incomplete data (3%) and 7 patients had psychiatric comorbidities (3%). For a flowchart of the study participants please see **Figure 2**.

Table 1 summarizes the main demographic characteristics and anthropometric measurements of the two examined groups. PwMS showed higher values of hip circumference compared to HC ($p < 0.014$), while there was a trend for waist circumference ($p < 0.069$) and no differences in terms of WHR and BMI. Among study participants only men in the HC group had BMI values belonging to the overweight range. No differences in terms of frequencies among the two groups were detected (**Figure 3**). Male HC subjects had WHR values above the cut-off, while all the other participants were normal weighted with normal WHR values. All subjects in our study were within the WC cut-off values.

Table 2 shows the main clinical characteristics and anthropometric measurements of the PwMS subgroups. PwMS in the disability group were older, had longer disease duration as well as higher FSS scores compared to PwMS in the no disability group. Waist circumferences and WHR were significantly different among the two subgroups.

Adherence to MeDi, Intake of Specific Food, Lifestyle in PwMS and HC

The majority of participants in each group adhered sufficiently to MeDi (60% in the group of PwMS and 56% in HC group). In the PwMS group, only one participant had an inadequate diet, while 38% poorly adequate and 2% completely adequate diet. Among HC, 2% had inadequate adherence to MeDi, 41% of participants showed poor adherence, while 1% followed a completely adequate diet. There were no significant differences in terms of adequacy to MeDi between PwMS and HC. Participants less adherent to MeDi showed higher smoking pack/years values in the whole sample ($p < 0.001$) as well in the PwMS ($p < 0.001$).

When we explored differences in terms of food intake frequency, we observed a tendency to a healthier diet in the group of PwMS, as they consumed more frequently fish and less frequently alcohol than HC ($p < 0.001$) (**Figure 4**).

PwMS showed overall lower IPAQ scores (2227.21 $\pm$ 3587.26) compared to HC (3701.33 $\pm$ 8338.35) [$p = 0.03$]. **Figure 5** shows the percentage of participants for each category according to groups.

In the group of PwMS there was a greater proportion of participants with a long-time smoking habit compared to HC (4.2 $\pm$ 8.6 vs. 1.0 $\pm$ 3.1, $p < 0.01$).

There were no differences in terms of adherence to MeDi between the two PwMS subgroups. However, when we compared the frequency of food intake, a greater proportion of participants

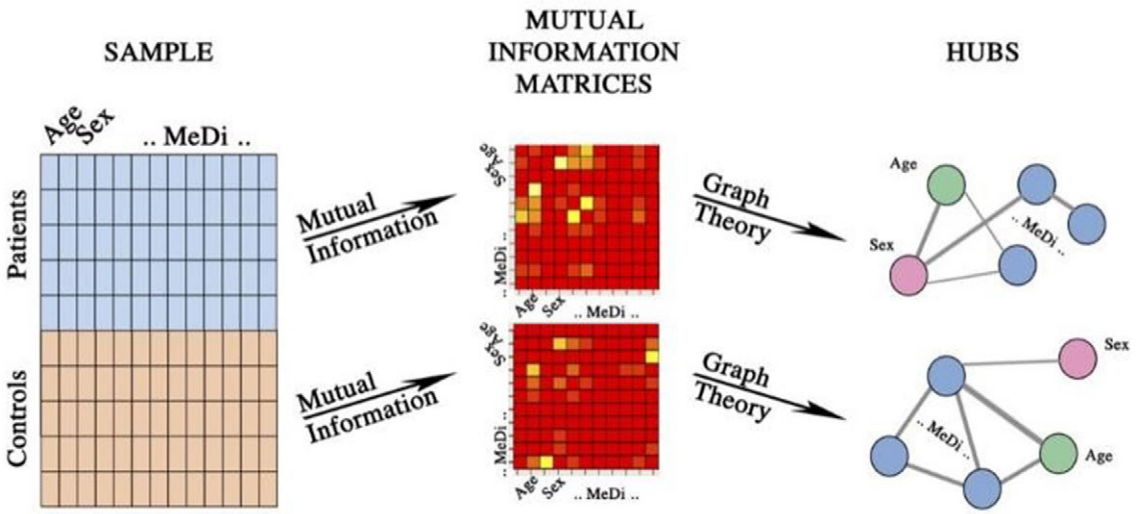
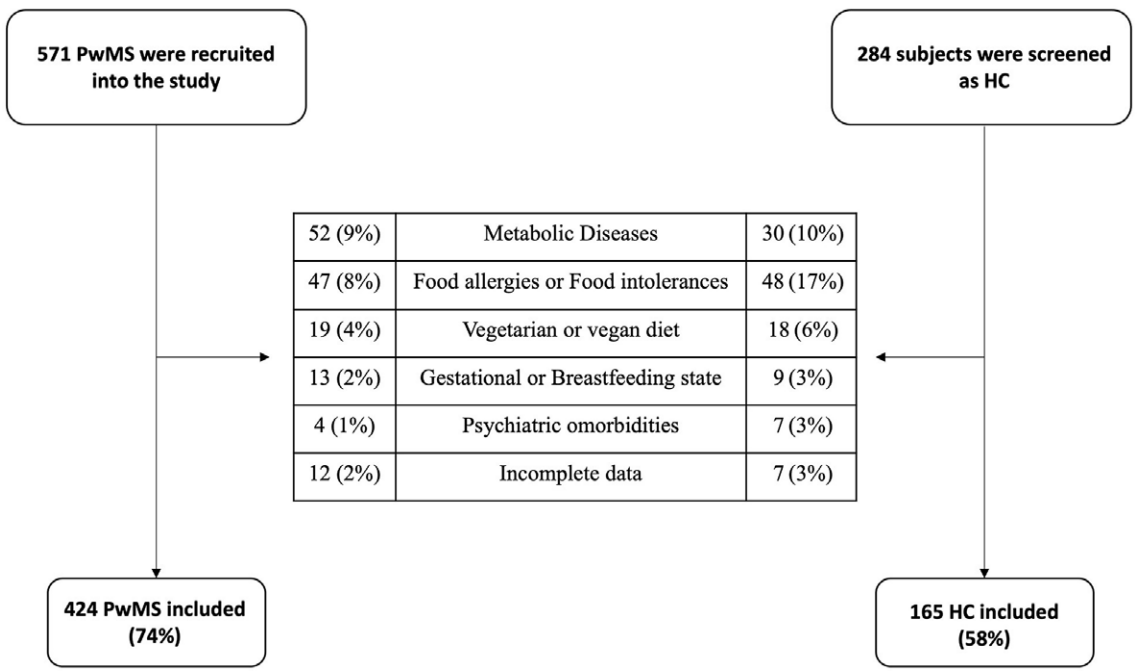


FIGURE 1 | Flowchart of the network analysis. Age, sex, and Mediterranean Diet items, such as fruit, vegetables, legumes, cereals, fish, meat, diary, olive oil, and alcohol, were collected for both people living with MS and healthy controls. From the two datasets the mutual information matrices were calculated and hubs were identified via graph theory.



52 (9%)	Metabolic Diseases	30 (10%)
47 (8%)	Food allergies or Food intolerances	48 (17%)
19 (4%)	Vegetarian or vegan diet	18 (6%)
13 (2%)	Gestational or Breastfeeding state	9 (3%)
4 (1%)	Psychiatric omorbidities	7 (3%)
12 (2%)	Incomplete data	7 (3%)

FIGURE 2 | Flowchart of the participants in the study. PwMS, People Living with MS; HC, Healthy Controls.

in the no disability subgroup consumed fish more frequently than patients in the disability subgroup ($p < 0.01$).

PwMS in the no disability subgroup engaged in physical activities more often and more intensely compared to those with disability (2667.8 ± 4263.6 vs. 1814.7 ± 2757.7, $p = 0.014$).

PwMS in the no disability group had lower values of smoking pack/years compared to those with disability (2.9 ± 6.4 vs. 5.5 ± 10.1, $p < 0.002$).

Food Network Analysis

We found no difference between mutual information matrices of PwMS and HC (**Figure 6**). When exploring each group separately, the network of PwMS identified fruit, vegetables, cereal and fish as hubs (**Figure 7A**), while HC's network identified meat and alcohol as hubs (**Figure 7B**).

Beta $= |0.32|$ was the minimum beta evaluable with a sample of 424 PwMS. Hubs were all inter-correlated, as shown in **Table 3**.

TABLE 1 | Demographical and anthropometric variables of the study participants.

	People living with MS (*N* = 424)	Healthy Controls (*N* = 165)	*P*-values
Female/Male (%)	284 (67)/140 (33)	112 (68)/53 (32)	0.8
Age, years [range]	42.6 (11.0) [18-74]	40.9 (14.5) [18-77]	0.127
BMI, kg/m^2	23.8 (3.9)	23.7 (3.5)	0.68
Female	23.3 (4.3)	22.9 (3.4)	0.33
Male	24.8 (3.0)	25.3 (3.1)	0.29
Waist circumference, cm	83.2 (12.3)	81.0 (14.8)	0.069
Female	80.0 (12.3)	76.8 (13.2)	**0.024**
Male	89.6 (9.5)	89.8 (14.2)	0.90
Hip circumference, cm	100.7 (8.8)	98.4 (13.2)	**0.014**
Female	100.4 (9.9)	98.7 (13.4)	0.18
Male	101.3 (5.6)	97.7 (12.6)	**<0.01**
Waist-hip ratio (WHR)	0.82 (0.08)	0.82 (0.1)	0.8
Female	0.79 (0.07)	0.78 (0.09)	0.10
Male	0.88 (0.07)	0.91 (0.08)	**0.022**

Values are expressed as mean. Standard Deviation (SD) is indicated in the round brackets. The minimum and maximum values of the measurements are indicated in the square brackets [range]. P-values refer to comparisons between groups; significance was reached if $p < 0.05$ and expressed in bold.

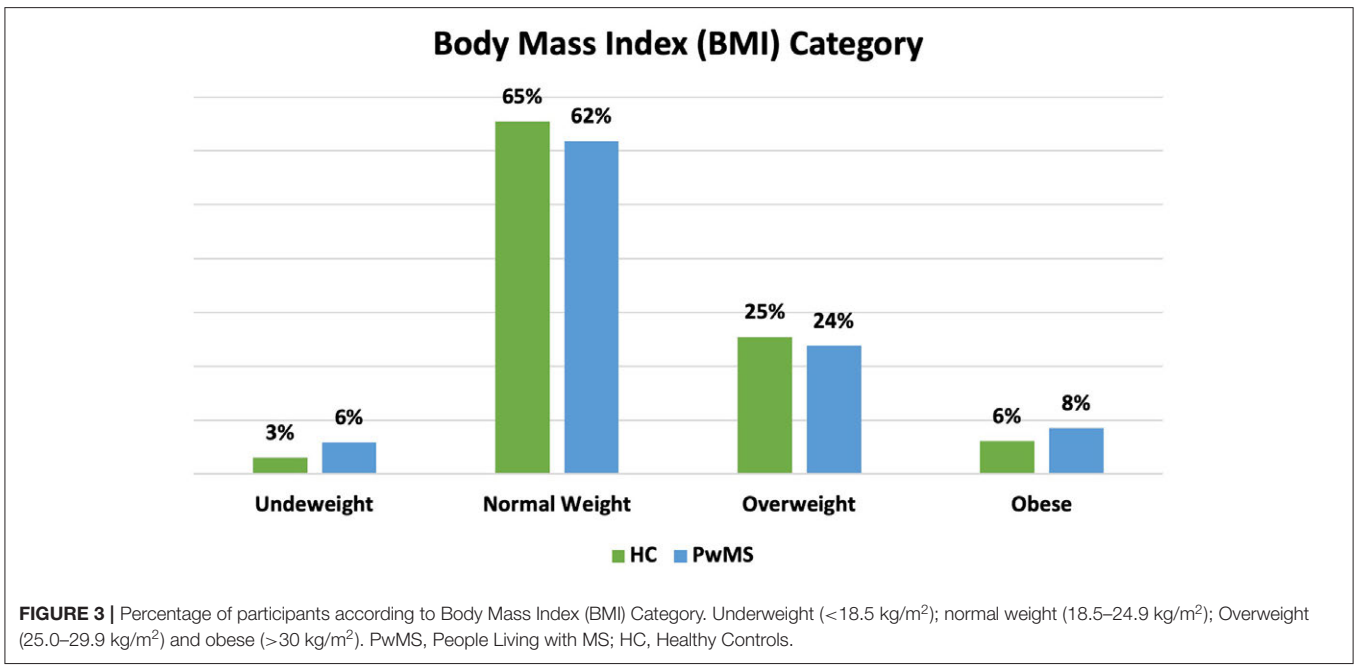


FIGURE 3 | Percentage of participants according to Body Mass Index (BMI) Category. Underweight (<18.5 kg/m^2); normal weight (18.5–24.9 kg/m^2); Overweight (25.0–29.9 kg/m^2) and obese (>30 kg/m^2). PwMS, People Living with MS; HC, Healthy Controls.

Significant positive Spearman correlations show monotonic association between variables, thus that the increase of one is paralleled by the increase of the other, thus eating habits of PwMS include a large intake of all the "positive foods" identified as hubs.

We found no difference between mutual information matrices of PwMS subgroups classified by EDSS or FSS.

Because of the collinearity, we did not perform a multiple regression including all the hubs in the same model, but rather a bivariate logistic regression for each hub to predict either EDSS or FSS groups. EDSS was predicted by the intake of vegetables (beta $= -0.36$, $p < 0.03$) and fish (beta $= -0.34$, $p < 0.02$). FSS was not predicted by any hub, e.g., fruit, vegetables, cereal and fish.

Relationship Between "Negative Foods" Lifestyle Factors and Disability

When testing the relationship between "negative foods" diet and disability, the model including smoking pack/year, IPAQ score and intake of "negative foods" predicted 6% of the variance in EDSS ($p < 0.001$), with smoking pack/year, exercise and diet being independent contributors (beta $= 0.190$, $p < 0.001$; beta $= -0.138$, $p < 0.004$; beta $= 0.098$, $p < 0.039$, respectively).

The model including smoking pack/year and IPAQ score predicted 4% of the variance in FSS ($p < 0.001$), with smoking pack/year and exercise being independent contributors (beta $= 0.135$, $p < 0.005$; beta $= -0.169$, $p < 0.001$, respectively).

TABLE 2 | Clinical and anthropometric characteristics of people living with MS involved in the study, according to level of disability.

	Total (*N* = 424)	EDSS ≤1.5 (*N* = 205)	EDSS ≥2 (*N* = 219)	*P*
Clinical characteristics				
Age, years	42.6 (11.0)	39.3 (10.5)	45.6 (10.7)	<0.001
Age of onset, years	31.4 (9.9)	30.1(8.7)	32.7 (10.8)	<0.01
Disease duration, years	12.0 (8.4)	10.1 (7.3)	13.8 (9.0)	<0.001
Therapies (no/first line/second line), %	20/60/20	20/64/16	20/54/26/	n.s.
Disease course, Relapsing Remitting (RR)/Secondary Progressive (SP)	402/22	205/0	198/22	<0.001
Median Expanded Disability Status Scale (EDSS) [range]	2.0 [0–5.5]	1.5 [0–1.5]	3 [2–5.5]	<0.001
Fatigue Severity Scale (FSS)	3.8 (1.7)	3.1 (1.6)	4.5 (1.5)	<0.001
Anthropometric characteristics				
BMI, kg/m^2	23.8 (3.9)	23.65 (4.05)	24.07 (3.94)	0.270
Waist circumference, cm	83.2 (12.3)	81.8 (11.9)	84.6 (12.6)	**0.021**
Hip circumference, cm	100.7 (8.8)	100.3 (8.55)	101.2 (9, 3)	0.281
Waist-hip ratio (WHR)	0.82 (0.08)	0.81 (0.78)	0.83 (0.08)	**0.013**

Values are expressed as mean, unless otherwise specified. Standard Deviation (SD) is indicated in the round brackets. The minimum and maximum values of the measurements are indicated in the square brackets. P-values refer to comparisons between groups; significance was reached if p < 0.05 and expressed in bold.
First line therapies: interferon, glatiramer acetate, dimethyl fumarate, teriflunomide.
Second Line therapies: fingolimod, natalizumab, ocrelizumab, alemtuzumab.

DISCUSSION

In our work we did not find significant differences in terms of adequacy to MeDi between PwMS and HC, nor significant differences regarding food intake within the study population besides fish and alcohol consumption. Even though most participants fell into the normal weight category value according to BMI and were adherent to MeDi, PwMS showed higher values of waits and hip circumferences compared to HC. The network analysis allowed us to unveil the relation between specific food items (fruit, vegetables, cereal and fish). In particular, PwMS showed an eating behavior characterized by the consumption of "positive" foods, whose intake appears highly intercorrelated and might partially contribute to physical disability level.

Adherence to MeDi, Intake of Specific Food, Lifestyle Habits

We found no significant differences in MeDi adequacy between PwMS and HC, in line with previous findings (25). However, although most of the participants in both groups were sufficiently adherent to MeDi, a very low percentage of participants were completely adherent to it (2% PwMS and 1% HC). Even though MeDi is the most used food model in Mediterranean countries, such as Italy, even the general population is not completely adherent to it, with younger age groups and smokers being less adherent (26). Accordingly, in our work participants less adherent to MeDi showed higher values of smoking pack/years and particularly, in the group of PwMS there was a higher percentage of participants with long-term smoking habits compared to HC. Generally, the category of smokers seems to have a less healthy lifestyle and incorrect eating habits than non-smokers (27).

When we explored differences in terms of food intake frequency, we observed a tendency toward a healthier diet in the group of PwMS, as they consumed more frequently fish and less frequently alcohol than HC. Despite the healthier diet, in our sample of PwMS higher values of waist and hip circumferences compared to HC were detected. WC is used to measure abdominal adiposity and high values are common among PwMS (28). While in our study population the BMI was in the range of accepted values and most of the PwMS fell in the normal weight category, there is evidence showing that an increase in circumferences values is associated with greater disability even in normal weight subjects (28). This is confirmed in our sample, where patients with higher level of disability showed significantly higher WC and WHR values in comparison with the no disability group. An excess of visceral or abdominal adiposity is one of the characteristics of the metabolic syndrome often related to other disorders such as diabetes, hyperlipidemia and hypertension (28). Metabolic and vascular comorbidities affect both neuroperformance and brain and gray matter volumes in MS, contributing to neurodegeneration and long-term disability (29).

Even though in our work we did not collect serological nutrional biomarker and such interpretations remain speculative, several evidences support the correlation between an excess of adipose tissue and the severity of MS. Firstly, an overweight or obese subject has a state of chronic inflammation characterized by an altered production of cytokines, such as IL-6, TNF-α, leptin, and a downregulation of anti-inflammatory molecules (30). Additionally, overweight negatively impacts disease course by modulating monocyte cell number through ceramide-induced DNA methylation of anti-proliferative genes (31). This increased state of inflammation of overweight/obese subjects is also observed within the Central Nervous System (CNS). In particular, a recent study has shown elevated levels of proinflammatory molecules (IL-6 and leptin) and reduced levels of the anti-inflammatory cytokine IL-13 in the cerebrospinal fluid

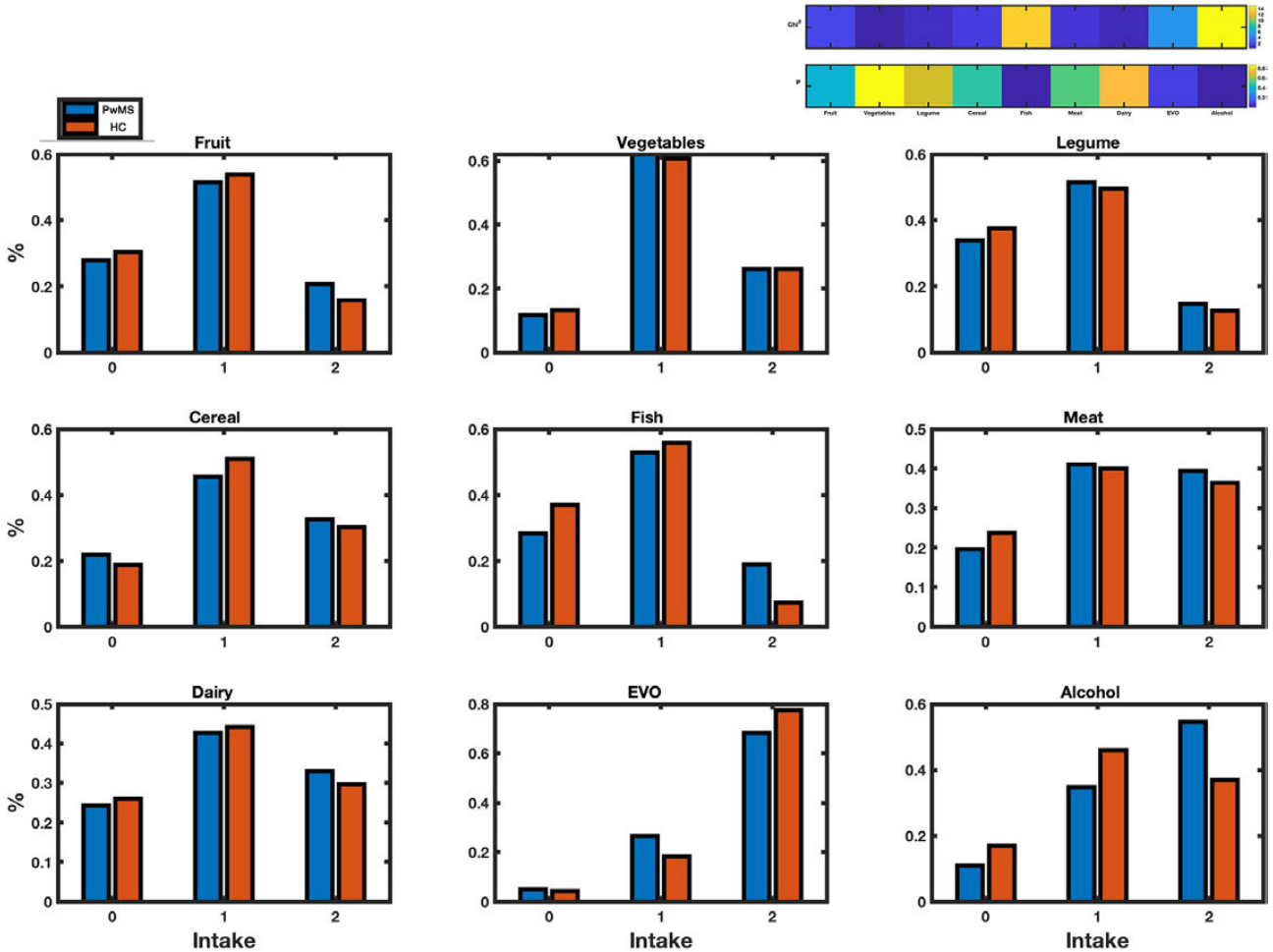


FIGURE 4 | Food intake frequency. Histograms of food intake frequency of people living with multiple sclerosis (PwMS, blue) and healthy controls (HC, orange), devised by intake scores (0, 1, 2). On the top right, heatmaps display X^2 and relative *p*-value of food intake difference between PwMS and HC. Significant difference (*p*-value<0.05) was found for fish and alcohol alone. Chi^2: X^2; EVO: olive oil.

(CSF) of obese MS patients, and patients with higher BMI (BMI > 30 kg/m^2) also had significantly higher EDSS values (30).

Excess of adipose tissue in PwMS might also affect daily clinical practice, as it can interfere with the response to drugs, possibly due to altered drug pharmacokinetics (32). Indeed, a study carried out on a population of adult patients, under interferon-beta (IFNβ) therapy, showed that overweight and obese patients have increased disease activity, as assessed by NEDA status (composite score of no evidence of disease activity). This result indicates that overweight and obesity may have an impact on IFNβ-treatment response (33). Based on these data, regular exercise by reducing visceral adiposity may play a key role in reducing inflammation. Finally, in line with previous evidence (34), PwMS were less physically active and showed overall lower IPAQ scores compared to HC.

Food Network Analysis

The food network analysis identified fruits, vegetables, cereals and fish as hubs in PwMS with meat and alcohol being hubs in HC, suggesting, in line with our analysis of food intake frequency, that PwMS tend to have a healthier diet than HC. It is possible that PwMS, having a chronic disease, are urged to pay more attention to what they eat than the general population. Indeed, a previous study has shown that patients, following an initial clinical diagnosis of CNS demyelination, tend to change their diet, increasing the amount of fruit and/or vegetables and following a low-fat diet (35). Additionally, a recent work has shown a trend toward reduced levels of storage lipids (fatty acids, cholesterol esters, triglycerides, and diglycerides) in the plasma of PwMS in comparison with HC (36). Additionally, in line with previous findings highlighting the potential effects of MeDi on MS course and disability (16) in our study participants higher consumption of vegetables and fish was inversely correlated to global motor disability, as assessed via EDSS. This relationship hints to a beneficial role of food hubs on disability outcomes but might also disclose an inverse causality.

Foods identified as hub of PwMS (fruits, vegetables, cereals and fish) are rich in fiber, vitamins, ω-3 polyunsaturated

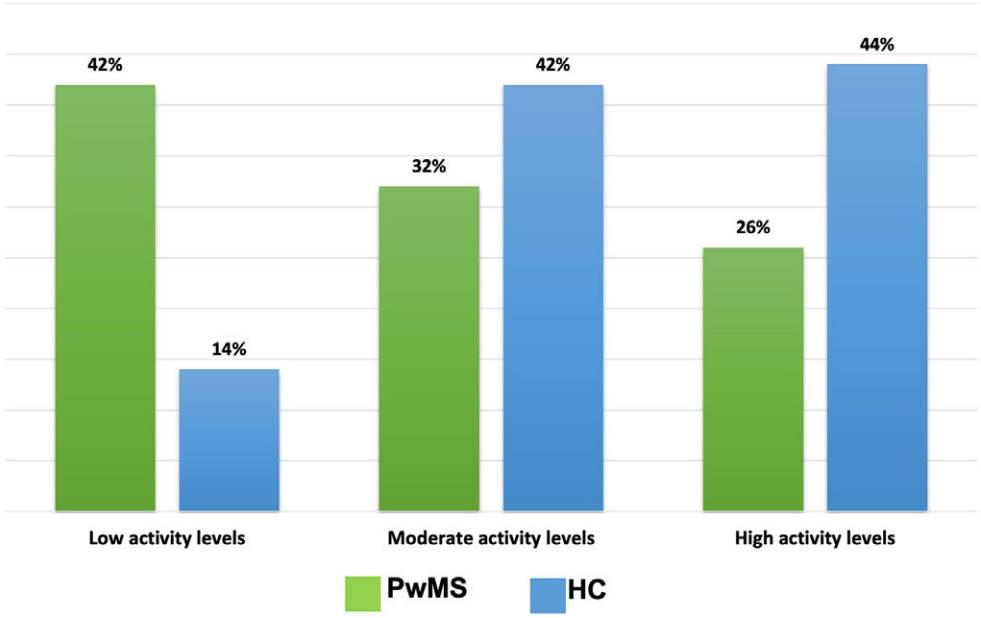


FIGURE 5 | Percentage of participants according to group for each category of International Physical Activity Questionnaire (IPAQ). PwMS, People Living with MS; HC, Healthy Controls.

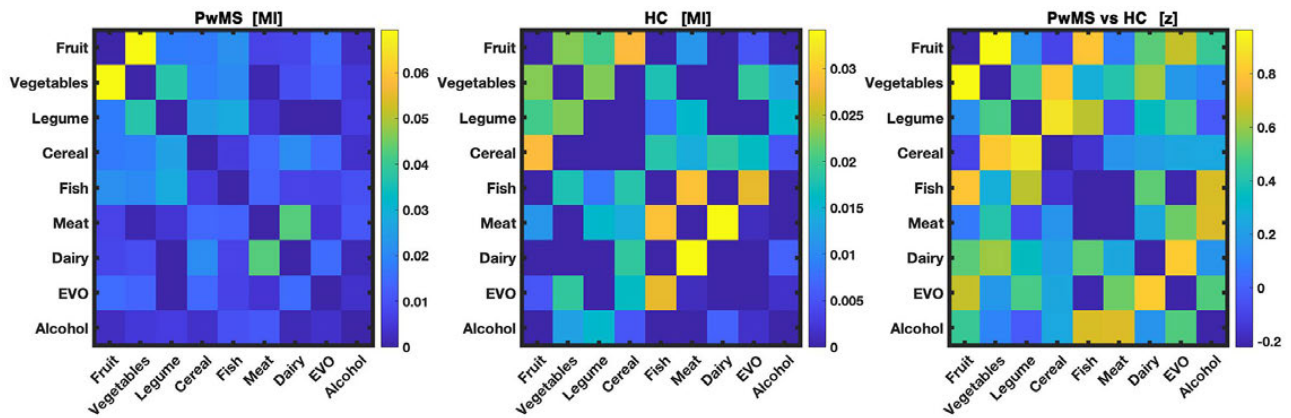


FIGURE 6 | Mutual information matrices. Mutual information matrices of people living with MS (PwMS) and healthy controls (HC), as well the matrix resulting by the 2-sample analysis to investigate differences between PwMS vs. HC and reporting the z-score, are displayed from the left to the right. PwMS vs. HC have no intake connectivity difference in any of the food-pair (z-score > 1.96).

fats or antioxidant molecules and possibly exert a protective effect on the disease (14, 37). In particular, antioxidant molecules such as vitamins C and E, plant polyphenol and carotenoids might contrast free radicals, with beneficial effects on the inflammatory response (37). Another mechanism by which hub foods that could influence the disease outcomes is the modulation of the microbiota (38). Specifically, foods with omega-3 polyunsaturated fatty acids (PUFA) and fiber can positively modify microbiota through the proliferation of bacteria with anti-inflammatory action. Both short-chain fatty acids (SCFA) obtained from the bacterial fermentation of dietary fibers, and PUFAs, contained in fish, have an anti-inflammatory function (39, 40). Nonetheless, we cannot exclude that disease-related symptoms such as mobility deficits and cognitive impairment in more disabled PwMS might have affected the access to healthier food, that usually require a more dedicated preparation rather than easy snacks or fast-foods, partly accounting for the observed relationship between food hubs intake and disability (41). However, in our sample of PwMS we have not recruited those with

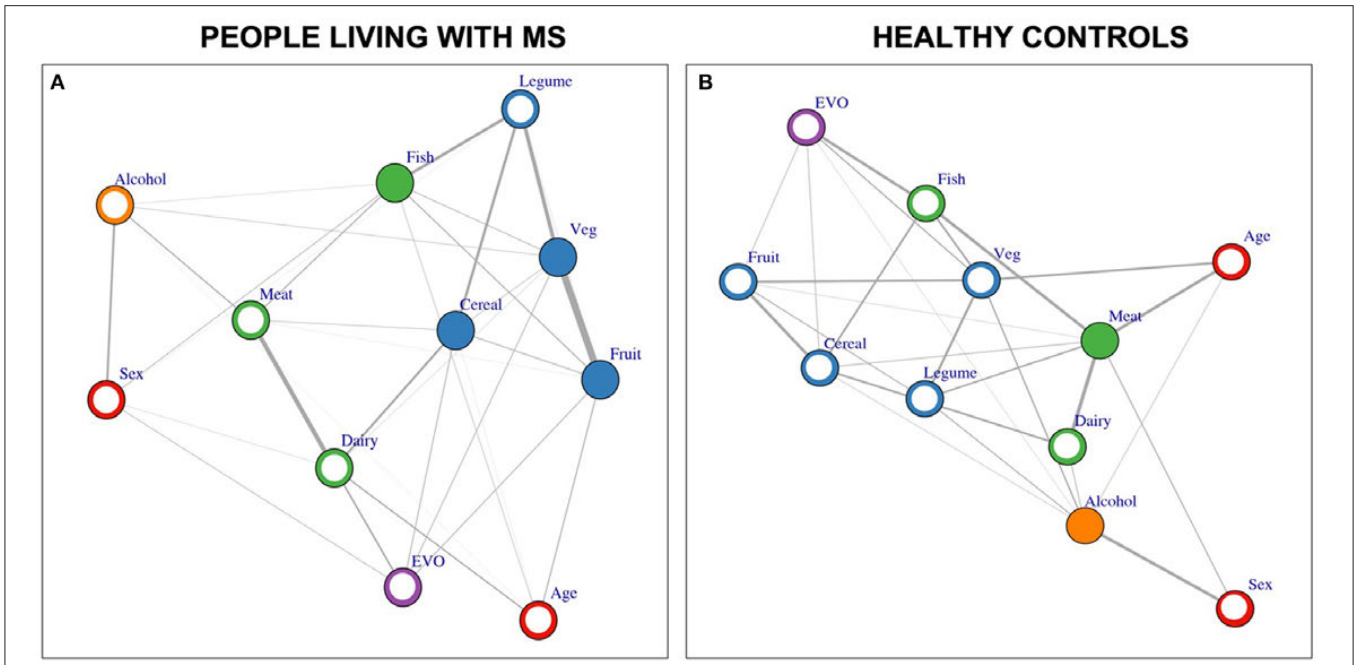


FIGURE 7 | Demographic and food networks. Demographic data, such as age and sex, and Mediterranean Diet food items, such as cereal, vegetables, fruit, diary, meat, fish, olive oil (EVO) are displayed as nodes of a graph, whose links (edges) are calculated as mutual information coefficients for either people living with MS **(A)** and healthy controls **(B)**. Hubs are the variables with the highest amount of connections with the other data and represent the mos influential items for the eating habits of people living with MS and controls. Link thickness mirrors the intensity of the mutual information coefficient between the linked nodes. Circles display nodes and are colored following the class, thus red if demographics, blue if with vegetal origin, green if with animal origin, purple for EVO, orange for alcohol. Full circles identify hubs.

TABLE 3 | Spearman correlation among MeDi item intake identified as hubs in people living with MS.

	Fruit	Vegetables	Cereal	Fish
Fruit	–	**0.33 (0.001)**	**0.13 (0.01)**	**0.22 (0.001)**
Vegetables		–	**0.19 (0.001)**	**0.21 (0.001)**
Cereal			–	**0.12 (0.01)**
Fish			0.12	–

To check for collinearity among hubs, we calculated Spearman correlation between couples of items, as reported in the table. Significant correlations are reported in bold font, relative p-value is shown between round brackets.

an EDSS higher than 5.5, also in order to restrain this potential bias.

Relationship Between "Negative Foods" Lifestyle Factors and Disability

Our *post-hoc* analysis disclosed an independent predictive role for "negative foods," exercise and smoking on motor disability. This finding suggests that not only the intake of "positive foods" is related to disability, but that the global composition of the dietary regimen as well as the lifestyle adopted potentially affect MS outcomes. Specifically, both smoking habit and low level of exercise were related to disability and fatigue independently from diet, as previously suggested (42, 43). Although reverse causality and simultaneity cannot be excluded when interpreting these associations, the relevance of a balanced dietary regimen, rich in "positive foods" and poor in "negative foods," associated to a healthy lifestyle, might explain why studies exploring the beneficial effects of isolated dietary supplementations are often unsuccessful (44).

Our work is not without limitation. First, the cross-sectional design of our work limited the ability to explore the temporal relationship between lifestyle habits and changes in disability and fatigue over time, preventing us from drawing conclusions about direct causality. Second, we used a qualitative questionnaire to assess the adequacy to MeDi and food intake, intrinsically limiting the exact estimation of nutrients intake. We did not measure serum markers (i.e., antiflammatory or antioxidant) that could better reveal the association between nutrition intake and disability or fatigue if inserted in the network analysis, but on the basis of our results, future more comprehensive study on the matter might lead to further elucidation. Moreover, the use of family members as part of the control group might have limited the possibility to reveal real differences in dietary and lifestyle habits with PwMS. We have tried to minimize this occurrence introducing another set of controls in the HC group (healthcare staff). Indeed, the choice of family members as control group lie on the basis that family peers share characteristics such as socioeconomic and educational level with their relatives, that are known to influence diet behaviors (45).

CONCLUSION

In conclusion, we identified a sufficient, although not optimal adherence to MeDi in our population. People living with MS showed a healthier dietary pattern than HC. Among food hubs, vegetables and fish were related to disability outcomes, which, in turn, were also predicted by intake of foods rich in saturated fats and alcohol, smoking and exercise. Our results confirm the association between diet, lifestyle and disability, suggesting that the modulation of these factors might affect MS outcomes. Further, our results suggest that, rather than the intake of a single food, the association of a food with the others is at the basis of the difference between PwMS and HC dietary habits. Future longitudinal studies applying integrated approaches should be planned to confirm these hypotheses, as the adoption of specific dietary regimens and exercise plans, could be used as complementary to the prescription of standard pharmacological therapies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee board of Sapienza University of Rome at Sant'Andrea Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FF, MP, CP, and SR: conceptualization. CP and SR: supervision and funding acquisition. FG, CP, and SR: project administration. All authors: methodology, formal analysis, investigation, data curation, writing, and have read and agreed to the published version of the manuscript.

FUNDING

This study was partially supported by Sapienza University of Rome Italy Research Initiation Project n. RP120172B4168A93. This funding source had no role in the design of this study and did not have any role during its execution, analyses, interpretation of the data, or decision to submit results.

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Conflict of Interest: MP discloses travel/meeting expenses from Novartis, Roche and Merck, speaking honoraria from HEALTH&LIFE S.r.l. and honoraria for consulting services from Biogen and research grants from Baroni Foundation. CP has served on scientific advisory boards for Actelion, Biogen, Genzyme, Hoffmann-La Roche Ltd, Merck, Novartis, Sanofi, Teva, and has received consulting and/or speaking fees, research support and travel grants from Allergan, Almirall, Biogen, Genzyme, Hoffmann-La Roche Ltd, Merck, Novartis, Sanofi and Teva; SR has received honoraria from Biogen, Merck Serono, Novartis and Teva for consulting services, speaking and/or travel support.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Neurotherapeutics (2018) 15:109–125
https://doi.org/10.1007/s13311-017-0588-x

REVIEW

The "Gut Feeling": Breaking Down the Role of Gut Microbiome in Multiple Sclerosis

Samantha N. Freedman[1,2] · **Shailesh K. Shahi**[2] · **Ashutosh K. Mangalam**[1,2]

Published online: 4 December 2017

Abstract Multiple sclerosis (MS) is a chronic neuroinflammatory disease of the central nervous system with unknown etiology. Recently, the gut microbiota has emerged as a potential factor in the development of MS, with a number of studies having shown that patients with MS exhibit gut dysbiosis. The gut microbiota helps the host remain healthy by regulating various functions, including food metabolism, energy homeostasis, maintenance of the intestinal barrier, inhibition of colonization by pathogenic organisms, and shaping of both mucosal and systemic immune responses. Alteration of the gut microbiota, and subsequent changes in its metabolic network that perturb this homeostasis, may lead to intestinal and systemic disorders such as MS. Here we discuss the findings of recent MS microbiome studies and potential mechanisms through which gut microbiota can predispose to, or protect against, MS. These findings highlight the need of an improved understanding of the interactions between the microbiota and host for developing therapies based on gut commensals with which to treat MS.

Key Words Multiple sclerosis (MS) · gut microbiome · immune response · experimental autoimmune encephalomyelitis (EAE) · host–microbe interaction · microbial metabolism

✉ Ashutosh K. Mangalam
ashutosh-mangalam@uiowa.edu

[1] Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA, USA

[2] Department of Pathology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic neuroinflammatory disease of the central nervous system (CNS) [1]. Clinical presentation varies among patients, which may include sensory, motor, and/or cognitive deficits, and is typically preceded by inflammatory and demyelinating lesions in the CNS white matter [1]. MS is thought to be an autoimmune disease caused by aberrant T-cell responses to myelin self-peptides [2]. Encephalitogenic T-cell responses in the CNS are followed by additional immune-cell infiltration, leading to inflammation, demyelination, and neurodegeneration [3]. The etiology of MS is complex, with both genetic and environmental factors playing major roles in disease pathogenesis. Genetic factors account for about 30% of disease risk (identical twins), with human leukocyte antigen (HLA) genes being the strongest genetic component [4, 5]. Although 70% of disease risk stems from nongenetic components, considerably less is known about environmental contributions to disease pathogenesis. Possible environmental contributing factors include smoking, exposure to the Epstein–Barr virus or other microbes, and low vitamin D levels due to insufficient exposure to sunlight/ultraviolet [3]. However, recent evidence suggests that the gut microbiota can make a major contribution to both susceptibility and protection from the disease.

Gut Microbiota

Throughout the twentieth century, the incidence of MS (and other autoimmune and allergic diseases) has increased in developed countries; a phenomenon that correlates inversely with the incidence of infectious diseases such as measles and polio [6]. This observation led to development of the "hygiene hypothesis" according to which individuals who live in

cleaner environments (developed countries where there is a strong emphasis on personal hygiene) have little exposure to orofecal microbes at an early age and are thus more likely to develop allergic and autoimmune diseases [6, 7]. The corollary is that individuals who live in developing countries and are exposed to a high orofecal microbial load are more likely to develop infectious diseases while being less likely to develop allergic and autoimmune diseases. However, in recent years the hygiene hypothesis has been revisited because advances in the microbiome field have suggested that clean hygienic practices, such as the chlorination of water, overuse of antibiotics, loss of green space, small family size, and common delivery by cesarean section, have resulted in alterations of host–microbial flora collectively termed the microbiota [8, 9]. Indeed, it is now appreciated that the microbiota has a crucial role in keeping the host in a healthy state.

Microbiota refers to the trillions of bacteria, viruses, and fungi that live inside and on the human body, and their collective genetic pool is termed the microbiome. Bacterial species colonizing the small and large intestines are referred to as the gut microbiota, and their role in health and diseases are being investigated as a primary driver of host physiology [10–12]. The gut microbiota functions like a bioreactor that influences nutrient uptake, food metabolism, energy homeostasis, and mucosal and systemic immune responses. A healthy gut microbiota is characterized by its diversity and resilience [13], and it helps to keep the host healthy in multiple ways, including maintenance of an intact intestinal barrier, inhibition of colonization by pathogenic organisms, and regulation of host physiology and immune responses [11, 14–16].

Alterations of the gut microbiota, resulting in changes in its metabolic network, perturb this homeostasis, and can result in intestinal and systemic disorders [10, 17, 18]. These alterations are reflected in fecal samples which are utilized to profile the gut microbiota because they provide an easily accessible source of biospecimens .

Gut Microbiota and Autoimmunity

Advancements in culture-independent analysis, through improved DNA sequencing technology, have led to remarkable progress in our understanding of the roles of the gut microbiota in autoimmune diseases. Microbiome profiling, using 16S rRNA and metagenomic shotgun sequencing technologies, has been performed for several autoimmune/autoinflammatory diseases, including MS [19–29], type 1 diabetes (T1D) [30, 31], inflammatory bowel disease (IBD) [32, 33], celiac disease [34, 35], and rheumatoid arthritis [36–38]. The consensus of these studies is that patients with autoimmune/autoinflammatory diseases exhibit microbial dysbiosis, i.e., an alteration in the composition of the microbial community characterized by a decrease in beneficial bacteria and an increase in harmful bacteria (pathobionts). A healthy mixture of commensal bacteria helps in maintaining homeostasis at mucosal surfaces in the gut and perturbation of this community can result in colonization by pathobionts, potentially promoting a proinflammatory environment and predisposing the host to inflammatory diseases [39]. How beneficial bacteria maintain a healthy state and/or how pathobionts might predispose to or accelerate autoimmune disease is unknown. However, considerable efforts are being made to determine whether alterations in the gut microbiota are the cause or consequence of autoimmunity.

Studies performed in germ-free (GF) mice have established that the gut microbiota is crucial for the development of a healthy immune system; mice born and raised in GF facilities lack well-developed immune cells and secondary immune organs [40]. The initial interaction between the host immune system and the gut microbiota is a major determinant of the composition of the gut flora [41, 42]. Constant cross-talk between immune cells, intestinal epithelial cells (IECs), and the gut microbiota results in up- or downregulation of inflammatory mediators from the host (pattern recognition receptors, e.g., Toll-like receptors) and microbes [toxins, e.g., lipopolysaccharide (LPS)] [43]. Thus, the balance between host immune responses and gut microbiota is intricate, and the host adapts to discriminate between beneficial commensal bacteria and pathogenic bacteria. IECs play a significant role in this discrimination; their tight junctions join adjacent cells to form a barrier between immune cells of the host and bacteria in the gut [11, 16]. This cross-talk between IECs, immune cells and the microbiota help in maintaining homeostasis at the mucosal surface. Interestingly, in pathological states, this barrier is breached, resulting in increased gut permeability and systemic distribution of bacterial products and/or the bacteria itself. Several autoimmune diseases are associated with a condition called “leaky gut syndrome”, which is characterized by increased gut permeability [44, 45].

The importance of the gut bacteria, their enzymes, and metabolites in human health and disease are actively being deciphered. Early research in the field points towards the gut microbiota playing an important role in host physiology by regulating numerous metabolic pathways. We discuss in the following sections 1) differences in microbial compositions of the gut between healthy controls and patients with MS; 2) how depletion or enrichment of particular gut microbes in patients with MS might predispose to autoimmunity; and 3) the possibility that reversal of such dysbiosis might protect against autoimmunity by regulating various metabolic pathways.

MS and the Gut Microbiota

Recently, several groups, including ours, profiled fecal microbiota in patients with MS in an attempt to determine the extent

to which the gut microbiome influences MS risk (Table 1). These studies revealed that patients with MS exhibit microbial dysbiosis [19–28].

The majority of MS microbiome studies have reported a reduced abundance of *Prevotella* and *Parabacteroides* belonging to the Bacteroidetes phylum in patients with MS *versus* healthy controls (Table 1) [19, 20, 24, 26, 28]. As highlighted in Table 1, several human microbiome studies in adult patients with MS (at least > 10 subjects) across different geographical regions (USA, Japan, and Italy) have shown either depletion of *Prevotella versus* healthy controls [19, 20, 26, 28] or enrichment of *Prevotella* after treatment with disease-modifying drugs [24]. A recent study analyzing duodenal biopsies from patients with MS reported that patients with active disease showed a lower abundance of *Prevotella* than healthy controls or patients in remission [28]. Among the 3 MS microbiome studies from the USA, 2 have shown a lower abundance of *Parabacteroides* in patients with MS *versus* healthy controls [19, 26]. Jangi et al. [24] demonstrated that the abundance of *Butyricimonas*, another member of the Bacteroidetes, is low. Finally, Miyake et al. [20] demonstrated that the abundance of *Bacteroides* is lower in patients with MS than in healthy controls. The demonstration of reduced abundance of bacteria from the Bacteroidetes phylum by several groups suggests that certain bacteria from this phylum might play an important role in protecting against the development of MS.

Table 1 Bacterial families (F), genera (G), and species (S) whose abundance is often higher or lower in patients with multiple sclerosis (MS) *versus* healthy controls

Bacteria	Abundance in MS *vs* healthy controls [ref.]
Bacteroides (G)	Decreased [20]
Parabacteroides (G)	Decreased [19, 26]
Prevotella (G)	Decreased [19, 20, 24, 28]
Butyricimonas (G)	Decreased [24]
Lachnospiraceae (F)	Decreased [22]
Blautia (G)	Increased [19]
Dorea (G)	Increased [19]
Streptococcus	Increased [20, 28]
Faecalibacterium (G)	Decreased [20, 25]
Eubacterium (G)	Decreased [20]
Clostridium (G)	Decreased [20, 25]
Ruminococcaceae (F)	Decreased [22]
Ruminococcus (G)	Increased [25]
Lactobacillus (G)	Decreased [19]
Coprobacillus (G)	Decreased [19]
Erysipelotrichaceae (F)	Decreased [19]
Veillonellaceae (F)	Decreased [19]
Collinsella (G)	Decreased [19, 24]
Adlercreutzia (G)	Decreased [19]
Slackia (G)	Decreased [24]
Acinetobacter (G)	Increased [26]
Bifidobacterium (G)	Increased [20, 22]
Eggerthella (G)	Increased [20]
Pseudomonas (G)	Increased [19]
Mycoplana (G)	Increased [19]
Haemophilus (G)	Increased [19]
Bilophila (G)	Increased [22]
Sutterella (G)	Increased [20]
Akkermansia (G)	Increased [24, 26, 27]

Certain bacterial genera belonging to the phylum Firmicutes have been found to be either enriched or depleted in patients with MS. However, some discrepancies regarding the association of Firmicutes with MS have been reported by different laboratories. For example, where we found an increase in the abundance of *Blautia* and *Dorea* (of the Lachnospiraceae family) in patients with MS [19], others observed an increases in the abundance of *Streptococcus* [20, 28] and *Ruminococcus* [25]. Also, where we observed a decrease in the abundance of *Lactobacillus*, *Coprobacillus*, Erysipelotrichaceae, and Veillonellaceae [19], others observed decreases in the abundance of Lachnospiraceae, Ruminococcaceae [22], and in *Clostridia* species [20, 25]. The observed discrepancies in the abundance of Lachnospiraceae and Ruminococcaceae could be due to many factors (discussed below), including specificity of 16S rRNA primers utilized for analysis of the microbiome and/or differences in the patient population. Details about the various primers and sequencing technologies utilized and their significance are discussed in a recent review [46].

With regard to the phylum Actinobacteria, we and Jangi et al. [19, 24] observed a decrease in the abundance of *Collinsella* in patients with MS. In addition, we found a reduced abundance of *Adlercreutzia* in patients with MS [19]. Although Jangi et al. [24] did not report a difference in *Adlercreutzia*, they did report a reduction in *Slackia*, another member of the Actinobacteria phylum. Other groups observed an increased abundance of *Bifidobacterium* [20, 22] and *Eggerthella* [20] in patients with MS.

In the case of Proteobacteria, the majority of the studies revealed an increase in the abundance of certain bacteria belonging to this phylum in patients with MS (Table 1). We observed an enrichment of *Pseudomonas*, *Mycoplana*, and *Haemophilus* in patients with MS [19], whereas others observed an increase in abundance of *Bilophila* [22], and *Acinetobacter* [26]. Two studies reported a modulation of *Sutterella* in patients with MS, with Miyake et al. [20] reporting a decreased abundance in patients with MS and Jangi et al. [24] reporting an increased abundance in patients with MS on disease-modifying therapies. Finally, 3 groups observed an enrichment of *Akkermansia*, of the Verrucomicrobia phylum, in patients with MS [24, 26, 27].

Caution must be made when interpreting the results in the aforementioned microbiome studies as considerable variability exists between each study of patients with MS. The results from the handful of studies that profiled the gut microbiome in healthy controls and patients with MS suggest that there is no "MS-associated gut microbial signature". Rather, the literature suggests that patients with MS exhibit dysbiosis, defined as an alteration of microbial architecture from healthy controls that shifts the immune balance towards an inflammatory phenotype [46]. This is supported by 2 recent studies showing that fecal transfer from patients with MS, but not healthy controls, to mice increased either disease incidence [27] or severity [26] of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Based on this, we hypothesize that patients with MS exhibit a general increase in proinflammatory bacteria, rather than exhibiting an increase or decrease in a specific set of bacterial genera. That being said, there are certain bacterial genera found to be depleted (*Prevotella*) or enriched (*Akkermansia*) in multiple cohorts of MS patients from different continents (Table 1). Therefore, the possibility for commonalities for certain gut bacteria in patients with MS is not completely inconceivable. Overall, caution must be taken when interpreting results as bacterial alterations in one set of patients with MS might not represent the gut microbial state of all patients.

Additional explanation for high variability among studies stems from the lack of standarized sequencing methodologies. Several 16S rRNA primers, specific for certain hypervariable regions within the 16S gene, exist and differential use of these primers may show bias for particular taxa [46]. Additionally, the gut microbiome has been shown to be influenced by genetics [47], diet [48], geographical location [49], and use of disease-modifying therapies [24, 27]. These are all potentially confounding factors and must be kept in mind when designing and interpreting gut microbiome experiments, not just for MS but for any disease/disorder of interest.

The above-described MS microbiome studies have shown that there are differences in the bacterial populations between patients with MS and healthy controls, with some consensus between different studies. The next step is to elucidate the functional significance of these differences, i.e., to determine the mechanism whereby modulation of the host immune response by gut bacteria might predispose to, or protect from, disease. A better understanding of these mechanisms will aid in developing therapies for treating or even curing MS based on gut commensal bacteria. Gut bacteria may affect host physiology through either their own components (e.g., cell wall, polysaccharide A, LPS) or microbial metabolites produced as a result of their metabolism of the host diet and/or other bacterial breakdown products (a process known as cross-feeding) [50, 51]. In the following sections, we discuss various metabolic pathways in which gut bacteria participate, their known effects on host immunity, as well neurons and/or glial cells, and how these metabolites might influence MS. The major metabolic pathways used by commensals that influence host immune responses are metabolism of short-chain fatty acids (SCFAs), bile acids, phytoestrogens, tryptophan, and choline (Table 2; Fig. 1).

SCFA Metabolism

Among the bacterial metabolic pathways, SCFAs are the most extensively studied in the context of host immunity [51–53]. SCFAs, abundant in high-fiber foods, are 1 to 6 carbons in length and produced through the fermentation of indigestible starches and complex sugars [52, 53]. The majority of SCFAs in the colon are acetic acid, propionic acid, and butyric acid [54]. SCFAs contribute to host immunity at both intestinal and extraintestinal locations, modulating cell processes either by interacting with certain G-protein coupled receptor and/or actively or passively transporting into the cytoplasm. Once inside the cytoplasm, SCFAs can regulate transcription factors and certain enzymes, such as histone deacetylases [50].

Within the colonic lumen, IECs represent the first line of defense as they are in direct contact with the gut microbiota and their metabolites. Besides serving as an energy source, SCFAs can modulate the immune-defensive functions of IECs by altering barrier integrity and cytokine production in response to inflammatory stimuli [52]. *In vitro* treatment of human IECs with SCFAs leads to a decrease in the induction of proinflammatory cytokines by inflammatory stimuli [55, 56] but induces production of interleukin (IL)-18, a proinflammatory cytokine involved in epithelial integrity and homeostasis [57]. Notably, treatment with acetate-producing *Bifidobacterium* improves barrier function against enterohemorrhagic *Escherichia coli* O157:H7 [58]. Similarly, direct treatment with SCFAs enhances barrier function by inducing mucin genes in goblet cells [50].

SCFAs can also modulate the phenotype and effector function of leukocytes in the gut. In general, SCFAs induce a tolerogenic phenotype in lymphocytes and antigen presenting cells [50, 54, 56]. Previous studies showed that SCFAs downregulate the expression of major histocompatibility complex class II, co-stimulatory molecules, and inflammatory cytokines by dendritic cells (DCs) and potentiate a regulatory phenotype in $CD4^+FoxP3^+$ T cells (Tregs) [50, 52, 59–61]. Several studies demonstrated that SCFAs induce the differentiation of both $FoxP3^+$ Tregs and IL-10-producing T cells *in vitro*, as well as *in vivo*, thereby maintaining mucosal homeostasis and protecting against colonic inflammation [50, 52, 54, 62–64]. Taken together, these results suggest that SCFAs induce a tolerogenic and immunosuppressive phenotype in the gut mucosa. It should be noted, however, that SCFAs have also been shown to potentiate a proinflammatory

Table 2 Bacterial families (F) or genera (G) that are depleted or enriched in patients with multiple sclerosis (MS), and their association with specific metabolic pathways

Bacterial metabolic pathway	Gut bacteria depleted or enriched in patients with MS	Reference for bacterial involvement in metabolic pathway
SCFA	*Clostridium* (G)	[53, 68]
	Faecalibacterium (G)	[53, 83]
	Eubacterium (G)	[53]
	Ruminococcus (G)	[53]
	Butyricimonas (G)	[24]
	Bacteroides (G)	[51, 83]
	Prevotella (G)	[67]
Bile acid	*Lactobacillus* (G)	[115, 116]
	Clostridium (G)	[117, 118]
	Erysipelotrichaceae (F)	[119]
	Parabacteroides (G)	[121]
	Acinetobacter (G)	[126]
	Bifidobacterium (G)	[127]
	Pseudomonas (G)	[123, 124]
	Bilophila (G)	[110]
Phytoestrogen	*Prevotella* (G)	[152]
	Parabacteroides (G)	[19]
	Adlercreutzia (G)	[136]
	Slackia (G)	[136]
	Lactobacillus (G)	[158]
Tryptophan	*Lactobacillus* (G)	[50]
Choline	*Acinetobacter* (G)	[199]
Mucin-degrading	*Akkermansia muciniphila* (G)	[32, 205–207]
	Bacteroides (G)	[207]
	Ruminococcus (G)	[207]
	Bifidobacterium (G)	[207]
	Dorea (G)	[207]

SCFA = short-chain fatty acid

phenotype in both IECs and immune cells [52]. Presumably, the precise phenotype elicited by SCFA treatment depends on one or several factors such as the SCFA used, the dose used, the duration of the treatment, and the model system utilized.

In addition to modulating the immune system, bacterial metabolites generated from the gut microbiota have the ability to directly modulate CNS function. For example, in both *in vivo* and *in vitro* models of Parkinson's disease, butyrate prevents neuronal degeneration and death [65]. Moreover, this modulation of CNS function may be direct, given that SCFAs are found in systemic circulation [66].

Several bacterial genera whose abundance is low in patients with MS are capable of participating in SCFA metabolism. These include *Clostridium*, *Faecalibacterium*, *Eubacterium*, *Ruminococcus*, *Butyricimonas*, *Bacteroides*, and *Prevotella* (Table 2) [51, 53, 67]. Consistent with their association with SCFA metabolism, these bacterial genera induce an anti-inflammatory phenotype in IECs and immune cells. *Clostridium* species (Firmicutes) are dominant gut commensals and are strongly associated with SCFA metabolism [68]. *Clostridium* clusters IV and XIVa (distinct cohorts of *Clostridium* groups based on phylogeny) are implicated in maintaining gut homeostasis by inducing FoxP3$^+$ Tregs, and their representation is reduced in patients who have MS, as well as active IBD [20, 68–71]. Several groups have demonstrated that immunosuppression by *Clostridium* involves SCFA production, as discussed by Nagano et al. [72]. The Clostridia family includes *Eubacterium*, *Ruminococcus*, and *Faecalibacterium* which have anti-inflammatory properties [53]. *Eubacterium* levels are low in stool samples from patients with IBD, and treatment with *Eubacterium* attenuates experimental colitis, presumably owing to its SCFA-producing abilities [73, 74]. Within the colon, *Ruminococcus* plays a crucial role in degrading resistant starch to SCFAs, and it leads to decreased proinflammatory cytokine production *in vitro* [75, 76]. *Faecalibacterium prausnitzii* is another commensal bacterium of the Firmicutes phylum, and low levels are associated with a higher risk of IBD [77, 78].

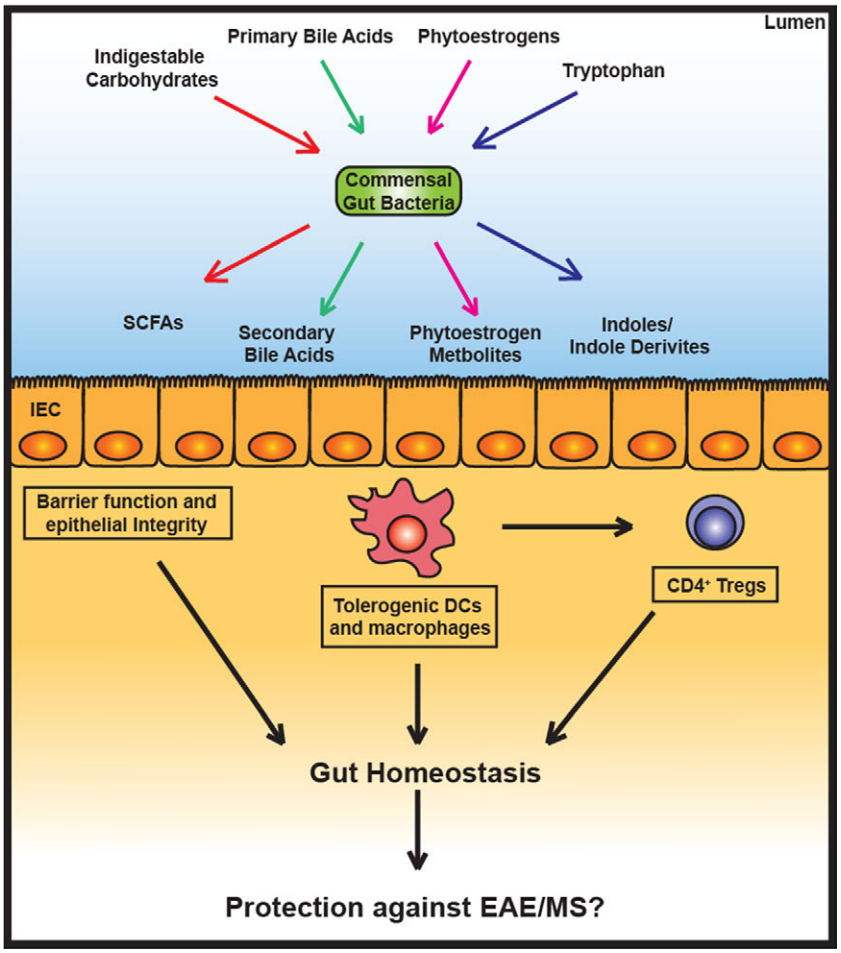

Fig. 1 Schematic of the bacterial metabolic pathways that influence the host immune system. Metabolism of indigestible carbohydrates, primary bile acids, phytoestrogens, and tryptophan by commensal gut bacteria results in production of metabolites such as short-chain fatty acids (SCFAs), secondary bile acids, phytoestrogen metabolites, and indoles/indole derivatives, respectively. These bacterial metabolites exert anti-inflammatory effects on mucosal immune cells, and has been demonstrated *in vitro* and *in vivo*. Bacterial metabolites can 1) promote barrier function and epithelial integrity; 2) induce tolerogenic dendritic cells (DCs), which trigger the polarization of $CD4^+$ T cells into interleukin (IL)-10 and/or transforming growth factor (TGF)-β-producing $FoxP3^+$ regulatory T cells (Tregs); and 3) directly induce IL-10 and/or TGF-β producing $FoxP3^+$ Tregs. This tolerogenic intestinal environment may influence the peripheral immune system, and thereby lead to protection against and/or amelioration of experimental autoimmune encephalomyelitis (EAE)/multiple sclerosis (MS). IEC = intestinal epithelial cell

Faecalibacterium prausnitzii supernatant induces anti-inflammatory effects in human peripheral blood mononuclear cells and a model of colitis based on induction by 2,4,6-trinitrobenzenesulfonic acid [79–81].

Bacteroides fragilis is a well-studied member of the *Bacteroides* genus and has been shown to induce IL-10-producing $FoxP3^+$ Tregs by producing polysaccharide A [82]. *Bacteroides fragilis* has also been shown to participate in SCFA metabolism [51]. *Bacteroides thetaiotaomicron* produces acetate, which is associated with modulation of mucus production in colonic epithelial goblet cells [83]. *Butyricimonas* is a SCFA-producing bacterium predominant in the gut, and its abundance correlates negatively with the expression of proinflammatory genes in T cells and monocytes from patients with MS [24]. *Prevotella* is a commensal bacterium that plays a dominant role in metabolizing xylans to SCFAs [67]; its abundance has been reported to be low in patients with MS [19, 20, 24, 28] and patients with rheumatoid arthritis (RA) [36]. Additionally *Prevotella histicola* has been shown to suppress EAE and collagen-induced arthritis in humanized mice [84, 85]. Although abundance of *Prevotella* in MS has been consistent with studies showing either a lower abundance [19, 20, 28] or an increase after treatment in patients with MS [24], the role of *Prevotella* in RA remains uncertain, as 1 study has reported a higher abundance of *Prevotella* in patients with early-onset RA [38]. It is possible that the apparent contradictions regarding the *Prevotella* results are due to differences in the specific species and strains utilized in the studies, and/or to differences in the patient populations [86]. Notwithstanding the uncertainty on a few points covered above, the majority of the

available data suggest that a reduction in SCFA-producing bacteria contributes to the proinflammatory state in patients with MS.

Using the EAE model, Haghikia et al. [87] demonstrated that SCFA treatment induces Tregs in the gut and ameliorates EAE. Chitrala et al. [88] showed that fecal transplant from CD44$^{-/-}$ donors to wild-type (WT) hosts ameliorated EAE, which they attributed to the increase in abundance of SCFAs in the stool. Mizuno et al. [89] also demonstrated that oral treatment with SCFAs or a high-fiber diet (results in high SCFA levels in the cecum) ameliorated EAE in C57Bl/6 mice, and this was accompanied by a greater frequency of Tregs in the draining lymph nodes [89]. The anti-inflammatory properties of SCFAs have been studied in other models of autoimmune diseases as well. For example, Marino et al. [90] demonstrated that nonobese diabetic mice receiving special diets designed to release particular SCFAs in the colon were less prone to develop spontaneous T1D [90]. In addition to their EAE findings, Mizuno et al. [89] demonstrated that oral administration of SCFAs ameliorates collagen-induced arthritis. It should be noted, however, that SCFA administration exacerbated K/BxN serum transfer arthritis; a model that, unlike collagen-induced arthritis, does not require adaptive immune responses and instead relies on innate immunity [89]. Thus, SCFAs may exert bimodal effects dependent on the cell type they are influencing. In a model of intestinal inflammation, dextran sulfate sodium-induced colitis, lack of the SCFA receptor GPR43, expressed on a variety of myeloid cell types, resulted in the development of more severe disease than in WT mice [91]. Moreover, in GF mice, which also exhibit exaggerated dextran sulfate sodium-induced colitis, treatment with SCFAs ameliorated intestinal inflammation [91]. Indeed, SCFAs have a demonstrated therapeutic potential in patients with IBD when given as an enema [92].

Collectively, the published findings on SCFA-producing bacteria suggest that they could influence immune responses involved in local or systemic pathophysiological states through their modulation of IECs and/or leukocytes (Fig. 1). The development of novel and more effective therapeutic interventions is likely to benefit from future assessment of the contribution of SCFA-producing bacteria to pathological states, such as MS.

Bile Acid Metabolism

Bile acids—steroid acids produced by the liver—aid in the digestion and absorption of dietary fats and fat-soluble vitamins [93]. Hepatocytes synthesize primary bile acids, which undergo a conjugation reaction with glycine or taurine before being secreted to the gallbladder [94]. After a meal, the gallbladder releases bile into the duodenum. In the distal ileum, conjugated bile acids are later reabsorbed by active transport and returned to the liver via the portal circulation to be utilized for subsequent digestions [94]. This process is termed enterohepatic circulation. While 95% of primary conjugated bile acids are efficiently returned to the portal circulation, the remaining 5% escape active transport and become substrates for bacterial metabolic reactions [93, 95]. The major microbial metabolic modifications, including deconjugation, the oxidation of hydroxy groups, and 7α/β dehydroxylation, result in the production of secondary bile acids [95]. Unlike the primary bile acids, which are conjugated, the secondary bile acids cannot participate in active reuptake. Some secondary bile acids are absorbed through the colon by passive diffusion, whereas others are excreted in feces [94]. Both primary and secondary bile acids can modulate host hormonal and immunological processes by stimulating nuclear receptor farnesoid X receptor (FXR) or cell-surface receptor G protein-coupled bile acid receptor 1 (GPBAR1 also known as TGR5) [54, 96].

Within the intestinal lumen, bile acids can influence barrier function and defense mechanisms via FXR^{+} and GPBAR^{+} IECs. A study using the biliary obstruction model of intestinal injury demonstrated that FXR plays a crucial role in protecting against bacterial overgrowth and the disruption of epithelial integrity [97]. In a separate study, feeding rats primary bile acids prior to bile-duct ligation led to a decrease in intestinal injury [98]. Similarly, GPBAR1$^{-/-}$ mice develop abnormal intestines characterized by altered tight junctions, irregular mucus-cell morphology, and increased susceptibility to colitis [99]. Furthermore, treatment of the human intestinal epithelial cell line Caco-2 with secondary bile acids resulted in decreased IL-8 production after stimulation with IL-1β [55].

Bile acids may also be involved in regulating leukocytes in the gut. FXR$^{-/-}$ mice exhibit a proinflammatory colonic phenotype and develop exacerbated trinitrobenzenesulfonic acid-induced colitis [100]. Additionally, macrophages from these animals exhibit enhanced proinflammatory cytokine response to stimulation with LPS [100]. This study also revealed that FXR agonists attenuated the expression of IL-1β and tumor necrosis factor (TNF)-α in LPS-stimulated murine lamina propria CD11b^{+} cells and human THP-1 cells [100]. Several other studies demonstrated that GPBAR1 agonists decrease nuclear factor kappa B (NF-κB) activity in macrophages and monocytes [101–103]. Recently, it was shown that GPBAR1$^{-/-}$ mice developed exacerbated colitis and enhanced recruitment of classically activated M1 macrophages [104]. In contrast, GPBAR1 agonist treatment alleviated colitis and shifted the polarization of lamina propria macrophages from M1 to alternatively activated M2 macrophages; a process reliant on IL-10 [104]. GPBAR1 agonists also inhibit the production of proinflammatory cytokines in primary human macrophages but not anti-inflammatory IL-10 [105]. The differentiation of monocytes into DCs in the presence of bile acids results in an IL-12 hypoproducing phenotype that is dependent on

GPBAR1 [106]. However, once DCs are terminally differentiated, they do not respond to GPBAR1 stimulation as the expression of this receptor is downregulated over the course of differentiation [106]. Further, bile retinoids (which are distinct from bile acids) imprint $CD103^+$ DCs within the lamina propria of the small intestine with retinol-metabolizing activities, enabling them to generate gut-tropic effector T cells and inducible Tregs [107]. Interestingly, both the retinoic acid receptor and FXR dimerize with retinoid X receptor, suggesting possible interplay between these 2 pathways [107]. Collectively, these studies indicate that bile acids have a central role in maintaining intestinal homeostasis.

The majority of these studies do not directly test the contribution of primary *versus* secondary bile acids. However, fecal samples from patients with IBD have increased and decreased abundance of primary and secondary bile acids, respectively. This suggests that a decrease in the amount of secondary bile acids may play a role in the proinflammatory state of patients with IBD [55]. Indeed, primary bile acids can stimulate *Clostridium difficile* growth and spore formation, whereas secondary bile acids inhibit outgrowth [93, 108]. Recent studies have shown that antibiotic treatment results in a higher primary to secondary bile acid ratio in fecal samples, which may promote *C. difficile* infection [93, 109]. Furthermore, primary bile acids generated from a high milk-fat diet promote the growth of *Bilophila wadsworthia*, leading to exacerbated colitis in genetically susceptible $IL\text{-}10^{-/-}$ mice [110]. These finding suggest that the ability of particular primary or secondary bile acids to promote or protect against disease is context dependent. Bile acids may also modulate CNS function directly. Primary and secondary bile acids are present in rat brain under homeostatic conditions, and may interact with $GPBAR1^+$ microglia [111, 112].

Several bacteria capable of bile acid metabolism are depleted (*Lactobacillus*, *Clostridium*, Erysipelotrichaceae, and *Parabacteroides*) or enriched (*Acinetobacter*, *Bifidobacterium*, *Pseudomonas*, and *Bilophila*) in patients with MS (Table 2) [19, 20, 22, 26]. *Lactobacillus*, *Clostridium*, Erysipelotrichaceae, and *Parabacteroides* have been shown to exert anti-inflammatory effects. *Lactobacillus* species are a common ingredient in a number of probiotics, and exert anti-inflammatory effects in both *in vitro* and *in vivo* settings [113]. THP-1 cells exposed to *Lactobacillus* supernatant produced lower levels of TNF-α [114], possibly owing to its participation in bile acid metabolism [115, 116]. Certain *Clostridium* species have also demonstrated anti-inflammatory activities via bile acid metabolism. *Clostridium scindens*, which belongs to cluster XIVa, protects against *C. difficile* infection by their production of secondary bile acids [117, 118]. Patients with MS are depleted of Clostridia clusters XIV (as well as IV), further supporting a role for producers of secondary bile acids in maintaining immune homeostasis in the gut [20]. The Erysipelotrichaceae family plays a major role in bile-acid metabolism [119], and some members exert anti-inflammatory effects on the host immune system [19, 120]. *Parabacteroides* can also generate secondary bile acids [121], and this might be responsible for its anti-inflammatory effects [122].

With regard to bile acid-metabolizing bacteria for which patients with MS are enriched, *Bilophila* and *Pseudomonas* promote proinflammatory responses. As mentioned above, *Bilophila* utilizes primary bile acids that promote colonic inflammation in genetically susceptible mice [110]. Furthermore, bile-acid signaling via FXR promotes *Pseudomonas* biofilm formation, and this likely contributes to its proinflammatory effects [123, 124]. Similarly, *Acinetobacter baumannii* promotes biofilm formation, and is an important pathogen associated with nosocomial infections [125]. Other members of the same genus have also been shown to participate in bile-acid metabolism [126]. Finally, *Bifidobacterium* expresses enzymes that are necessary for bile-acid metabolism [127]. Although interactions between bacteria and bile acids can result in proinflammatory effects, the majority of studies indicate that bile-acid signaling pathways promote anti-inflammatory responses. In support of this, 2 separate studies showed that the activation of either FXR or GPBAR1 suppresses lymphocyte and myeloid cell activation, resulting in an attenuation of EAE [128, 129]. Collectively, these studies support the notion that bile-acid metabolism plays a major role in immune regulation both within and outside of the intestine (Fig. 1). Future work distinguishing the roles of primary *versus* secondary bile acids in modulating immune responses in EAE/MS will aid in our understanding of the contribution of this metabolic pathway to disease.

Phytoestrogen Metabolism

Phytoestrogens are plant-derived compounds that are present in diet and can be categorized into 4 major classes: isoflavones, prenylflavonoids, coumestans, and lignans [130, 131]. Gut bacteria metabolize isoflavones and lignans into equol and enterolactone, respectively [130, 131]. Isoflavones are most abundant in soybeans/soy products and lignans are most abundant in flaxseed [132]. Phytoestrogens are structurally similar to mammalian estrogen and can be either agonistic or antagonistic to estrogen receptors (ER), with a preference for ERβ [133, 134]. Gut bacteria play a critical role in the metabolism and bioavailability of lignans [135] and isoflavones [136] by providing the enzymes necessary for their conversion to enterolactone and equol, respectively. After gut bacteria hydrolyze and deglycosylate phytoestrogens, the resulting metabolites are more readily absorbed by the intestine [130, 136]. From there, they may be transported to the liver to have systemic effects on immune modulation.

Phytoestrogens may have an impact on various aspects of immunity, both in the intestine and systemically. With regard to the colonic lumen, studies on IECs (*in vitro*) have shown that they modulate host-cell behavior, as both primary intestinal cells and IEC cell lines express ERβ [137]. Several studies have demonstrated that isoflavones suppresses the production of proinflammatory cytokines and exert protective effects on barrier function in IEC cell lines [138, 139]. Isoflavones also inhibit LPS-induced nitric oxide production and inducible nitric oxide synthase expression in a dose-dependent manner [140], and downregulate NF-κB and extracellular signal–regulated kinase activation [141]. Lignans also have an immunosuppressive effect on IECs, reducing their expression of monocyte chemoattractant protein-1 in response to LPS treatment [142].

Phytoestrogens may also modulate the phenotype of immune cells in the gut. Isoflavones and lignans suppress macrophage/monocyte activity by inhibiting reactive oxygen species release, cyclooxygenase-2 and NF-κB activation, and proinflammatory cytokine production after LPS stimulation *in vitro* [139, 143–146]. DCs express ERs, and therefore their behavior may also be modulated by phytoestrogens [147]. Similar to their effects on macrophages/monocytes, phytoestrogens downregulate LPS-induced production of proinflammatory cytokines and antigen-presenting machinery in DCs [139]. Phytoestrogens may also influence Treg function, as treatment with them increases FoxP3, transforming growth factor-β, and cytotoxic T lymphocyte-associated protein 4 expression in splenocytes [148]. Genistein, an abundant isoflavone, is a known tyrosine kinase inhibitor, and, at high concentrations, may inhibit lymphocyte proliferation [130]. Phytoestrogen metabolites, which are found in circulation, may be able to access the CNS and protect against neurodegenerative insults as various regions in the CNS express ER [134, 149–151]. These observations suggest that bacterial metabolites not only modulate immune function, but also may influence the CNS directly.

Patients with MS have a reduced abundance of *Prevotella*, *Parabacteroides*, *Adlercreutzia*, *Slackia*, and *Lactobacillus*, that have the ability to metabolize phytoestrogen compounds (Table 2) [19, 24]. *Prevotella* species were shown to metabolize phytoestrogens into beneficial metabolites [152], and this may contribute to its anti-inflammatory effects [84, 153]. The ability of *P. histicola* to induce Tregs and suppress EAE in humanized mice [85] suggests that metabolism of phytoestrogen by *Prevotella* might be one of the major mechanism of disease suppression. *Parabacteroides* metabolize phytoestrogens and their presence is correlated with remission in patients with Crohn's disease [19, 122, 154]. *Adlercreutzia equolifaciens* and *Slackia* are other equol producers in the gut [136, 155, 156]. *Lactobacillus*, a major milk fermenter, can confer anti-inflammatory effects on immune cells, possibly through its bioconversion of phytoestrogens [157, 158]. Given these findings, it is reasonable to hypothesize that phytoestrogens and their metabolites might confer protection in EAE and/or MS by inducing anti-inflammatory responses. Several studies have demonstrated that therapeutic administration of phytoestrogens (genistein and daidzein) ameliorates EAE, which is correlated with a decrease in the production of proinflammatory cytokines and an increase in the production of anti-inflammatory cytokines [159–164]. Phytoestrogens are also protective in the nonobese diabetic mouse model of T1D [165] and were associated with shifts in the gut microbiome [166]. A recent study by Berer et al. [27] demonstrated that mice transplanted with fecal samples from healthy human controls, which developed a lower frequency of spontaneous EAE than mice transplanted with MS fecal samples, harbored a higher abundance of *Adlercreutzia* then MS fecal-treated mice. This suggests that phytoestrogen metabolism may confer protection in EAE [27]. However, a direct role of equol and enterolactone (end metabolites of phytoestrogen) in EAE/MS is unknown. Studies are currently underway in our laboratory to decipher their role in modulation of immune responses and EAE.

Aside from their effects on autoimmune models, phytoestrogens are well known for their health benefits in treating menopausal symptoms and various diseases such as cardiovascular diseases, type 2 diabetes, and cancer [130, 167–169]. However, the results of clinical trials for the therapeutic use of phytoestrogens have been mixed [170, 171]. A possible explanation for the failure of phytoestrogens to provide health benefits in clinical trials is the loss of specific commensal bacteria in the gut required for the conversion of these phytoestrogens into beneficial metabolites such as equol and enterolactone. Based on our data and published reports, we propose that metabolism of phytoestrogen into equol and enterolactone by microbes of the gut plays a critical role in maintaining immune homeostasis because they induce anti-inflammatory responses (Fig. 1).

Tryptophan Metabolism

Tryptophan, an essential amino acid present in a variety of protein-based foods, is metabolized by certain gut bacteria into indole and indole derivatives and these can modulate the effectiveness of both the intestinal barrier and immune cells by activating the aryl hydrocarbon receptor (AhR) or pregnane X receptor [50, 172]. AhR plays a major role in regulating mucosal immune responses, as demonstrated by microbial perturbations in $AhR^{-/-}$ mice [173]. In addition, the generation of AhR ligands by commensal bacteria confers protection from inflammation and leads to modulation of the expression of both pro- and anti-inflammatory genes [172].

Tryptophan metabolites strengthen barrier function, increase mucin production, and attenuate TNF-α-mediated

production of proinflammatory cytokines [174, 175]. Activation of AhR by tryptophan metabolites induces expression of the IL-10 receptor on IECs, suggesting an anti-inflammatory phenotype [176]. AhR activation also influences intestinal epithelial integrity indirectly through activation of group 3 innate lymphoid cells (ILC3), which require AhR for complete functionality and to produce IL-22, a cytokine essential for maintaining barrier integrity [177]. Tryptophan metabolites also activate pregnane X receptor, which helps maintain integrity of the gut barrier and enhances the healing of intestinal wounds *in vivo* [178, 179]. Tryptophan metabolites may also modulate immune cell function via AhR, as its expression in $CD11c^+$ DCs is critical for immune homeostasis [180]. Further, AhR is required for the expression of indoleamine 2,3-dioxygenase, an immunosuppressive enzyme involved in tryptophan metabolism [181]. Given that indoleamine 2,3-dioxygenase-expressing DCs can limit effector responses and promote the expansion of Tregs [182], AhR activation modulates tolerogenic DC function, possibly by bacterial-derived metabolites. Indeed, tryptophan catabolites from gut bacteria confer mucosal homeostasis by engaging AhR [183]. In addition to modulating immune function, indole metabolites may modify neural function directly [184].

Lactobacillus, which patients with MS are depleted of, is well known for its involvement in tryptophan metabolism (Table 2) [19, 50]. Transfer of *Lactobacillus* to mice genetically susceptible to colitis ($CARD9^{-/-}$) resulted in attenuation of inflammation in an AhR-dependent manner [185]. With regard to EAE, rats highly resistant to EAE harbor a diverse group of *Lactobacillus* species [186], and mice administered such a mixture exhibited milder EAE accompanied by IL-10-producing Tregs [187]. Furthermore, we observed that suppression of EAE by *P. histicola* in HLA transgenic mice was associated with an increase in the abundance of *Lactobacillus* [85]. In addition to Tregs, other gut-associated immune cells can exert regulatory function in EAE. Kadowaki et al. [188] demonstrated that transfer of $CD4^+$ induced intraepithelial autoreactive T cells from myelin oligodendrocyte glycoprotein-specific T-cell receptor transgenic (2D2) mice suppressed EAE. Gut environmental stimuli that contribute to the development of these cells include gut microbiota and gut microbiota-derived dietary compounds such as Ahr ligands [188]. Furthermore, administration of microbial tryptophan metabolites to mice reduced CNS inflammation and EAE, and patients with MS have lower circulating levels of AhR agonists [189]. These studies highlight the potentially significant role of tryptophan metabolites in MS.

Choline Metabolism

Gut bacteria can modulate lipid levels through their effects on choline metabolism [172]. Choline is a water-soluble nutrient essential for cell signaling, the structural integrity of cell membranes, neurotransmission, and biosynthetic reactions [190, 191]. Certain gut bacteria metabolize dietary choline into an intermediate precursor, trymethylamine, which is further metabolized in the liver to generate trimethylamine N-oxide (TMAO) [172]. Most of the studies examining the relationship between the gut microbiota and choline metabolism have been performed in the contexts of obesity, atherosclerosis, and cardiovascular disease. However, obese individuals have an increased risk of MS development [192], and obesity has been shown to modulate host physiology by influencing the gut microbiota and systemic inflammation [193, 194].

The molecular mechanisms underlying TMAO-induced pathogenicity are poorly understood because its receptor is unknown [172]. TMAO exacerbates atherosclerosis, and its levels are elevated in patients with cardiovascular disease and obesity [195, 196]. In the atherosclerotic $ApoE^{-/-}$ mouse model, supplementation of diet with high levels of choline promotes atherosclerosis and the formation of lipid-rich macrophages (foam cells) [197]. Additionally, in mice, atherosclerosis susceptibility can be transferred from donor to host by fecal transplantation [198]. *Acinetobacter baumannii* participates in choline metabolism, and the abundance of *Acinetobacter* is high in patients with MS (Table 2) [26, 199]. Thus, choline-metabolizing bacteria potentially play a role in MS pathogenesis by modulating lipid levels and/or obesity.

Mucin Degradation

The mucus layer of the digestive tract, secreted by goblet cells, consists of a heterogenous population of glycoproteins (mucins), salts, lipids, and other proteins such as immunoglobulins and growth factors [200, 201]. The mucus layer sits at the interface between the intestinal epithelium and the lumen, providing a selective barrier against damage, coming into direct contact with bacteria. The mucus layer is also an adhesive surface that is colonized and used as an energy source by commensal bacteria [200, 201]. Thus, the mucus layer is a crucial physical layer between bacteria and the host cells, and serves as a scaffold for the attachment of commensal bacteria. The colonic epithelium of GF mice has a significantly thicker mucus layer than that of its WT counterparts, owing to lack of mucin degradation by commensals and subsequent water retention [200]. Indeed, a certain amount of mucin degradation by commensal bacteria is an important aspect of homeostatic mucus turnover [200]. However, excessive mucin degradation may allow direct exposure of luminal antigens to the intestinal immune system, and/or provide by-products for other pathogenic bacteria to scavenge [202]. Whether mucin degradation is protective or pathogenic for the host depends on the bacterium utilizing the pathway and the context of the model system.

Goblet cells are an integral part of the mucosal immune system, as they not only provide mucus, which separates host cells and the external environment; they also provide luminal antigens to DCs of the lamina propria in the small intestines [203, 204]. Interestingly, only tolerogenic $CD103^+$ DCs were found to be capable of participating in this interaction. Thus, it may be possible that the modulation of goblet cell function by mucin degradation can influence the anti-inflammatory aspects of the intestinal immune system.

Several bacterial species that are capable of degrading mucins are increased in patients with MS including *Akkermansia muciniphilia Ruminococcus*, *Bifidobacterium*, and *Dorea* [19, 22, 25–27]. Whether these bacteria exhibit proinflammatory activities by mucin degradation is controversial. Whereas the presence of *A. muciniphilia* exacerbates colonic pathology in *Salmonella typhimurium*-induced intestinal inflammation [205], it has also been shown to exert anti-inflammatory properties and correlate negatively with IBD, T1D, and obesity [32, 206, 207]. *Ruminococcus gnavus*, however, is present at high levels in patients with IBD and may promote dysbiosis [32]. *Bifidobacterium* can induce different levels of expression of pro- and anti-inflammatory cytokines, depending on the strain [208]. *Dorea*, which does not directly metabolize mucins but feeds on by-products released by mucin degradation, is associated with IBD [207, 209]. Further, ceratin species of *Dorea* might be pro-inflammatory as they can induce interferon-γ [210]. Collectively, these studies suggest that mucin-degrading bacteria may promote inflammation in patients with MS, depending on the composition of the bacterial community.

Other Mechanisms: Molecular Mimicry

Molecular mimicry is defined as significant structural homology between a given microbial antigen and self-antigen, resulting in aberrant immune responses to the latter peptide following exposure to the microbe [211]. Although molecular mimicry is proposed as a possible cause of autoreactive T-cell activation, conclusive supporting experimental evidence has been elusive. Certain components of the gut microbiota have been shown to share sequence similarities with encephalitogenic myelin peptides, suggesting that gut bacteria may contribute to MS through molecular mimicry [212]. *Pseudomonas*, which is present at high levels in patients with MS [19], has structural similarity to myelin basic protein (a dominant CNS antigen) and can activate myelin basic protein-specific T-cell clones [213]. Peptides from *Escherichia coli*, a common gut commensal, have been shown to provoke MS-like disease in a mouse model expressing a humanized T-cell receptor transgene [214]. However, the biological relevance of these findings to patients with MS is unclear.

Concluding Remarks

Recent studies showing that patients with MS are enriched or depleted of certain bacteria highlight the importance of the gut microbiota in the development of this disease. Gut microbiota may participate in MS pathogenesis by modulating host immunity through the regulation of multiple metabolic pathways (including those for SCFAs, bile acids, phytoestrogens, tryptophan, choline) and mucin degradation. The participation of certain bacteria in more than one metabolic pathway adds a layer of complexity, requiring an understanding of potential cross-talk between bacterial metabolites and host immune responses. For example, the addition of isoflavones to anaerobic bacteria from fecal samples results in the enrichment of SCFAs, as well as equol [215]. *Lactobacillus*, *Prevotella*, and *Parabacteroides* are examples of such bacteria because they can induce production of SCFAs in addition to metabolizing phytoestrogens. Similarly, *Lactobacillus* can participate in the metabolism of SCFAs, phytoestrogens, and tryptophans. Future studies examining these aspects in a single experiment might help to better understand cross-talk among bacteria and various metabolic pathways in the context of MS. However, the majority of microbiome studies are based on a single time point in patients with MS with established disease and thus it is possible that some of the reported changes in the microbiota are due to inflammation itself. Future studies should be based on longitudinal collection of samples to determine whether disease relapse is associated with presence of certain gut bacteria. Additionally, the colonization of GF mice with gut bacteria that are associated positively or negatively with MS can be tested for the ability to either induce disease or protect against it.

Acknowledgments This work was supported by funding from the National Multiple Sclerosis Society (RG 5138A1/1T), the Carver College of Medicine at the University of Iowa (pathology pilot grant), a Carver Trust Medical Research Initiative Grant, and the Mayo Clinic Center for Individualized Medicine. S.N.F. was supported on institutional training grant (T32AI007485 to Gail Bishop).

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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Review

Vitamin D as a Nutri-Epigenetic Factor in Autoimmunity—A Review of Current Research and Reports on Vitamin D Deficiency in Autoimmune Diseases

Artur Mazur [1], Paulina Frączek [2,*] and Jacek Tabarkiewicz [2,3]

[1] Institute of Medical Sciences, Medical College of Rzeszow University, University of Rzeszów, 35-310 Rzeszow, Poland
[2] Department of Human Immunology, Institute of Medical Sciences, Medical College of Rzeszow University, University of Rzeszów, 35-310 Rzeszow, Poland
[3] Centre for Innovative Research in Medical and Natural Sciences, Medical Faculty, University of Rzeszów, 35-310 Rzeszow, Poland
* Correspondence: pafraczek@ur.edu.pl

Abstract: Epigenetics is a series of alterations regulating gene expression without disrupting the DNA sequence of bases. These regulatory mechanisms can result in embryogenesis, cellular differentiation, X-chromosome inactivation, and DNA-protein interactions. The main epigenetic mechanisms considered to play a major role in both health and disease are DNA methylation, histone modifications, and profiling of non-coding RNA. When the fragile balance between these simultaneously occurring phenomena is disrupted, the risk of pathology increases. Thus, the factors that determine proper epigenetic modeling are defined and those with disruptive influence are sought. Several such factors with proven negative effects have already been described. Diet and nutritional substances have recently been one of the most interesting targets of exploration for epigenetic modeling in disease states, including autoimmunity. The preventive role of proper nutrition and maintaining sufficient vitamin D concentration in maternal blood during pregnancy, as well as in the early years of life, is emphasized. Opportunities are also being investigated for affecting the course of the disease by exploring nutriepigenetics. The authors aim to review the literature presenting vitamin D as one of the important nutrients potentially modeling the course of disease in selected autoimmune disorders.

Keywords: nutrition; epigenetics; autoimmunity; vitamin D

Citation: Mazur, A.; Frączek, P.; Tabarkiewicz, J. Vitamin D as a Nutri-Epigenetic Factor in Autoimmunity—A Review of Current Research and Reports on Vitamin D Deficiency in Autoimmune Diseases. *Nutrients* **2022**, *14*, 4286. https://doi.org/10.3390/nu14204286

Academic Editor: Carsten Carlberg

Received: 12 September 2022
Accepted: 11 October 2022
Published: 14 October 2022

1. Introduction to Epigenetic Modifications

The history of epigenetics is linked with the study of evolution and development. The first steps taken to understand epigenetics were based on observing the differentiation and specialization of cells and the adjustment of their metabolic functions with the knowledge that every somatic cell in the body contains the same genetic material. Since the beginning of interest in the topic of epigenetics, its definition has been changing and becoming more specific, leading to its current understanding as the presence of patterns of gene expression that can be inherited, and which are the foundation for changes in the phenotype. The pattern of these gene expressions is modified due to the influence of various factors, including the external environment (such as pollutants and radiation) and lifestyle (such as tobacco smoking, alcohol consumption, or inadequate diet). From studies on Agouti rodents and bee populations to studies involving a famine-exposed population during World War II, we have learned that food—the amount and type of nutrients—has an undeniable long-term effect on the lives of offspring, exerted by epigenetic mechanisms. As already mentioned, some of these mechanisms are hereditary, so epigenetic marks can potentially persist during development and be passed from offspring to offspring, which also affects the lives of grandchildren and possibly further generations [1,2].

Regulation of gene expression by activating or silencing genes is accomplished through simultaneous mechanisms that include i.a. DNA methylation, histone modification, and profiling of non-coding RNA. The process of DNA methylation is mediated by the DNA methyltransferase (DNMT) family, which transfers methyl groups on DNA strands. There are four known patterns of methylation, with the most widely studied being the methylation of CpG islands located in promoter regions of genes, and similar methylation of CpG island shores. Other patterns are methylation in gene bodies, and in repetitive sequences, which protects chromosomal integrity. Methylation results in silencing the gene. It can be done either by blocking the availability of heterochromatin for transcription in the promotor region or in the gene body itself. Among the DNMT family, we can distinguish subtypes, involved in methylation during embryonic development (de novo methylation), such as DNMT3A and DNMT3B, as well as those involved in methylation of hemimethylated sites generated during DNA replication, such as DNMT1. There is also a ten-eleven translocation (TET) enzyme family, described as having a role in the DNA active demethylation process. TET1-3 are dioxygenases catalyzing 5-methylcytosine oxidation and promoting cytosine demethylation. The TET enzymes group has been especially studied in stem cell differentiation and early development, and more recently in carcinogenesis in multiple solid cancers [3].

Histones are proteins responsible for packing and ordering DNA. The modification processes such as methylation, demethylation, acetylation, deacetylation, phosphorylation, ubiquitination, SUMOylation, and ADP-ribosylation reorganize the structure of chromatin, thus regulating gene expression. Acetylation is performed by histone acetyltransferases (HATs) and results in higher gene expression, while deacetylation is catalyzed by histone deacetylases (HDACs) and results in gene suppression predominance. These two best-known histone modifications occur on lysine residues in the N-terminal tail. It is worth noting that the region and the number of histone methylations may result in various effects—silencing or expression of genes. At the same time, the co-occurrence of DNA methylation and histone methylation can have different resultant effects. The methylation status of histones depends on dynamic processes determined by the activity of methyltransferases (HMTs) and demethylases (HDMs). An example of HDM can be amine oxidase lysine-specific demethylase 1 (LSD1/KDM1A), which was described to affect the clinical outcome and recurrence risk in various cancers, including colon cancer [4]. It is worth noting that depending on the histone substrate, H3K4me2/me1 or H3K9me2/me1, the result of the LSD1/KDM1A activity can be gene repression or activation, respectively [5].

Noncoding RNAs (ncRNAs) are sequences of infrastructural and regulatory functions with no protein product. Among regulatory ncRNAs, there are microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and long non-coding RNAs (lncRNAs), each with a slightly different regulatory function—modulating the transcriptional and post-transcriptional expression or repression of genes, regulating chromatin formation, histone modifications, and DNA methylation [6].

The impact of vitamin D has been noted in various epigenetic phenomena in recent years, mainly in the context of a search for the pathogenetic basis of cancer. In complex machinery of interactions, the genes responsible for proper vitamin D signaling may themselves undergo epigenetic modifications. Due to the long CpG islands in the promoter regions of genes essential for vitamin D-dependent signaling, these genes are susceptible to methylation and may be silenced. The interplay between the VDR gene product, its ligands, coactivators, corepressors, chromatin modifiers, remodelers, and the genes encoding these elements, creates a complex structure that is a likely substrate for the development of pathology. In terms of DNA methylation status, there was a negative correlation found between 1,25(OH)2D levels and CpG methylation in adenomatous polyposis coli—a tumor suppressor related to colon cancer [7]. In further studies, vitamin D seemed to alter colorectal cancer risk by mediating the Wnt regulatory genes—a strong negative association of vitamin D intake with DKK1 and Wnt5a methylation has been noted [8]. 1,25(OH)2D was also found to reduce DNA methylation of the e-cadherin promoter, or even promote DNA

demethylation in human breast cancer [9,10]. The effect of vitamin D on the demethylation process was comprehensively described in the work of Fetahu et al. The authors gathered available data from studies describing the effect of VDR signaling on the expression of genes regulating oncogenic processes in patients with colorectal cancer and several other tumors. Attention was drawn to the cross-regulatory effect between VDR and histone demethylase activity. 1,25(OH)2D was found to increase the expression of the lysine-specific demethylase 1 and 2, JARID2, KDM5B, and KDM6B histone demethylases, while inhibiting others—KDM4A, KDM4C, KDM4D, KDM5A, KDM2B, JMJD5, JMJD6, and PLA2G4B. The effects of such broad actions are diverse, although they have been related, especially the inhibition of the KDM4 family, to genome stability, and the upregulation of KDM6B has been attributed to a possible antiproliferative role. [5,11] Higher vitamin D levels may also have an indirect influence on histone demethylases by mRNA [12,13].

A proper balance between epigenetic modifications is essential for embryonic development, division, differentiation, and maturation of all cells, including the cellular component of the immune system. As epigenetics has become a keenly explored topic in understanding the basis of cancer, its involvement is also being increasingly studied in the context of autoimmune diseases (ADs). The evidence so far has shown that epigenetic dysregulated modifications, including DNA methylation, histone modification, and ncRNAs, are involved in the pathogenesis of several ADs. Epigenetic mechanisms have also been discussed as a potential source of abnormal signaling interactions in various conditions, as well as the substrate of susceptibility of some individuals to developing different ADs [14–16].

2. Involvement of Vitamin D in Immunomodulation

Vitamin D in its most common form, cholecalciferol, is a micronutrient crucial for human health. Supplied from the diet or synthesized in the epidermis from 7-dehydrocholesterol, it has to be modified to obtain biological activity. Through progressive hydroxylation it is converted into 25-hydroxycholecalciferol (25(OH)D), and then the active form, 1,25-dihydroxycholecalciferol (1,25(OH)2D), a hormone that performs numerous functions. Interestingly, the conversion from 25(OH)D to 1,25(OH)2D occurs mainly in the kidneys with the CYP27B1, an enzyme stimulated by parathyroid hormone; however, the hydroxylation can be performed in other cells and tissues. Such an extrarenal hydroxylation, in various epithelial cells, parathyroid glands, and macrophages, is under the control of cytokines (e.g., TNFα or IFNγ) [17]. Circulating in the blood, 1,25(OH)2D binds with the specific vitamin D receptor (VDR), found in many tissues of the body, i.e., bone (osteoblasts and chondrocytes), muscle, adipose tissue, skin, epithelium, kidneys, pancreas, pituitary gland, as well as in immune cells: lymphocytes T, lymphocytes B, monocytes, natural killer cells, and antigen-presenting cells (APC) [18]. Apart from maintaining the calcium-phosphate balance and thus role in the correct structure and function of bones, vitamin D also takes a number of actions modulating immunological processes, which explains its interest in the context of autoimmunity. After binding to the VDR, vitamin D forms a heterodimer with the retinoid X receptor (RXR). The obtained complex activates the vitamin D response element and recruits enzymes responsible for histone acetylation. The ongoing structural changes in chromatin induced by the obtained complex result in targeted gene regulation [19]. More than 900 genes involved in both innate and adaptive immunity, as well as many other physiological interactions are under the control of 1,25(OH)2D and intracellular VDR connection. A deficiency of vitamin D has been therefore associated with a wide range of diseases, including cardiovascular, neuromuscular, and metabolic disorders, hypertension, neoplasm, as well as infectious and autoimmune diseases [20,21]. In terms of the innate response, vitamin D is proven to activate NK cells and NKT cells modulating the secretion of cytokines, such as IFN-γ and IL-4, with simultaneous suppression of macrophage activation and the domination of the M2 "anti-inflammatory" macrophages. On the other hand, the antimicrobial activity of macrophages induced by vitamin D is known—both the increased phagocytosis of *M. tuberculosis* and *P. aeruginosa*, as well as the induction of cathelicidin and

defensin secretion [22–24]. The protective function of 1,25(OH)2D through genomic and non-genomic mechanisms has been recently studied in COVID-19 patients. Worth noting is the non-genomic activity of vitamin D against SARS-CoV-2, involving active inhibition of the replication machinery of the virus. According to Qayyum et al., vitamin D and the inhibitory action of luminesterol on the M^{pro} viral protease and the viral RNA-dependent RNA polymerase may play a significant role in an active fight against SARS-CoV-2 infection and thus diminish the severity of COVID-19 progression in patients [25,26]. The infectious stimulus and the type of cell-producing cytokine seem to be crucial for the impact of vitamin D on the production of IL-8. 1,25(OH)2D may increase IL-8 production and thus the ability of neutrophils to respond to invading pathogens by recruiting additional neutrophils to the site of infection. However, vitamin D has been also described to decrease IL-8 release in hyperinflammatory macrophages [27]. Vitamin D is known to inhibit monocyte production of proinflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α. It also restrains the differentiation and maturation of dendritic cells with decreased expression of MHC class II molecules, co-stimulatory molecules, and IL-12, important in self-tolerance [28]. Studies show that dendritic cells can metabolize vitamin D for the programming of T cells. What is more, 1,25(OH)2D may also interact with dendritic cells directly and influence their migration and capacity to instruct T cells and hence to initiate, fine-tune, or suppress immune responses [29]. The role in promoting proliferation and effector functions of immunosuppressive T regulatory cells is especially explored. Vitamin D supplementation may increase Treg/CD3 ratios in both healthy individuals and patients with autoimmune disorders as well as the T regulatory cells function [30]. Vitamin D is responsible for the activation and proliferation of lymphocytes, and the differentiation of Th lymphocytes, resulting in a more balanced Th1/Th2 response that limits the development of self-reactive T cells preventing inflammation and autoimmunity [31]. This includes regulation of the Th17 lymphocyte population through an alternative pathway of 20-hydroxy- and 20,23-dihydroxyvitamin D synthesis involving related orphan receptors α and γ (RORα and RORγ). This is possible as RORα and RORγ have also been identified as major regulators of the said lymphocytes [32]. Moreover, the differentiation of B lymphocytes into plasma cells and the production of antibodies is under the inhibitory influence of vitamin D. It should be remembered that, although naïve B cells have a relatively small expression of VDR mRNA, the authors of the present study observed a three- and fourfold increase in VDR expression in the excited state with the use of anti-CD40/IL-21 and anti-IgM/anti-CD40/IL-21 [33]. In addition, vitamin D-mediated signaling inhibits the formation of memory B cells as well as immunoglobulin secretion in activated B cells. The IgA, IgG, and IgM production is suppressed, while data concerning IgE secretion are conflicting, which may be accounted for by the modulatory role of vitamin D and IL-4-dependent increase in IgE immunoglobulins due to Th2 skewing [34]. Broad immunomodulatory effects on cell populations of the immune system and their functional changes were presented by Cyprian et al., and earlier by Mora et al. [35,36]. The mechanisms described above are summarized in Figure 1.

Maintaining proper vitamin D levels is therefore crucial for physiological immune functions. At the same time, according to some sources, vitamin D deficiency is nowadays considered the most common medical condition, affecting more than a billion people worldwide [37]. A 25OH2D of <50 nmol/L or 20 ng/mL is defined as vitamin D deficiency. It affects the European population in varying degrees—from <20% of the population in Northern Europe, 30–60% in Western, Southern, and Eastern Europe (even around 90% in Poland) [38], and up to 80% in the Middle East. Similar trends with the range of 20–90% have been reported for Australia, India, Africa, South America, Turkey, and Lebanon, suggesting that vitamin D deficiency is a problem for both developing and developed countries [39]. Severe deficiency (serum 25OHD < 30 nmol/L or 12 ng/mL) is found in >10% of Europeans and in >20% of the populations of India, Tunisia, Pakistan, and Afghanistan [40]. These figures may vary by ethnicity in different regions of the countries listed, as well as by age groups, with lower vitamin D levels occurring in children and the elderly [37,41].

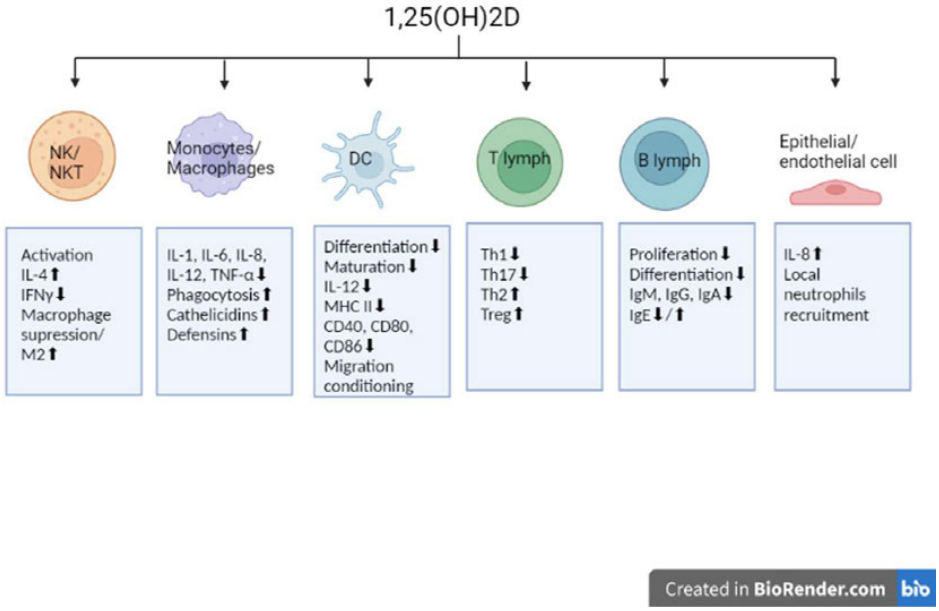

Figure 1. Immunomodulatory effect of 1,25(OH)2D on selected cells. NK, natural killer; NKT, natural killer T; DC, dendritic cell; T lymph, T lymphocyte; B lymph, B lymphocyte. Created with BioRender.com.

Knowing that the vitamin D status of the fetus and the newborn is completely dependent on the vitamin D levels in maternal blood, we better understand the importance of adequate supplementation during pregnancy and lactation in terms of proper modulation of the child's immune responses [42]. Studies in various countries have shown that vitamin D deficiency in pregnant women and infants is common, affecting 4% to 60% of the former and 3% to 86% of the latter [43–45]. In fact, adequate vitamin D levels determine the normal course of pregnancy from a very early stage, due to the necessary tolerance of the semi-allogenic fetus by the mother's immune system. Observational studies have linked vitamin D deficiency with preeclampsia, altered placental vascular pathology, abnormal fetal growth patterns, as well as the risk of preterm delivery [46].

In children, vitamin D deficiency is classically associated with the occurrence of rickets. In recent decades this disease remains a significant public health disorder despite the fortification of food. This is mainly due to the prevalence of the described deficiency. While skeletal symptoms are the most recognizable, it is the extraskeletal complications, hypocalcemic seizures, and cardiomyopathy that are the most devastating and cause the reported fatalities [47]. Newborns with vitamin D deficiency usually do not have overt defects in skeleton or calcium metabolism, while deficiency has been linked to a higher risk of a number of disorders in this age group: respiratory distress syndrome, lower respiratory tract infections, food sensitivities, asthma, autism, schizophrenia, and type I diabetes [42,48–50].

3. Vitamin D Deficiency and Epigenetic Dysregulations in Autoimmunity

In autoimmune conditions, the immune system misidentifies the host's own cells and tissues as foreign elements, which results in developing an immune response against them. According to a commonly accepted definition, in the background of ADs are certain genetic predispositions of an individual, which are superimposed by external factors—the so-called triggers that cause the disease to manifest. Undoubtedly, external factors can affect the epigenetic mechanisms described earlier and thus contribute to the development of ADs. Numerous interactions between epigenetic modifications and ADs, both systemic and organ-specific, have been identified. These correlations have been described in rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), Sjögren's syndrome (SjS), inflammatory bowel disease (IBD), multiple sclerosis (MS), type 1 diabetes (T1D), and others [16]. Reports of low serum vitamin D predicting the course of ADs in the future have

been published [28]. Vitamin D has also been shown to facilitate the progression of existing autoimmune diseases. In the study with undifferentiated connective tissue disease (UCTD) patients, the mean vitamin D level was significantly lower in the group that progressed to a definitive disease [51]. Disease activity has also been shown to correlate inversely with vitamin D in many but not all studies.

3.1. Systemic Autoimmune Diseases

SLE is believed to be the most studied autoimmune disorder correlated with epigenetic modifications. Among patients with ADs, a higher prevalence of vitamin D deficiency was commonly observed in SLE patients, which may be due to increased photosensitivity or possible renal complications of the disease, disrupting the effective hydroxylation of 25OHD [28,35]. At the same time, lower 25OHD levels found in SLE patients suggest that vitamin D deficiency may be a risk factor for the disease. The studies have also found higher SLE disease activity measured with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) associated with lower levels of vitamin D [52]. Similar correlations between low levels of vitamin D and disease activity and severity have been observed in other ADs such as MS and RA [28]. In their broad review, Mazzone et al. presented the importance of epigenetic alterations in ADs, including SLE patients. Attention was drawn to the methylation status of specific genes, such as CD11a (ITGAL), perforin (PRF1), CD70 (TNFSF7), and CD40LG (TNFSF5) in T lymphocytes as a factor in SLE pathogenesis and development. Histone modification patterns and the role of ncRNAs in SLE have been investigated to a lesser extent than DNA methylation. In general, the hypomethylation also observed in SLE is a result of the restricted availability of methyl donors and/or disruption of DNMT1 activity. Therefore, a balance between methyl donor, S-adenosylmethionine (SAM) as a factor dependent on dietary micronutrients, such as folate, zinc, methionine, choline, and vitamins, and DNMT1 seems to be the key to avoiding SLE flares [53]. A recent meta-analysis by Yang et al. evaluated the effect of VDR gene polymorphisms on susceptibility to developing SLE. As the researchers point out, links have been demonstrated between polymorphisms and different populations: between the VDR ApaI polymorphism and the susceptibility of the general population, between the VDR BsmI polymorphism and the susceptibility of the African and Caucasian populations, and between the VDR FokI polymorphism and the susceptibility of the African population [54]. The aforementioned polymorphisms—ApaI, BsmI, and FokI—are among the four polymorphic sites within the VDR gene, in addition to the TaqI polymorphism. The ApaI and BsmI sites are located in the intron portion of the gene, FokI in exon-2 is responsible for the creation of an alternative transcription initiation site, while TaqI in exon-9 is the result of a silent T to C substitution. All the mentioned sites can disrupt vitamin D metabolic pathways [55]. Although referenced study detected no significant correlation between TaqI polymorphisms and susceptibility to SLE in the populations assessed, further explorations concerning this conclusion need to be conducted. Dietary interventions in SLE are a frequently addressed topic of research since in this group of patients it was quite common to find vitamin and mineral deficiencies. Proper supplementation provides antioxidant, anti-inflammatory, and immunomodulatory effects, and thus makes it possible to reduce the severity or prevent the disease [53]. Currently, a diet rich in vitamins and minerals, and mono- and polyunsaturated fatty acids with moderate energy consumption preventing obesity and potentially cardiovascular disease is recommended to control the inflammatory findings of the disease and the complications and co-morbidities resulting from SLE therapy [56].

Although the incidence of RA does not begin to increase until after the age of 25, due to interesting reports of correlations between single nucleotide polymorphisms (SNPs) in vitamin D metabolic pathway genes and susceptibility to RA, it was decided to include it in this review. Multiple epigenetic factors in the pathogenesis and progression of the disease have already been identified. They include the role of RA synovial fibroblasts (RASFs), which through epigenetic modifications become a source of numerous pro-inflammatory cytokines. A participatory global hypomethylation in RASFs, associated with the acute course

of the disease, has also been described before [57]. In a study by Tian-Ping Zhang et al., ten SNPs in vitamin D metabolic pathway genes (CYP2R1, CYP24A1, VDR, and CYP27B1) were genotyped in an RA patient and a control group. The results showed that CYP2R1 and CYP27B1 genetic variations were associated with the genetic background of RA, while altered VDR and CYP27B1 methylation levels were related to the risk of RA [58]. The study also confirmed that vitamin D deficiency is prevalent among RA patients, and the 25OHD level is significantly lower compared with healthy controls. Subsequent studies yielded similar findings in this regard. At the same time, in view of previous reports of an increased risk of RA among Caucasians due to VDR gene polymorphisms and the FokI variant, an analysis of the association of four selected VDR gene polymorphisms (BsmI, FokI, ApaI, and TaqI) with susceptibility to RA in the Lithuanian population was conducted. However, the genetic analysis did not reveal RA susceptibility in the study group. Instead, a significant inverse correlation between vitamin D levels, DAS28, CRP, and HAQ scores was noted, indicating an association of vitamin D deficiency with increased disease activity and disability scores in these patients [59].

In SjS, the influence of vitamin D levels is still controversial. On the one hand, the lack of UV exposure as part of the treatment of cutaneous manifestations of the disease has been postulated as a risk factor for vitamin D deficiency, while on the other hand, the clinical picture of the disease and at least some of its manifestations appear to be reliant on vitamin D deficiency [60]. The deficiency has been linked to the appearance of peripheral neuropathy and non-Hodgkin lymphoma (NHL) in patients with SjS, which translates into mortality in this AD [61]. In terms of the impact on the nervous system, vitamin D was described to participate in the biosynthesis of neurotropic factors, production of enzymes for neurotransmitter synthesis, inhibition of inducible nitric oxide synthase (iNOS) synthesis as well as increasing levels of glutathione and gamma-glutamyl-transpeptidase [60,62]. Therefore, a correlation is being sought between vitamin D deficiency in patients with SjS and the severity of sensory or motor-sensory neuropathy, as well as abnormal corneal innervation and potentially more severe discomfort on the ocular surface [63]. Ro/SSA and La/SSB antibodies are the key serological findings associated with a congenital cardiac block in neonates of mothers with SjS. The vitamin D status was also studied as a potential factor for cardiac block development in children of SjS patients. Based on the observation of seasonal influence on the development of anti-Ro and anti-La positive congenital cardiac block, Ambrosi et al. suggested that low levels of vitamin D during 18 to 24 weeks of pregnancy could be involved in the pathogenesis of this disease. They found that average vitamin D levels for each month of pregnancy inversely correlated with the proportion of congenital heart block pregnancies [60,64,65]. Epigenetic studies are still needed to describe the complexity of primary SjS. As outlined in a recent review analysis by Imgenberg-Kreuz J, within the HLA genes we find the strongest genetic association with the occurrence of pSS. Attention is also drawn to the role of DNA hypomethylation and interferon-induced gene overexpression [66]. An association was also sought between the occurrence of VDR polymorphisms and susceptibility to primary SjS. In the currently only study on a Hungarian group evaluating this phenomenon, no such association was found for the BsmI, ApaI, TaqI, and FokI polymorphisms [67].

In patients with SSc—a chronic disease with vasculopathy, and visceral and cutaneous fibrosis—vitamin D deficiency of $\leq$30 ng/mL has been reported in up to more than 87% of cases [68]. Poor vitamin status seems to be related to a more aggressive disease with multivisceral and severe organ involvement—especially pulmonary and cardiac involvement. When comparing 25OHD levels, Groseanu et al. found no difference between diffuse and limited subtypes of the SSc [69]. In the systemic literature review by Schneider et al. the authors conclude that despite the vast literature on vitamin D deficiency and SSc incidence, it remains unclear if hypovitaminosis is an epiphenomenon or if it actually determines an increase in susceptibility and a worse prognosis for this complex disease. Analyses to date have postulated the involvement of impaired VDR signaling with reduced expression in fibroblasts from SSc patients and overactivation of TGF-β signaling. The study by Juan

Li et al. selected as many as eight single nucleotide polymorphisms (TaqI, FokI, ApaI, BsmI, Cdx2, BglI, Tru9I, and rs11168267) of the VDR gene and evaluated the association between their occurrence and susceptibility to SSc. It has been shown that ApaI and BglI genotypes may be important in the pathogenesis of SSc, while no significant association was found for the other single nucleotide polymorphisms. At the same time, no association was observed between the polymorphisms studied and the clinical picture of SSc [70]. Thorough research on the clinical effects of vitamin D supplementation in SSc patients as well as the clarification of vitamin D commitment in SSc pathogenesis is still needed [71].

The vitamin D status and the VDR function have also been of interest in studies on IBD. The expression level of VDR is high in the intestine, and therefore the role of surveillance of cell proliferation, barrier function, and immunity has been attributed to it. Vitamin D deficiency, low VDR expression, and dysfunction of vitamin D/VDR signaling have been observed in patients with Crohn's disease (CD) and ulcerative colitis (UC), and they were found to be related to the activity in both diseases [72]. Studies have shown that a low level of intestinal epithelial VDR is accompanied by a reduction in Atg16l1, an IBD risk gene, and a regulator of autophagy, which leads to dysbiosis and impaired autophagic responses [73]. Moreover, impaired vitamin D/VDR signaling fails to regulate the proper expression of several components of tight junctions and adherent junctions, which causes disruption of the mechanical barrier of the intestine and leads to a less effective mucosal healing process in murine models [74]. In a systematic electronic search by Vernia et al., the authors stated that vitamin D deficiency in IBD is multifactorial, resulting from inadequate sun exposure, unnecessary dietary restrictions, and, in some instances, impaired absorption of nutrients—a possible effect of an ongoing inflammation. Emerging evidence suggests that vitamin D deficiency may be implicated in a more aggressive disease behavior and an impaired response to biological therapy [75]. Deficiency of 25OHD has been associated with more hospitalizations, flare-ups, use of steroids, and escalating treatment [76]. Therefore, as vitamin D supplementation may prove to be one of the important elements of therapeutic management in IBD patients, reliable evidence is being sought to determine the dose of vitamin D effective for intervention. A limitation of most of the available data attempting to elucidate the molecular mechanisms of vitamin D action, including the effect on the maintenance of the mucosal barrier in the intestinal lumen, is that it comes from studies conducted in preclinical settings, making it difficult to translate the results into clinical management. The intestinal microbiota, which is a component of the vitamin D–IBD axis, should not be forgotten either. The ratio of commensal to pathogenic bacteria and its effect on the VDR was one of the elements studied within this topic [77,78]. The interplay of bacterial metabolites, butyrate, and lithocholic acid, as well as bacterial enzymes (e.g., capable of activating vitamin D) and growth factors on VDR signaling and the course of inflammation, has also been explored [75]. Guidelines on the practical use of probiotics as valuable components of treatment in IBD are also expected in the future [79].

3.2. Organ-Specific Autoimmune Diseases

The increasing incidence of type I diabetes mellitus (T1DM) has led to an active search for information on the interplay between diet and epigenetics in this disease. Understanding the diet–epigenome axis with vitamin D as one of the nutritional factors involved could potentially allow new diagnostic and therapeutic approaches for patients with T1DM. The pathogenesis of T1DM highlights known environmental factors associated with a higher risk of its development, including infections, dietary factors, advanced maternal age, psychological stress, antibiotic use, mode of delivery, and steroid intake [80]. Viral infections—especially by group B coxsackieviruses or echoviruses—have also been long considered as one of the most likely trigger candidates for T1DM [81]. Diet and nutrients as potential triggers of T1DM are still being studied with some interesting yet inconclusive insights. Evaluated dietary factors involve breastfeeding, early intake of cows' milk, solid foods (fruit, root vegetables, gluten, and non-gluten-containing cereals, and eggs), and vitamin D [82]. Most of the cohort studies showed that breastfeeding and vitamin D had

protective effects, whereas bovine milk and the early introduction of gluten were risk factors [83]. As Cerna et al. noted, these risk factors support the hypothesis that general antigenic stimulations are more important than actual antigens in the disease process. Combining these compounds with an immature immune response and insufficient tolerance in the gut, as well as a predisposition to inflammation due to a deficiency of long-chain polyunsaturated fatty acids, typical of the western diet, the hypothesis seems credible [84]. The concept of the influence of the gut microbiota on increasing or decreasing the risk of developing autoimmune diseases, including T1DM, is consistent with the above observations. Although the topic of the effect of breast milk on T1DM is controversial and one can also find sources in the literature that have not shown its protective role in the disease, it is believed that breastfeeding reduces intestinal epithelial permeability by preventing triggering factors [85,86]. In the past, vitamin D deficiency has been linked to the occurrence of T1DM, among others, based on epidemiological data showing that countries at northern latitudes had a high prevalence of T1DM [83]. Compared to healthy individuals, patients with T1DM also had lower 25OHD values [87–90]. This correlation was also supported by reports that vitamin D supplementation lowers the risk of developing T1DM, and that when supplemented appropriately (more than 30 ng/mL), vitamin D can help preserve residual ß-cell and insulin secretion, as well as improve glycemic control and insulin sensitivity [91,92]. A historical large-scale birth cohort study in Finland evaluated the effect of vitamin D supplementation on rickets and the development of type 1 diabetes and found an 80% reduction in the risk of T1DM in children who received >2000 IU of vitamin D per day compared to children receiving less or no vitamin D supplementation [93]. However, in a study by Bierschenk et al. comparing serum levels of 25OHD in patients with type 1 diabetes, first-degree relatives, and controls, the level of 25OHD was found to be low in all groups, and not specifically associated with T1D. The authors concluded that the uniform suboptimal 25OHD levels, despite residence in a zone with abundant sunshine, support additional dietary vitamin D fortification practices [94]. Important conclusions in terms of prenatal prevention of T1DM were reached in their study by Marjamäki et al. They found that maternal intake of vitamin D from food or supplements during pregnancy was not associated with advanced beta cell autoimmunity/type 1 diabetes or type 1 diabetes itself in Finnish offspring having increased genetic susceptibility to T1D [95].

Attention is also drawn to the role of the VDR and its polymorphism in T1DM. In recent years, a number of studies have examined the association of VDR gene polymorphisms with T1DM risk in different populations, with conflicting results. A recent meta-analysis of 39 case-control studies by Zhai et al. rejected any significant association between VDR gene polymorphisms and T1DM risk in the overall results. At the same time, the results of subgroup analysis revealed significant negative and positive associations between FokI and BsmI polymorphisms and T1DM in Africans and Americans, respectively. The authors emphasize that compared to the previous meta-analysis from 2014 by Tizaouia et al., apart from the association of VDR genetic polymorphisms with T1DM risk in different ethnic groups, the overall analysis was almost the same despite including further studies [96,97]. It is not entirely clear where all the differences in the observed vitamin D–diabetes axis of interactions come from. As Kohil et al. explain, some of these discrepancies can be attributed to differences in the type of supplement used (i.e., cholecalciferol, alpha-calcidiol, or calcitriol), the dose of the vitamin, the age group of the study participants, and/or the duration of diabetes [80]. Apart from the VDR polymorphism, the authors highlighted the potential role of CYP27B1 polymorphisms with a favorable genetic background for various autoimmune disorders including T1DM [82]. Knowing that the immune cells are also expressing CYP27B1 and thus modulate the immune response, the studies previously performed on the European population (German, British, and Polish) provide significant data concerning a potential decrease in the availability of the active form of vitamin D in people with the CYP27B1 promoter C(-1260)A polymorphism [98–101].

The pathogenesis of autoimmune thyroid diseases (AITDs) has been suggested to be multifactorial, including genetic, environmental, and hormonal factors, such as vitamin D

deficiency [102]. A recent review by Lee et al. focused on understanding the role of immune-related genes and thyroid-specific genes gathered in the group of AITD susceptibility genes. Although the topic of vitamin D metabolism and the genes and epigenetic mechanisms affecting it was not directly addressed in the paper, the undoubted involvement of IFN-α in triggering thyroid autoimmunity was highlighted [103]. Due to the conflicting results of previous studies on VDR polymorphism and the incidence of AITDs, a meta-analysis of eight studies with a total number of participants exceeding 1000 with AITDs was conducted. Four of the most commonly described polymorphisms of the VDR gene—BsmI, FokI, ApaI, and TaqI—were evaluated, showing that the BsmI or TaqI polymorphism is significantly associated with AITD risk. However, the authors noted important limitations of the analysis related to the omission of gene-environment interactions, the lack of a uniform definition of the control group, and the limited number of studies and their participants, especially in the context of patient ethnic diversity [104]. Vitamin D deficiency is highly prevalent in endocrine disorders and its supplementation appears to have beneficial effects, as described in one of the recent reviews by Galușca et al. In an analysis of the literature on Hashimoto's disease, low vitamin D production appeared to be associated with higher anti-thyroid peroxidase (anti-TPO) antibody titers and thyroid volume, while supplementation was addressed in connection with a reduction in antibodies levels in some studies. In addition, some researchers indicated a gradual decrease in thyroid-stimulating hormone (TSH) levels with supplementation. The serum concentration of vitamin D showed a significantly lower value in Graves' disease patients who were not in remission compared to those who were. Moreover, in a randomized prospective study, the thyroid volume and the degree of exophthalmos revealed a statistically significant correlation with vitamin D levels [105,106].

MS is an inflammatory disease characterized by neurodegenerative events and autoimmune attacks on myelin in the central nervous system (CNS), leading to varying degrees of recurrent or progressive neurological disorders. Epigenetic changes play an important role both in the development of myelination and remyelination and in the pathogenesis of some neurodegenerative diseases, including MS, Alzheimer's, Parkinson's, and Huntington's diseases [107]. Relapses of MS usually occur in the absence of a defined trigger; however, it has been described that MS symptoms vary throughout the year, suggesting that environmental factors may act as the above-mentioned triggers, and influence the overall susceptibility to MS and its progression [108]. Vitamin D is one of the best-described environmental factors for MS. It has been found to modulate Th17 autoimmunity through transcriptional suppression of the pro-inflammatory cytokine IL-17, via recruitment of histone deacetylase 2 to the *IL17A* promoter region. Moreover, 1,25(OH)2D may change the expression of genes that modify histones [107,108]. In a comprehensive review by Sintzel et al., the authors state that there is increasing evidence indicating a causal relationship between low vitamin D levels and the risk of MS as well as greater clinical activity and brain MRI-confirmed activity in MS patients [109]. Interventional studies conducted so far on limited groups of MS patients have shown that a high supplemental dose of vitamin D (10,400 IU/day) is safe and exhibits pleiotropic immunomodulatory effects. A reduction was found in the proportion of interleukin-17+CD4+ T cells, CD161+CD4+ T cells, and effector memory CD4+ T cells with a concomitant increase in the proportion of central memory CD4+ T cells and naive CD4+ T cells. These effects were not observed in the group of low-dose supplementation (800 IU/day) [110]. More research is needed to confirm whether high-dose supplementation of vitamin D in MS patients is truly beneficial in the context of changes in calcium levels and prolonging the time to disease progression to the secondary progressive phase. The search for a link between VDR polymorphisms and MS is still a topic under investigation. However, information on this relationship is heterogeneous, likely influenced by matched research groups. In a recent retrospective study involving more than 200 patients with relapsing-remitting multiple sclerosis and more than 800 Caucasian healthy controls, no influence of the ApaI, BsmI, Cdx2, and TaqI was found. However, a significant effect of the VDR FokI (rs2228570) on the development of MS was demonstrated [111]. It should be noted, however, that in an earlier large meta-analysis,

despite notable inconsistencies between the reports evaluated, a significant association between TaqI polymorphism and MS susceptibility was detected, while the effect of the BsmI polymorphism on increasing the risk of MS was detected only in the Asian population. Interestingly, it was also found that the ApaI VDR polymorphism may have a protective function, reducing MS risk in the Asian population [112]. The process of DNA methylation should also be mentioned as a regulator of processes in MS pathogenesis. In addition to the hypomethylation of FOXP3 or IL-17 in T cells, the methylation pattern of the alternative VDR promoter, which is located in exon 1c, has also been studied. This gene regulatory element was previously found to be hypomethylated in other immune cells; however, in a study by Ayuso et al., a different, moderate pattern of methylation in the T cells population was found. What is more interesting, in the group of relapsing-remitting multiple sclerosis patients the methylation of the exon 1c promoter was increased even more. The search for the significance of this VDR promoter methylation pattern in MS patients, as well as in patients with other autoimmune diseases, is a challenge for further research [113].

4. Current Clinical Trials Using Vitamin D in Autoimmune Diseases

Literature sources to date point to the need for vitamin D supplementation to compensate for vitamin D deficiency, which should be pursued in all individuals, not just those with autoimmune disorders. However, even the guidelines for the overall vitamin D supplementation dose in the community are not standardized. While in the United Kingdom, guidelines indicate 400 IU per day as the recommended dose for adults and children over the age of 4, the Endocrine Society recommends a daily supplementation dose of 600–1000 IU depending on age, and in cases of deficiency, 2000 IU per day, even to 50,000 IU per week. It seems that prevention and treatment of different conditions require different concentrations of vitamin D. Cutoff values in vitamin D concentration supported by high-level evidence studies are still not available. However, it appears that for interventions in bone mineral density, dental health, fracture risk, or tumorigenesis, benefits are obtained starting at 25OHD concentrations of 30 ng/mL [114]. For tuberculosis prevention, in vitro studies have indicated a cholecalciferol concentration of 4 μg/mL as adequate to slow proliferation in culture. This is a much higher concentration, but potentially achievable due to local hydroxylation performed by macrophages. At the same time, in vivo studies did not provide a clear conclusion [114,115]. A number of research and review papers, including those cited in this paper, point to the need for robust randomized controlled trials on large groups of patients to determine a dose of vitamin D supplementation that would demonstrate long-term benefits with a safety profile in ADs. In their study, Fletcher et al. point out other important uncertainties regarding the supplementation dose used. Indeed, it is still unclear what serum 25OHD level is optimal for immune function. It is possible that different levels of 25OHD are optimal for innate antimicrobial and antiviral responses relative to anti-inflammatory effects. It is also possible that the level of 25OHD beneficial in AD differs from that considered appropriate for maintaining skeletal health [116]. Prominent studies in the search for supplementation doses in people with ADs are those evaluating intervention with vitamin D in patients with MS. Notable are the exceptionally high daily doses of vitamin D (from 10,000 to 40,000 IU/day) used in these clinical trials, which has proven safe as a combined 25OHD–interferon-beta therapy. Only a few of the studies (presumably on a sufficient study group) have shown promising results in terms of improved MRI results, although baseline results were not achieved [117]. The literature also emphasizes the importance of the risk of vitamin D toxicity, especially in the face of hazardous internet trends, encouraging MS patients to use ultra-high doses of vitamin D of 80,000–100,000 IU daily [109]. For other ADs, positive effects of vitamin D are also sought, but lower supplementation doses are usually considered. A recent interventional study in patients with SLE showed benefits in disease activity and fatigue after 25OHD supplementation as follows: 8000 IU daily for 4 weeks, followed by 2000 IU daily maintenance for patients with vitamin D insufficiency, or 8000 IU daily for 8 weeks, followed by 2000 IU daily maintenance in vitamin D-deficient patients [118]. Another missing piece is solid

evidence for vitamin D use in autoimmunity prevention. As a model on this subject, a recent study with 25,871 participants, supplemented with a placebo, omega-3 fatty acids, or vitamin D (2000 IU/day), should be mentioned here. Vitamin D supplementation, with or without omega-3 fatty acids, was shown to reduce the incidence of autoimmune diseases in this cohort by 39% after 3 years of follow-up and by 22% after 5 years [119]. It is therefore suggested that vitamin D use may be considered as prevention against ADs, but may also require long periods of supplementation. Despite the availability of studies based on animal models, as well as numerous studies of vitamin D and specific autoimmune diseases in humans, high-quality randomized trials should be pursued in this area as well [116].

Listed below are some interesting clinical trials using vitamin D in patients with ADs. All were selected based on a search of clinicaltrials.gov and include registered, currently ongoing studies. They may inspire further research efforts and be the basis for new dietary recommendations in the management of autoimmune diseases in the future.

- "Pilot Study of OMEGA-3 and Vitamin D in High-Dose in Type I Diabetic Patients (POSEIDON)"—NCT03406897. A recruiting interventional open-label study with an estimated 56 participants. The authors aim to evaluate the efficacy and safety of a treatment regimen based on omega-3 fatty acids and vitamin D in patients with T1D. The authors hypothesize that the used combination administered to patients with new or established forms of the disease will be safe and preserve insulin secretion [120];
- "Early High-Dose Vitamin D and Residual β-Cell Function in Pediatric Type 1 Diabetes"—NCT05270343. Not yet recruiting interventional open-label study with an estimated 198 participants. The aim of the project is to evaluate the effect of high-dose vitamin D supplementation on early T1D in children and adolescents. Patients simultaneously require intensive insulin therapy [121];
- "Effect of Vitamin D Supplement on Disease Activity in SLE"—NCT05260255. The purpose of the ongoing study is to evaluate the effect of vitamin D supplementation on SLE activity (Systemic Lupus Erythematosus Disease Activity Index 2000). A recruiting interventional double-blind study with an estimated 100 participants. The study is also expected to assess IL-6 levels and anti-dsDNA titers at specific intervention intervals [122];
- "The Vitamin D in Pediatric Crohn's Disease (ViDiPeC-2) (ViDiPeC-2)"—NCT03999580. The aim of the study is to evaluate the effectiveness of high-dose vitamin D supplementation in children with CD. The researchers expect to achieve a reduction in the frequency of relapses and improved patient quality of life [123];
- "High Dose Interval Vitamin D Supplementation in Patients with Inflammatory Bowel Disease Receiving Biologic Therapy"—NCT04331639. A recruiting interventional open-label study with an estimated 50 participants. Vitamin D will be administered concurrently with IBD biologic therapy every 4–8 weeks. The researchers aim to evaluate, using laboratory tests and a questionnaire, inflammatory markers as well as markers of bone health after intervention with vitamin D [124];
- "Longitudinal Effect of Vitamin D3 Replacement on Cognitive Performance and MRI Markers in Multiple Sclerosis Patients"—NCT03610139. A recruiting interventional single-blind study with an estimated 162 participants. The researchers hypothesize that high-dose vitamin D supplementation will result in improvements in cognitive performance at 6 and 12 months after supplementation [125].

5. Conclusions

The above literature review represents a collection of studies and observations to date on the epigenetic function of vitamin D in autoimmune diseases. The latest available reports exploring the epigenetic basis of selected disease entities and the involvement of vitamin D receptor relay in the most commonly considered epigenetic mechanisms, as well as the possible benefits of vitamin D supplementation in patients, are analyzed. An effort was made to present the sometimes contradictory reports in an accessible manner. Using the clinicaltrials.gov database, the ongoing interventional clinical trials evaluating

the utility of vitamin D in ameliorating the course of autoimmune diseases and improving quality of life were presented. Further challenges in addressing the broad topic of vitamin D in autoimmunity were also identified.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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122. Available online: https://clinicaltrials.gov/ct2/show/NCT05260255 (accessed on 4 September 2022).
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Effect of probiotics supplementation on disease progression, depression, general health, and anthropometric measurements in relapsing-remitting multiple sclerosis patients: A systematic review and meta-analysis of clinical trials

Shahrzad Mirashrafi,Seyedeh Zahra Hejazi Taghanaki,Faezeh Sarlak,Amir Reza Moravejolahkami,Mohammad Ali Hojjati Kermani,Mohsen Haratian,

Background

Probiotics may have a promising role in chronic autoinflammatory diseases. The current systematic review and meta-analysis investigated the effects of probiotics on disease progression, depression, general health, and anthropometric measurements in Relapsing-Remitting Multiple Sclerosis (RRMS) patients.

Methods

The English literature search was performed using PubMed, Scopus, Web of Science, and the Central Cochrane Library through January 2021. Random effect models were used to synthesise quantitative data by STATA$_{14}$.

Results

From a total of 152 identified entries, four trials were included in quantitative synthesis (n = 213; 106 as intervention, 107 as control). An additional six studies with the same structure and different markers were also systematically reviewed. The pooled effect size showed that Expanded Disability Status Scale (EDSS) (WMD = −0.43; 95% CI = −0.65, −0.20; *P* < .001), Beck Depression Inventory-Ⅱ (BDI-Ⅱ) (WMD = −3.22; 95% CI = −4.38, −2.06; *P* < .001) and General Health Questionnaire (GHQ) (WMD = −4.37; 95% CI = −6.43, −2.31; *P* < .001) were improved following probiotics supplementation. However, body weight and body mass index did not statistically change.

Conclusion

Our findings revealed that probiotics supplementation can improve disease progression, suppress depression, and general health in MS patients; although, further investigations may be needed.

Multiple Sclerosis and Related Disorders 37 (2020) 101427

Contents lists available at ScienceDirect

Multiple Sclerosis and Related Disorders

journal homepage: www.elsevier.com/locate/msard

Review article

The multiple sclerosis gut microbiota: A systematic review

Ali Mirza[a], Jessica D. Forbes[b,c,f], Feng Zhu[a], Charles N. Bernstein[b], Gary Van Domselaar[c,d], Morag Graham[c,d], Emmanuelle Waubant[e], Helen Tremlett[a,*]

[a] Djavad Mowafaghian Centre for Brain Health, Faculty of Medicine (Neurology), University of British Columbia, Vancouver, BC, Canada
[b] Department of Internal Medicine, University of Manitoba, University of Manitoba IBD Clinical and Research Centre, Winnipeg, MB, Canada
[c] National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada
[d] Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada
[e] University of California San Francisco, San Francisco, CA, United States
[f] Department of Laboratory Medicine & Pathobiology, University of Toronto, ON, Canada

ARTICLE INFO

Keywords:
Multiple sclerosis
Gut
Microbiome
Microbiota
Bacteria
Systematic review

ABSTRACT

Background: To systematically review and synthesize the literature on the multiple sclerosis (MS) gut microbiota composition as compared to persons without MS.
Methods: We systematically searched MEDLINE, EMBASE, and Web of Science databases for relevant published articles (2008–2018).
Results: Of 415 articles identified ten fulfilled criteria. All studies used a case-control design, six sourced participants from the US, two Germany, one Italy, and one Japan. Nine focused exclusively on adults and one on children, totaling 286 MS and 296 control participants. Over 90% of cases had relapsing-remitting MS; disease duration ranged from 10.6 ± 6.5 months to 15.3 ± 8.6 years (mean ± SD). Nine studies examined stool and one evaluated duodenal mucosa. Diverse platforms were used to quantify microbes: Illumina MiSeq, Roche 454, microarray, and fluorescence in situ hybridization. None of eight studies reported a significant alpha-diversity differences between cases and controls. Two of seven studies reported a difference in beta-diversity ($P \leq 0.002$). At the taxa-level, ≥2 studies observed: lower relative abundance of *Prevotella, Faecalibacterium prausnitzii, Bacteroides coprophilus, Bacteroides fragilis*, and higher *Methanobrevibacter* and *Akkermansia muciniphila* in MS cases versus controls. Exposure to an immunomodulatory drug (IMD), relative to no exposure, was associated with individual taxonomic differences in three of three studies.
Conclusion: Gut microbiota diversity did not differ between MS cases and controls in the majority of studies. However, taxonomic differences were found, with consistent patterns emerging across studies. Longitudinal studies are warranted to elucidate the relationship between IMD exposure and differences in the gut microbiota composition.

1. Introduction

A growing body of evidence points to the gut microbiota playing a role in immune-mediated, neurological disease, such as multiple sclerosis (MS) (Rooks and Garrett, 2016; Tremlett et al., 2017). An important initial step to understanding the relationship between the gut microbiota and MS is to survey the gut microbial community. Recent studies on MS have focused on surveying the gut bacterial (and archaeal) communities. A common goal has been to investigate whether there are differences in the MS gut microbiota composition between MS cases and controls, measured as the overall microbiota composition (diversity) and relative abundance of individual resident microbes. Some studies also assessed potential effect modifiers (or confounders), such as exposure to an immunomodulatory drug (IMD) used to treat MS.

We conducted a systematic review in order to comprehensively collate the body of evidence surrounding the relationship between the gut microbiota and MS. Our objective was to include published articles in which the gut microbiota profiles had been compared between individuals with and without MS.

2. Methods

Our systematic review was designed to address the following

* Correspondence to: Djavad Mowafaghian Centre for Brain Health, Faculty of Medicine (Neurology), University of British Columbia, Room S126, UBC Hospital, 2211 Wesbrook Mall, Vancouver, BC V6T 2B5, Canada.
E-mail address: helen.tremlett@ubc.ca (H. Tremlett).

https://doi.org/10.1016/j.msard.2019.101427
Received 10 July 2019; Received in revised form 6 September 2019; Accepted 30 September 2019

specific questions: (1) Does the gut microbiota composition differ between MS cases and controls (participants without MS), as assessed by (a) diversity metrics and/or by (b) taxa-level relative abundances? (2) What are the main effect modifiers (confounders) identified to date in studies evaluating the gut microbiota in MS?

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Moher et al., 2010). The systematic review protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) database (registration no. CRD42018089173) and the protocol is in accordance with the PRISMA-P (2015) guidelines (Shamseer et al., 2015).

2.1. Eligibility criteria

Original research articles assessing the potential gut microbiota differences in diversity or taxonomic relative abundance between MS cases and controls (individuals without MS) were eligible for inclusion. We included any of the following study designs: cohort, cross-sectional, case-control, and comparative cohort studies. Other study designs were excluded, such as intervention studies (unless pre-intervention samples were available for cases/controls) and studies without a control group (e.g., case series). No restrictions were imposed with respect to the age of study participants, geographical location or setting (e.g., community or hospital), or disease course(s) under study (e.g., relapsing-onset, primary progressive).

2.2. Literature search strategy

We performed a systematic literature search of MEDLINE, EMBASE, and Web of Science databases for original research articles published in English between January 1st, 2008 and February 8th, 2018, which we subsequently updated to June 19th, 2018. At the time of publication, a final update to August 24th, 2019 was included and reported as an addendum. The search strategies for each database are shown in Fig. 1A. We did not include conference abstracts, unpublished work or the grey literature (e.g., presentations, posters, websites or dissertations).

2.3. Eligibility assessment, data extraction, and quality assessment

The literature search results were uploaded to Mendeley for screening. Titles and abstracts were screened based on the study inclusion and exclusion criteria by two independent reviewers (AM and JF). Any disagreement was resolved between the reviewers. The full texts of all screened-in abstracts were then retrieved and assessed for eligibility and relevant information retrieved were extracted by one reviewer (AM), as outlined in Appendix 1. Two independent reviewers (AM and JF) assessed the risk of bias in individuals studies specific to our current systematic review-related questions using the US's National Institutes of Health (NIH) tool for Quality Assessment of Case-Control Studies (NIH National Heart, Lung, and Blood Institute). No study was excluded based on the risk of bias (rated as good, fair, and poor), in part because a study could be assigned as 'poor', inferring a high risk of bias, if the microbiota quantification platform was not valid to address our study question(s).

3. Results

3.1. Study and participant characteristics

Of 415 articles identified (based on titles and abstracts), ten fulfilled criteria for inclusion (Cantarel et al., 2015; Miyake et al., 2015; Chen et al., 2016; Cree et al., 2016; Tremlett et al., 2016; Jangi et al., 2016; Cosorich et al., 2017; Swidsinski et al., 2017; Berer et al., 2017; Cekanaviciute et al., 2017). A flow chart outlining the screening process

A. Literature Search Strategies

MEDLINE search - Ovid interface

1 exp Multiple Sclerosis/
2 multiple sclerosis.mp.
3 1 or 2
4 microbiota/ or gastrointestinal microbiome/ or microbial consortia/
5 (microbiome or microbiota).mp.
6 ((gut or intestin*) adj3 (microbi* or flora or microflora)).mp
7 4 or 5 or 6
8 3 and 7
9 8 not (animals/ not humans.sh.)
10 limit 9 to english language
11 limit 10 to last 10 years
12 review.pt.
13 11 not 12

EMBASE search - Ovid interface

1 exp Multiple Sclerosis/
2 multiple sclerosis.mp.
3 1 or 2
4 microflora/ or bacterial flora/ or feces microflora/ or exp intestine flora/ or exp microbiome/
5 (microbiome or microbiota).mp.
6 ((gut or intestin*) adj3 (microbi* or flora or microflora)).mp
7 4 or 5 or 6
8 3 and 7
9 8 not ((exp animal/ or nonhuman/) not exp human/)
10 limit 9 to english language
11 limit 10 to last 10 years
12 review.pt.
13 11 not 12

Web of Science

(TS=("multiple sclerosis") AND TS=((microbiome OR microbiota) OR ((gut or intestin*) NEAR/3 (microbi* OR flora OR microflora)))) AND LANGUAGE: (English) AND DOCUMENT TYPES: (Article)
Indexes=SCI-EXPANDED, ESCI Timespan=2008-2018

B. PRISMA 2009 Flow Diagram

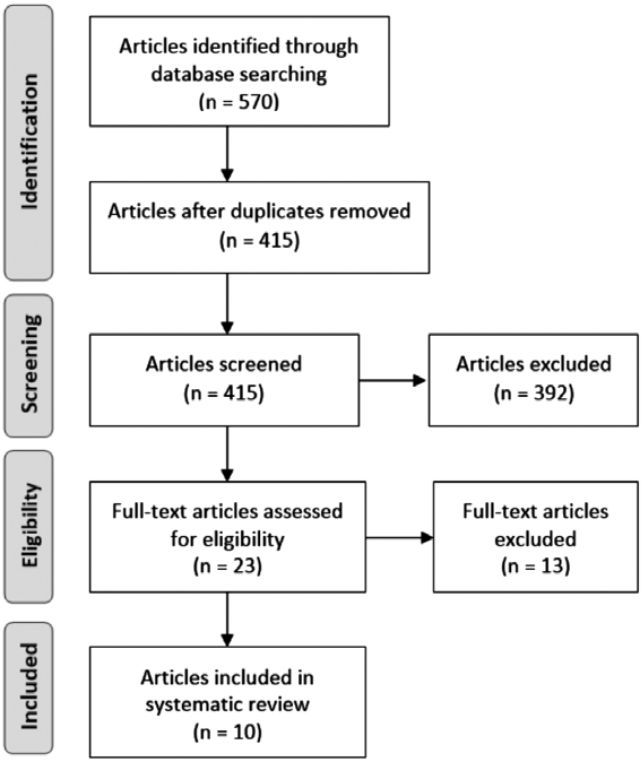

Fig. 1. Literature search strategies and PRISMA flow diagram.
A. Systematic literature search strategies of MEDLINE, EMBASE, and Web of Science databases for original research articles. B. Selection of articles for inclusion in the systematic review of the multiple sclerosis microbiota (2008–18).

is provided in Fig. 1B (Moher et al., 2010). All ten studies used a case-control design for a total of 286 MS cases and 296 controls. Nine studies enrolled adults, of which one focused solely on twins discordant for MS, and one enrolled children. Nine studies collected stool samples from

Table 1
The multiple sclerosis gut microbiota: summary of the included studies.

First Author, Year of Publication, Country	Study Design	Biological Sample Type	Number of Participants (MS / Control)	Age (Years) Near Time of Sample Collection (MS / Control)	Race & Ethnicity (MS / Control)	Body Mass Index (kg/m^2, MS / Control)	Diet Assessement	NIH Quality (or 'Risk of Bias') Assessement
Cantarel, 2015, United States	Intervention, case-control, pilot study	Stool	7 / 8	Median 42 (30–48) / 38 (29–51)	White: 7 (100%) / 8 (100%) Hispanic: 0 (0%) /0 (0%)	MS and controls combined range: 18 - 30	NA	Fair
Miyake, 2015, Japan	Case-control	Stool	20 / 40	36.0 ± 7.2 / 28.5 ± 9.8	NA	NA	NA	Fair
Chen, 2016, United States	Case-control	Stool	31 / 36	42.9 ± 10.6 / 40.3 ± 7.3	NA	28.0 ± 6.3 / 27.8 ± 4.7	NA	Fair
Cree, 2016, United States	Case-control	Stool	16 / 16	54 ± 11.8 / 54 ± 15.8	White: 13 (81%) / 11 (69%) Black: 0 (0%) / 2 (13%) Asian: 1 (6%) / 2 (13%) Other: 2 (13%) / 1 (6%)	23.3 ± 3.59 / 23.8 ± 4.10	Yes; Block Dietary Data Systems (NutritionQuest©)	Fair.
Tremlett, 2016, United States	Case-control, pilot study	Stool	18 / 17	12.5 ± 4.44 / 13.5 ± 3.08	White: 9 (50%) / 13 (77%) Hispanic: 8 (44%) / 6 (35%)	22.2 ± 5.66 / 22.8 ± 7.10. Missing 1	Yes; Block Kids Food Screener (NutritionQuest©)	Fair
Jangi, 2016, United States	Case-control	Stool	60 / 43	49.7 ± 8.50 / 42.2 ± 9.61	White: 58 (97%) / 43 (100%) Black: 2 (3%) / 0 (0%) Hispanic: 1 (2%) / 0 (0%)	27.2 ± 4.7 / 26.4 ± 6.3	Yes; method not reported.	Fair
Cosorich, 2017, Italy	Case-control	Duodenal mucosa tissue	19 / 17	41 ± 2 / 48 ± 3	NA	NA	Yes; method not reported.	Fair
Swidsinski, 2017, Germany	Intervention, case-control	Stool	10 / 14	NA	NA	NA	NA	Poor / High risk of bias. Taxa quantification method for assessing our study questions is obsolete
Berer, 2017, Germany	Co-twin, case-control	Stool	34 / 34	MS and controls combined: 41.3 ± 10.8 (21–63)	NA	NA	Yes; method not reported.	Fair
Cekanaviciute, 2017, United States	Case-control	Stool	71 / 71	40.7 ± 11.9 (19–64) / 44.6 ± 14.4 (22–71). Missing 9 controls and 4 cases	NA	NA	NA	Fair

Characteristics of the study participants relate to the time of microbiota sample collection (typically stool). Jangi et al. (2016) were missing clinical data for 8 subjects; they did not report which clinical data was missing. Continuous data are expressed as mean ± SD. NA, not available; MS, multiple sclerosis; BMI, body mass index; NIH, National Institutes of Health.

participants while one collected duodenal mucosal samples. Characteristics of the included studies are summarized in Table 1. Study quality was 'fair' for nine articles and 'poor' for one. The study identified as poor used an obsolete method (fluorescence *in situ* hybridization; FISH) for characterizing the gut microbiota, thus making it a high risk of bias in the context of our systematic review-specific questions (Swidsinski et al., 2017).

Across the nine studies which reported participant demographics, females predominated. Overall the number (%) of females/males were: 182/104 (64%/36%) for cases and 181/111 (61%/38%) for controls (sex was missing for four controls in one study (Cekanaviciute et al., 2017)). The average ages of cases and controls ranged from 12–54 and 13–54 years, respectively across studies (age was missing for nine controls and four cases in one study (Cekanaviciute et al., 2017)). Across the four studies that reported race and/or ethnicity, the majority of participants were Caucasian, totaling 87/101 (86%) of MS cases and 75/84 (89%) of controls. Six studies recruited participants from the United States, two from Germany, one from Italy, and one from Japan. The most commonly reported lifestyle factors were diet (five studies) and BMI (five studies), both diet and BMI were available in three studies (Table 1). Diet was collated via a validated food frequency questionnaire in two US-based studies; three did not specify. Both diet metrics and BMI (based on descriptive group means) were similar when cases were compared to controls, except for one study, which, for MS cases, observed a higher intake of specific dietary elements (i.e., carotenoids) (Cree et al., 2016).

The majority (267/286; 93%) of MS cases had a relapsing-remitting disease course at the time of sample collection. Five studies used the McDonald 2010 criteria for MS diagnosis, one used the Poser criteria and four did not specify (Poser et al., 1983; Thompson et al., 2018). Five studies reported the disease duration of the MS cases, which ranged, on average from 0.9–15.3 years (Table 2).

Medication exposure status was reported in nine of ten studies; all focused on IMDs or immunosuppressants (IMS) used to treat MS. Overall, 118 (43%) of MS cases ($n = 276$) were exposed to an IMD or IMS near the time of sample collection; 64 (23%) to β-interferon, 35 (13%) glatiramer acetate, 18 (7%) other IMDs, and 1 (<1%) IMS (azathioprine). IMD or IMS exposure was typically defined as exposed in the two or three months prior to the time of microbiota sample collection. The pediatric study included IMD naïve MS cases, explicitly defined as never exposed to an IMD (Tremlett et al., 2016). Of the seven studies that reported use of systemic corticosteroids, 11/229 (5%) of MS cases were exposed to systemic corticosteroids near the time of sample collection (Table 2).

3.2. Sample handling and sequencing procedures

Eight of the ten studies mentioned at least some aspects of the methods related to sample collection. A US-based group sampled participant's microbiota using culture swabs, another US-based group collected stool directly into a dry container, and the Italy-based group collected mucosal biopsies into a culturing solution containing antibiotics (Chen et al., 2016; Cosorich et al., 2017; Cekanaviciute et al., 2017). Four studies shipped stool samples overnight (shipped with ice with the exception of one study) while four groups stored or processed the sample the same day of the collection (Cantarel et al., 2015; Cree et al., 2016; Tremlett et al., 2016; Jangi et al., 2016). Samples not sequenced the same day of collection were stored at −70 °C or −80 °C prior to DNA extraction (Cantarel et al., 2015; Miyake et al., 2015; Chen et al., 2016; Tremlett et al., 2016; Jangi et al., 2016; Berer et al., 2017; Cekanaviciute et al., 2017).

Seven studies sequenced various regions of the 16S rRNA gene using a Next-Generation Sequencing (NGS) platform (either Illumina MiSeq or Roche 454). One study used both Roche 454 and Illumina MiSeq (Table 3) (Berer et al., 2017). Two studies used a DNA microarray platform only (Affymetrix PhyloChip G3) and one used FISH to probe and profile the 16S rRNA genes (Cantarel et al., 2015; Cree et al., 2016; Swidsinski et al., 2017). The 16S rRNA variable-region gene sequenced varied between studies. The method of generating 'Operational Taxonomic Units' (OTUs) also varied. OTUs are clusters of similar sequences—typically >97%—that are assumed to represent a single taxon; for 16S rRNA, OTUs typically have genus-to-species level of taxonomic specificity. The different methods of generating OTUs among the studies that used an NGS platform include: *de novo* OTU clustering (four studies) and closed reference OTUs (three studies). Despite these differences, all studies that used an NGS platform clustered the 16S rRNA sequences into OTUs based on a 97% similarity threshold (Table 3).

The twin cohort study also sequenced the gut metagenome (the collective genomes of all the gut microbes in a stool sample), although authors reported that none of the results reached significance, and no data was provided (Berer et al., 2017).

3.3. Gut microbiota diversity

Eight of ten studies assessed the gut microbiota diversity (alpha- or beta-diversity), Table 4. None of the alpha-diversity metrics, e.g., the number of different species within a sample, differed significantly between cases and controls. Out of seven studies that calculated beta-diversity (the number of microbial species that are not the same in two different environments), two studies reported a difference in a beta-diversity metric, between cases and controls (Table 4) (Miyake et al., 2015; Chen et al., 2016). No studies reported whether potential confounders, such as IMD exposure, were relevant to the significant alpha- or beta-diversity findings.

IMD exposure and gut microbiota diversity were assessed in two US-based studies (Cantarel et al., 2015; Tremlett et al., 2016). Alpha-diversity did not differ between IMD exposed and unexposed MS cases in either study, although beta-diversity did differ in the pediatric MS study (Table 4) (Tremlett et al., 2016).

3.4. Taxa-level findings

Taxa-level differences between cases and controls were reported as follows: phylum level (seven studies), genus (seven studies), and species/OTU level (eight studies). In total, two genera and four species (none of which reached significance at the phylum level) similarly differed in their relative abundances between MS cases compared to controls in a consistent direction for two or more studies (Table 5). Specifically, at the genus level, lower relative abundances of *Prevotella* were observed in three studies and a higher relative abundance of the Archaea *Methanobrevibacter* was observed in two studies. At the species level, there was a higher relative abundance of *Akkermansia (muciniphila)* and *Faecalibacterium prausnitzii*, which was observed in four studies each. Also, a lower relative abundance of *Bacteroides coprophilus* was reported in three studies and a lower *Bacteroides fragilis* abundance was reported in two studies (Table 5). Different statistical tests were used to assess differences in OTU abundance, including: models based on the negative binomial distribution (four studies), t-tests (Welch or Student, three studies), Wilcoxon rank sum test (two studies) and ANOVA (one study, Table 6).

Three studies compared gut microbiota taxa- or OTU-level findings according to IMD exposure status via a sub-group analysis (Cantarel et al., 2015; Tremlett et al., 2016; Jangi et al., 2016; Cosorich et al., 2017; Berer et al., 2017). When IMD unexposed MS cases were compared to controls (Table 5), a higher relative abundance of the following taxa was found *Methanobrevibacter* (two studies, with a combined total of 37/60 cases/controls) (Tremlett et al., 2016; Jangi et al., 2016) and *A. muciniphila* (four studies, with 125/148 cases/controls) (Tremlett et al., 2016; Jangi et al., 2016; Berer et al., 2017; Cekanaviciute et al., 2017). Lower relative abundances were observed for *Prevotella* (two studies, with 99/114 cases/controls), (Jangi et al.,

Table 2
Clinical characteristics of the participants with MS in the included studies.

First Author, Year of Publication	Age Onset of MS (Years)	MS Disease Duration (Years)	Diagnostic Criteria	EDSS Near Time of Sample Collection	MS Disease Course	IMD/IMS Exposed Near Time of Sample Collection	Number of Participants Exposed to a Specific IMD/IMS drug						Systemic Corticosteroids
				0[c]			Interferon-β	Glatiramer Acetate	Natalizumab	Rituximab	Fingolimod	Azathioprine	
Cantarel, 2015	NA	NA	McDonald	3.0 or less	RR 7 (100%)	5 (71%)	0	5 (71%)	0%	0	0	0	0[a]
Miyake, 2015	NA	8.8 ± 6.0	McDonald	NA	RR 20 (100%)	9 (45%)	9 (45%)	0	0%	0	0	0	5 (25%)
Chen, 2016	35.4 ± 10.4	NA	McDonald	< 6, $n = 22$; > 6, $n = 2$	RR 31 (100%)	20 (65%)	14 (45%)	1 (3%)	5 (16%)	0	0	0	NA
Cree, 2016	NA	15.3 ± 8.6	NA	NA	RR 9 (56.3%), SP 4 (25%), PP 3 (18.8%)	5 (31%)	0	0	0	5 (31%)	0	0	NA
Tremlett, 2016	12.1 ± 4.7	0.88 ± 0.54	McDonald	(range 0–4.0) < 2.0, n=7; 2.0 to < 3.0, n=8; >3.0, n=3	RR 18 (100%)	9 (50%)[b]	3 (17%)	5 (28%)	1 (6%)	0	0	0	6 (33%)[b]
Jangi, 2016	NA	12.8 ± 8.3	NA	1.2 (1.0 SD)	RR 60 (100%)	32 (53%)[c]	18 (30%)	14 (23%)	0	0	0	0	0[a]
Cosorich, 2017	NA	NA	Poser	(range 0–5.5) 0.0 to < 2.0, n=8; 2.0 to < 3.0, n=4; 3.0 to < 6.0, n=7	RR/NEDA 9 (47%), RR/EDA 10 (53%)	19 (100%)	7 (37%)		9 (47%)	0	0	3 (16%)	0[c]
Swidsinski, 2017	NA	NA	NA	NA	RR 10 (100%)	NA	NA	NA	NA	NA	NA	NA	NA
Berer, 2017	28.0 ± 9.0	13.2 ± 9.6	McDonald	NA	CIS 3 (9%), RR 22 (65%), SP 7 (21%), PP 2 (6%) PP 2 (6%)	19 (56%)[c]	13 (38%)	1 (3%)	4 (12%)	0	0	1	0[c]
Cekanaviciute, 2017	NA	NA	NA	NA	RR 71 (100%)	0 (0%)[c]	0	0	0	0	0	0	0[c]
Total						**118 (43%)**	**64 (23%)**	**35 (13%)**	**10 (4%)**	**5 (2%)**	**3 (1%)**	**1**	**11 (5%)**

Unless otherwise stated, characteristics shown relate to the time of microbiota sample collection. The frequency of exposures to an immunomodulatory drug, immunosuppressant and systemic corticosteroids at the time of microbiota sample collection were typically within 2 or 3 months prior to sample collection. One study did not report treatment status. Systemic corticosteroids are not included in the number of MS cases exposed to IMD/IMS (column 7). All percentages are rounded to the nearest whole numbers. Continuous data are expressed as mean ± SD. NA, not available; MS, multiple sclerosis; RR, relapse-remitting multiple sclerosis; SP, secondary progressive multiple sclerosis; PP, primary progressive multiple sclerosis; CIS, clinically isolated syndrome; RR-EDA, evidence of disease activity in patients with relapse-remitting multiple sclerosis; NA, not available; RR-NEDA, no evidence of disease activity in patients with relapse-remitting multiple sclerosis; F, female; M, male; EDSS, Expanded Disability Status Scale. IMD, Immunomodulatory drug; IMS, immunosuppressant; [a]Exposed within 1 month; [b]Exposed within 2 months; [c]Exposed within 3 months.

Table 3
Technical and computational methods used to process and quantify the microbiota.

First Author, Date of Publication	Microbiota Quantifying Instrument, 16S rRNA Region	Method Generating OTUs	Normalization of Sample Sequence Depth
Cantarel, 2015	DNA microarray (Affymetrix PhyloChip)	The array contains representative sequences from OTUs, which were clustered at 95% similarity	NA
Miyake, 2015	Roche 454, V1-V2	Individual taxa analysis: Closed reference OTUs at 97% similarity. Diversity analysis: *De novo* OTU clustering at 96% similarity	Rarefied to 3000 sequence per sample
Chen, 2016	Illumina MiSeq,V3-V5	*De novo* OTU clustering at 97% similarity using IM-TORNADO	Rarefied to 10,000 sequence per sample
Cree, 2016	DNA microarray (Affymetrix PhyloChip)	NA	NA
Tremlett, 2016	Illumina MiSeq, V4	*De novo* OTU clustering at 97% similarity using QIIME	Rarefied to 201,546 sequence per sample
Jangi, 2016	Roche 454, V3-V5;Illumina MiSeq, V4	*De novo* OTU clustering at 97% similarity using Mothur	NA
Cosorich, 2017	Roche 454, V3-V5	*De novo* OTU clustering generaed using QIIME	At least 3000 sequence per sample
Swidsinski, 2017	Fluorescence *in situ*hybridization (FISH)	NA	NA
Berer, 2017	Roche 454, V3–V5	Closed reference OTUs with 97% similarity using QIIME	Diversity analysis: Rarefied to 10,975 sequences per sample.Individual taxa analysis: variance-stabilizing transformation
Cekanaviciute, 2017	Illumina MiSeq, V4	Closed reference OTUs with 97% similarity using QIIME	Diversity analysis: Rarefied to 10,000 sequence per sampleIndividual taxa analysis: variance-stabilizing transformation

Closed-reference OTU assignment assigns query sequences to OTUs generated from an external reference database, whereas sequences in *de novo OTU clustering* are clustered against one another. The two common methods for normalizing read counts were *rarefying* and *scaling.* Rarefying refers to randomly discarding reads in each sample until each sample has an equal number of sequences. Scaling often includes transforming, e.g., variance stabilizing transformation. OTU, operational taxonomic units; NA, not available; V, variable region of 16S rRNA gene; IM-TORNADO, Illinois Mayo Taxon Organization from RNA Dataset Operations; Qiime, Quantitative Insights Into Microbial Ecology.

2016; Cekanaviciute et al., 2017) *B. coprophilus* and *B. fragilis* (two studies, with 80/87 cases/controls) (Tremlett et al., 2016; Cekanaviciute et al., 2017). Findings for *F. prausnitzii* were mixed (Tremlett et al., 2016; Cekanaviciute et al., 2017).

4. Discussion

We reviewed the recent literature (2008–2018) on the MS gut microbiota composition. Of the ten studies comparing the gut microbiota between MS participants and controls, the majority found no major differences in the overall composition of the gut microbiota in children or adults with MS relative to controls, as judged by alpha- or beta-diversity measures. Instead, subtle differences in the gut microbial communities were generally observed. At least two or more studies reported a higher relative abundance of *Akkermansia* and *Methanobrevibacter* and a lower relative abundance of *Prevotella, Bacteroides (coprophilus* and *fragilis)* and *Faecalibacterium prausnitzii* for MS cases relative to controls. Studies were generally too modest in size to adequately assess potential effect modifiers (confounders) such as drug exposure relevant to diversity or taxa-level findings.

4.1. Microbiota diversity

No study observed a significant difference in alpha-diversity between MS cases and controls and the majority found no differences in beta-diversity. However, the metrics and methods used varied across studies. While this makes comparisons challenging, the use of different diversity measures was not unexpected, as no single index perfectly summarizes local diversity (Morris et al., 2014). Beta-diversity specifically quantifies the variation in the taxonomic composition between samples; two of seven studies reported a significant difference between MS cases and controls (Miyake et al., 2015; Chen et al., 2016). However, it remains possible that findings were affected by IMD exposure; differences in beta-diversity were found in one study when IMD exposure was examined (Tremlett et al., 2016).

4.2. Individual taxa

We identified several taxa that differed in their relative abundance between MS cases and controls across two or more studies, though their role in MS are largely unknown. *Methanobrevibacter,* an archaeal anaerobe and methanogen, was enriched in MS cases relative to controls (Gaci et al., 2014). Consistent with this are the higher methane breath test results observed in MS patients compared to controls (Jangi et al., 2016). However, enrichment of methanogens has also been associated with constipation (Gaci et al., 2014; Falony et al., 2016; Vandeputte et al., 2016), a condition which is common in MS. *A. muciniphila* was also enriched in MS cases relative to controls. A similar relationship was reported in Parkinson's disease (Bedarf et al., 2017; Collado et al., 2007; Heintz-Buschart et al., 2018; Hill-Burns et al., 2017). *A. muciniphila* has been shown to elicit a pro-inflammatory T lymphocyte response *in vitro*; however, *in vivo* studies using mouse models of MS have so far failed to elicit a similar reponse (Cekanaviciute et al., 2017). Intriguingly, *A. muciniphila* may be beneficial in the setting of obesity or metabolic disorders, by supporting metabolic health and improving the intestinal barrier (Greer et al., 2016; Plovier et al., 2017; Schneeberger et al., 2015). These context-specific observations highlight the complexity of the gut microbiome, and a need to understand the underlying biology.

The remaining taxa identified—all of which are also common commensal bacteria of the human gastrointestinal tract—were all lower in relative abundance for MS cases relative to controls. *Faecalibacterium prausnitzii* is known for mitigating inflammation and may be depleted in the gut of individuals with other diseases, such as inflammatory bowel disease and irritable bowel syndrome (Liu et al., 2017; Lopez-Siles et al., 2017; Prosberg et al., 2016; Wright et al., 2015).

Two studies reported conflicting relative abundances, within each study, for several OTUs classified as *F. prausnitzii* (Table 5) (Tremlett et al., 2016; Cekanaviciute et al., 2017). These conflicting OTUs may actually be two different recently discovered phylogroups within the species *F. prausnitzii* which have recently been identified (Lopez-Siles et al., 2017). The two phylogroups share 97% 16S rRNA gene sequence similarity but have different metabolic properties.

Table 4
Gut Microbiota Diversity: MS cases versus controls.

First Author, Date of Publication	Diversity Metric	MS vs Controls (Main Analyses)		IMD-Related Subgroup Analyses	
		Statistical Test Used	Main Findings (R and P-values)	IMD Exposed MS vs IMD Unexposed MS	IMD Unexposed MS vs Control
Cantarel, 2015	β-diversity (weighted UniFrac)	PERMANOVA	$P = 0.74$	$P = 0.66$	NA
Miyake, 2015	α-diversity (richness: Choa1)	Welch's test	$P > 0.05$	NA	NA
	α-diversity (diversity: Shannon index)	Welch's test	$P > 0.05$	NA	NA
	β-diversity (weighted UniFrac)	ANOSIM	$\mathbf{R = 0.24, P \leq 0.0009}$	NA	NA
	β-diversity (unweighted UniFrac)	ANOSIM	$\mathbf{R = 0.21, P \leq 0.002}$	NA	NA
Chen, 2016	α-diversity (richness: observed OTUs)	NA	$P = 0.73$	NA	NA
	α-diversity (diversity: Shannon index)	NA	$P > 0.05$	NA	NA
	β-diversity (Bray-Curtis)	PERMANOVA	$\mathbf{P < 0.001}$	NA	NA
Cree, 2016	NA	NA	NA	NA	NA
Tremlett, 2016	α-diversity (evenness)	Mann-Whitney	$P > 0.2$	NA	$P > 0.05$
	α-diversity (richness)	Mann-Whitney	$P > 0.2$	NA	$P > 0.05$
	α-diversity (Faith's phylogenic diversity)	Mann-Whitney	$P > 0.2$	NA	$P > 0.05$
	β-diversity (Canberra)	PERMANOVA	$P > 0.05$	$\mathbf{P = 0.016}$	NA
Jangi, 2016	α-diversity (diversity: Shannon index)	Wilcoxon rank-sum test	$P > 0.05$	NA	NA
	β-diversity (weighted UniFrac)	AMOVA	$P > 0.05$	NA	NA
	β-diversity (unweighted UniFrac)	AMOVA	$P > 0.05$	NA	NA
	β-diversity (Bray-curtis)	AMOVA	$P > 0.05$	NA	NA
Cosorich, 2017	α-diversity (richness: observed OTUs)	Student's *t*-test	$P > 0.05$	NA	NA
Swidsinski, 2017	NA	NA	NA	NA	NA
Berer, 2017	α-diversity (Faith's phylogenetic diversity)	NA	$P > 0.05$	NA	NA
	β-diversity (weighted UniFrac)	NA	$P > 0.05$	NA	NA
Cekanaviciute, 2017	α-diversity (richness: Choa1)	NA	NA	NA	$P > 0.05$
	β-diversity (unweighted UniFrac)	NA	NA	NA	$P > 0.05$

Diversity tests that were statistically significant ($P < 0.05$) are in bold. Blank cells indicate a diversity test result was not reported. Not all diversity metrics were reported for every alpha-diversity measure. IMD Exposed: MS cases exposed to an immunomodulatory drug within 3 months of sample collection. IMD, Immunomodulatory drug; ANOSIM, The ANalysis Of SIMilarity; PERMANOVA, PERmutational Multivariate ANalysis Of VAriance; AMOVA, Analysis of MOlecular Variance; NA, not available.

Table 5
Key findings from taxa-level relative abundances: MS cases versus controls *(all MS cases* versus *controls are initially shown, regardless of immunomodulatory drug (IMD) exposure. In addition, findings from IMD unexposed MS cases relative to controls are also depicted*).

		First Author, Year of Publication					
Genus	Comparison	Miyake, 2015	Tremlett, 2016	Jangi, 2016	Swidsinski, 2017	Berer, 2017	Cekanavic-iute, 2017
Methanobrevibacter	MS vs Control					NS	
	IMD Unexposed MS vs Control						
Prevotella	MS vs Control			NS		NS	
	IMD Unexposed MS vs Control						
Species	Comparison						
Akkermansia muciniphila	MS vs Control					NS	
	IMD Unexposed MS vs Control						
Bacteroides coprophilus	MS vs Control					NS	
	IMD Unexposed MS vs Control						
Bacteroides fragilis	MS vs Control					NS	
	IMD Unexposed MS vs Control						
Faecalibacterium prausnitzii	MS vs Control					NS	
	IMD Unexposed MS vs Control						

Taxa ↓ in MS vs control; Taxa ↑ in MS vs control; Mixed findings; NS Not significant; Not reported

Taxa listed here are differentially significant across 2 or more studies. Red and green cells indicate a lower and higher relative abundance, respectively. Empty cells indicates that the respected study did not report the differential abundance of the respected taxa between the two groups. 'MS vs Control' refers to all of the MS participants included in the study, regardless of IMD exposure, compared to the control participants. 'IMD unexposed MS vs Control' refers to the MS cases unexposed to an IMD at the time of microbiota sample collection, compared to the controls. Genus *Akkermansia*is represented as *Akkermansia muciniphila*. The 4 studies that compared taxa-level findings between IMD unexposed MS cases vs. controls are (1st author, num. MS cases vs. controls): Tremlett, $n = 9$ vs. $n = 17$; Jangi, $n = 28$ vs $n = 43$; Berer, $n = 17$ vs. $n = 17$; Cekanaviciute, $n = 71$ vs. $n = 71$. If a study reported a higher and lower relative abundances with the same taxonomy assignment, the cell was split into two colors, as was the case for *Faecalibacterium prausnitzii*. If a study reported a taxonomy assignment confidence score, only scores of 95% or better were included in the table. All taxa are statistically significant ($P < 0.05$) after FDR adjustment. NS, not significant; IMD, immunomodulatory drug; untr, IMD untreated; tr, IMD treated; MS, multiple sclerosis; NS, not significant.

Future studies planning to assign taxonomy may find it helpful to further classify *F. prausnitzii* into phylogroups when possible to better resolve the mapping contradiction and serve as a better discriminating biomarker (Lopez-Siles et al., 2017).

The effects of *Prevotella* may differ by species. *P. histicola* has been shown to suppress or prevent disease activity in a mouse model of MS (Mangalam et al., 2017). Interestingly, the relative abundance of *Prevotella* was lower in relapsing-remitting MS patients with 'evidence of

Table 6
Operational taxonomic unit (OTU)-level findings: MS cases versus controls.

First Author, Date of Publication	Differential Abundance Statistical Method	Total OTUs Generated	Number of OTUs or Species Differentialy Abundant After Multiple-Testing Adjustment
Cantarel, 2015	Wilcoxon rank sum test, with Bonferroni correction	NA	Total 359 OTUs
Miyake, 2015	Welch's *t*-test, with Benjamini-Hochberg correction.	130 OTUs	Total 21 OTUs (2 OTUs enriched, 19 OTUs reduced)
Chen, 2016	Wilcoxon rank-sum test, with Benjamini-Hochberg correction	NA	NA
Cree, 2016	ANOVA test, with Bonferroni correction	2621 OTUs	0 OTUs (277 OTUs before adjustment)
Tremlett, 2016	Negative binomial regression, with Bonferroni correction	25,134 OTUs	Total 323 OTUs (160 OTUs enriched and 163 taxa OTUs reduced MS).
Jangi, 2016	DESeq2, with Benjamini and Hochberg correction	Roche 454: 4317 OTUs. 426 OTUs (after filtering). MiSeq: 10,620 OTUs. 1191 OTUs (after filtering)	NA
Cosorich, 2017	t-Student test	NA	None statistically significant
Swidsinski, 2017	t-Student test	NA	NA
Berer, 2017	DESeq2, with Benjamini and Hochberg correction	NA	None statistically significant
Cekanaviciute (2017)	DESeq2, with Benjamini and Hochberg correction	1462 OTUs (after filtering)	247 OTUs (161 reduced in MS and 86 enriched in MS)

Different statistical tests were used to assess differences in OTU abundance. DESeq2 is a differential gene expression analysis based on the negative binomial distribution. Filtering is the process in which undesired (e.g., unreliable) OTUs are removed. Often when filtering is used, OTUs present in less than 5% of samples are discarded. In some cases, it was not possible to determine if the total OTU count was filtered or not before reporting. ANOVA, Analysis of variance; NA, not available.

disease activity' relative to those with 'no evidence of disease activity' in one study (Cosorich et al., 2017). *Prevotella* species were also present in the oral microbiota of new-onset rheumatoid arthritis participants, but not in controls, suggesting a possible role in this autoimmune inflammatory condition (Scher et al., 2012).

B. fragilis is thought to benefit human health by, for example, breaking down dietary fibers to produce short-chain fatty acids and anti-inflammatory polysaccharides (Lukiw, 2016; Ochoa-Repáraz et al., 2010). *B. fragilis* is also considered a pathobiont, having an inflammatory pathogenic potential via its production of endotoxins (Lukiw, 2016). While there is limited relevant literature for *Bacteroides coprophilus,* this species merits further investigation for its potential role in MS.

Experimental studies allude to a pro-inflammatory MS gut microbiota. Neurological symptoms were exacerbated in animal models of MS when stool from individuals with MS was transplanted into the gut of mice with spontaneous or induced autoimmune encephalomyelitis, further supporting the association of the gut microbiota with MS (Berer et al., 2017; Cekanaviciute et al., 2017; Procaccini et al., 2015).

4.3. Potential confounders

Of the few confounding factors assessed to date in MS, exposure to disease-modifying drugs appears to be a likely candidate. However, most studies were too modest in size to formally assess the effect of confounders, hence much remains unknown. For example, at least 20 samples per group have been suggested in order to detect differences in taxonomic relative abundances (Weiss et al., 2017). Exposure to several medications as well as stool consistency (a reflection of gut transit time) are considered important factors in explaining microbiome variation and are related to MS itself (Falony et al., 2016; Vandeputte et al., 2016; He et al., 2018; Maier et al., 2018). A cross-sectional study assessing the differences between the gut microbiota of MS cases treated with either dimethyl fumarate (DMF; $n = 33$ cases) or glatiramer acetate (GA, $n = 60$) relative to IMD naïve cases ($n = 75$) was published in 2018, and although did not fulfill our inclusion criteria (due to absence of controls without MS), it is worthy of comment (Katz Sand et al., 2019). Authors reported that MS cases exposed to either DMF or GA had lower relative abundances of the *Lachnospiraceae* and *Veillonellaceae* families compared to IMD naïve cases (Katz Sand et al., 2019).

Constipation (reflecting a slow gut transit time) is common in MS, may influence the gut microbiota composition and contribute to a pro-inflammatory local environment. Microbiota from chronically constipated individuals was demonstrated to damage the intestinal barrier and further contribute to constipation (Cao et al., 2017). An enrichment of *A. muciniphila* and *Methanobrevibacter* spp. could be related to a slow gut transit and constipation, as shown in other conditions, including Parkinson disease (Gaci et al., 2014; Falony et al., 2016; Vandeputte et al., 2016; Cao et al., 2017; Chia et al., 1995; Stocchi and Torti, 2017). Methanogens may thrive in a gut with reduced motility, and may contribute to a slow colonic transit by augmenting methane production which acts as a neuromuscular transmitter, and has been shown to slow bowel movement (Gaci et al., 2014; Vandeputte et al., 2016; Pimentel et al., 2006). Understanding how these factors relate to MS and the gut microbiota's composition and function may clarify a possible causal role of the gut microbiota in MS. Alternatively, findings might point towards an opportunity to modify the gut microbiota to improve outcomes in MS. Future studies could assess stool consistency using proxy markers, such as the Bristol Stool Scale (Koh et al., 2010). Polypharmacy is common in MS and ideally, all recent drug exposure should be captured (Falony et al., 2016; Maier et al., 2018). The relationship between the IMDs used to treat MS and the gut microbiota is particularly intriguing and could provide additional mechanistic insights. Sufficiently powered, prospective longitudinal studies are needed to better understand the complex and likely dynamic relationship between MS, the gut microbiome, comorbidities, medication exposure, diet, and other lifestyle factors.

4.4. Heterogeneity of study design

Heterogeneity in the microbiota composition across studies may relate to differences in the sourcing of cases and controls and their characteristics, including: the broad age range of participants (from children to adults); (Falony et al., 2016) MS course (2 studies included participants with primary or secondary progressive MS (Cree et al., 2016; Berer et al., 2017)) and disease duration (which ranged from a few months to decades); host geographic location (although all studies were from largely westernized populations) and ethnicity (He et al., 2018; Deschasaux et al., 2018; Gaulke and Sharpton, 2018; McDonald et al., 2018). The degree to which these differences contribute to variation in the gut microbiota are complex, context specific and is not fully understood (He et al., 2018). For example, although it is possible that individuals with a progressive disease course might differ in terms of their gut microbiota composition from those with a relapsing disease course, insufficient data and cases were available. Further, teasing apart the effects of age, disease duration, accrual of comorbidities and medication exposures from the underlying disease course would likely require access to a sizable cohort of individuals with progressive and relapsing MS.

Technical methods for quantifying and analyzing the gut microbiota also differed, including the choice of: quantification instrument, 16S rRNA sequences regions(s), and gastrointestinal tract site sampled. Computational methods also differed, including: the bioinformatics pipeline used to generate OTUs, OTU abundance normalization, and statistical tests employed. While the method of generating OTUs varied, all studies using an NGS platform generated OTUs at the same taxonomic resolution by clustering the 16S rRNA sequences based on a 97% similarity threshold.

4.5. Strengths and limitations

The strengths of this review include its systematic, reproducible approach, and pre-registered protocol. Our systematic review also provides insights into the heterogeneity in microbiome study design including an overview of the differences in the computational pipelines. However, all studies included were relatively modest in size, with the total number of available data pertaining to 286 MS cases and 296 controls from a limited number of regions in the world. It remains possible that associations have been missed, particularly with the lower abundant taxa. For simplicity, we only reported findings on taxa that were similarly observed across two or more studies. Further, all studies were considered together, including one which sampled duodenal mucosal tissue. It remains possible that different physiological niches in the gastrointestinal tract will harbor distinct microbiota communities of relevance in MS. Interrogation of the gut microbiota in MS was primarily conducted using 16S rRNA sequencing which is typically unable to assign taxonomy below species level and is incomplete at low taxonomic ranks. It was not possible to match and compare individual OTUs identified across studies. We found no published study investigating the virome or mycobiome (fungi microbiome) in MS.

4.6. Concluding remarks

To our knowledge, this article is the first to systematically review the scientific literature investigation the link between the gut microbiome and MS. Despite the modest cohort sizes, diversity in the geographical location of participants and sample processing and bioinformatics pipelines used, consistent patterns are emerging: several taxa were similarly identified as being over or underrepresented in MS versus controls. A better understanding of a possible causal role of the microbiota in either facilitating the onset of MS, or outcomes in MS, including perpetuating comorbidities will facilitate our ability to

harness the microbiome to affect positive change in MS.

5. Addendum

5.1. Summary of articles published between June 20th 2018 and August 24th, 2019

The systematic literature search was updated on August 24th, 2019 by one reviewer (AM). Of 122 new articles identified, three fulfilled the inclusion criteria, one from the US, one from Canada, and one from China (Cekanaviciute et al., 2018; Forbes et al., 2018; Zeng et al., 2019).

Briefly, the US-based study included stool samples from 25 RRMS cases, all unexposed to IMDs or corticosteroids 3 months prior to stool collection (80% were women, mean age = 44.0 years), and 24 controls (12.5% women, mean age = 49.3 years). All bacteria as well as the spore-forming bacterial fractions alone were sequenced at the 16S rRNA V4 region, using the Illumina NextSeq platform and clustered into closed-reference OTUs (97% sequence similarity). No differences were observed in alpha-diversity (Chao1) or beta-diversity (unweighted UniFrac) between cases and controls for either the spore-forming bacteria or total bacteria. Differences between MS cases and controls in the relative abundance of OTUs of spore-forming bacteria fractions including: lower *Ruminococcus gnavus, Ruminococcus bromii, Veillonella dispar,* and a higher relative abundance of *Propionibacterium acnes, Staphylococcus epidermidis, Clostridium perfringens, and Clostridium citroniae* were reported (all $P < 0.05$). Taxa-level differences between cases and controls among total bacteria and the effects of possible confounders were not reported (Cekanaviciute et al., 2018).

The Canada-based study included stool samples from 19 MS cases (average age = 47.3 years; 14 were women; MS course was not reported) and 23 healthy controls (average age = 32.4 years; 12 were women). In addition, the authors included 20 Crohn's disease cases, 19 ulcerative colitis cases, and 21 rheumatoid arthritis cases (not reported here). DNA extracts were sequenced at the 16S rRNA V4 region using the Illumina MiSeq platform, clustered into *de novo* OTUs (97% sequence similarity). Because the dataset had an unusually low abundance of Gram-negative bacteria, the differential taxa abundance testing included only OTUs from Gram-positives phyla (i.e., OTUs within the phyla *Firmicutes, Actinobacteria*, and *Tenericutes*). The author's main goal was to compare across all the immune-mediated diseases relative to controls. Here, we report only the comparisons between the MS cases and controls. Alpha-diversity (i.e., Chao1, ACE, Shannon index, and Simpson diversity index, based on all phyla) did not differ between MS cases and controls. Beta-diversity was not directly compared between MS cases and controls. Significant differences between MS cases and healthy controls in the relative abundance of gram-positive bacteria at the genera level included: Lower *Butyricicoccus, Dialister, Faecalibacterium* (consistent with a previous finding (Miyake et al., 2015)), *Fusicatenibacter, Gemmiger, Lachnospira, Sporobacter*, and *Subdoligranulum* in MS cases relative to controls and higher *Actinomyces, Eggerthella, Anaerofustis, Clostridium* group III, *Clostridium* group XlVa, *Clostridium sensu stricto, Faecalicoccus, Streptococcus* and *Turicibacter* in MS cases relative to healthy controls ($P < 0.05$, Kruskal–Wallis test and Dunn's post hoc tests for multiple comparisons, with FDR correction) (Forbes et al., 2018).

The third study included stool samples from 34 RRMS cases (21 unexposed and 13 exposed to an immunosuppressant [e.g., azathioprine, methotrexate, or mycophenolate]) and 34 healthy controls from China (in addition to 34 individuals with neuromyelitis optica spectrum disorder). DNA extracts were sequenced at the 16S rRNA V3-V4 region using the Illumina MiSeq platform and clustered into *de novo* OTUs (97% sequence similarity). Alpha-diversity (i.e., Chao1, Shannon index, and Simpson diversity index) did not differ between all MS cases and healthy controls. Although beta-diversity did differ for both exposed MS cases and unexposed MS cases compared to healthy controls, (using a non-phylogenetic gain distance metric, $P < 0.01$ and $P < 0.05$, respectively; Mann−Whitney U test), the authors used an unconventional approach, by comparing distance values computed from the first principal coordinate of a principal coordinate analysis. The relative abundance of *Prevotella* was lower for the MS cases compared to controls, consistent with findings from prior studies in MS (exposed MS cases vs. controls, $P < 0.05$ and unexposed MS cases vs. controls, $P < 0.0001$; Mann−Whitney U test). As in the prior Canadian study (Forbes et al., 2018), the relative abundance of *Streptococcus* was higher for the MS cases compared to controls, (exposed MS cases vs. controls, $P < 0.001$ and unexposed MS cases vs. controls, $P < 0.0001$) (Zeng et al., 2019).

5.2. Summary comments

The new articles support the conclusion that there is no major differences in the overall composition of the gut microbiota in individuals with MS relative to controls, as assessed using alpha- or beta-diversity measures. Two studies consistently identified two taxa that differed in their relative abundance between MS cases and controls: lower *Faecalibacterium* and higher *Streptococcus* in MS cases relative to controls (Forbes et al., 2018; Zeng et al., 2019). The former observation concurs with a finding from a study in our systematic review, the latter represents a 'new' observation.

Author contributions

AM and HT contributed to conception and design of the study, acquisition, analysis and interpretation of data, and drafted the first version of the manuscript, tables and figures. JF, FZ, CB, GV, MG, and EW contributed to acquisition and interpretation of data and provided critical review of the manuscript.

Potential conflict of interest

HT is the Canada Research Chair for Neuroepidemiology and Multiple Sclerosis. Current research support received from the National Multiple Sclerosis Society, the Canadian Institutes of Health Research, the Multiple Sclerosis Society of Canada and the Multiple Sclerosis Scientific Research Foundation. In addition, in the last five years, has received research support from the Multiple Sclerosis Society of Canada (Don Paty Career Development Award); the Michael Smith Foundation for Health Research (Scholar Award) and the UK MS Trust; speaker honoraria and/or travel expenses to attend CME conferences from the Consortium of MS Centres (2013, 2018), the National MS Society (2014, 2016, 2018), ECTRIMS (2013, 2014, 2015, 2016, 2017, 2018, 2019), Biogen Idec (2014), American Academy of Neurology (2013, 2014, 2015, 2016, 2019). All speaker honoraria are either declined or donated to an MS charity or to an unrestricted grant for use by HT's research group.

AM, FZ and JF were funded through research grants held by HT. AM received funding from the Multiple Sclerosis Society of Canada.

CB, GV, MG, and EW have no conflict of interest to report.

Acknowledgements

The study authors received funding from The Multiple Sclerosis Scientific and Research Foundation, EGID: 2636 (PI: Tremlett). Ali Mirza was supported by an endMS Doctoral Studentship Award from the Multiple Sclerosis Society of Canada, EGID: 3246.

Appendix 1. Information retrieved and extracted from each included study

1. *Publication information*: name of author(s) and year of publication,
2. *Study information*: study design, geographic location of recruits (e.g., National Center of Neurology and Psychiatry Hospital, Tokyo, Japan), MS diagnosis criteria (e.g., McDonald criteria), and eligibility criteria, and enrolment period.
3. *Case-control demographics and characteristics close to the time of microbiota sample collection:* age, sex, race/ethnicity, MS disease course (e.g., relapsing-remitting onset MS or primary progressive), case or control status, MS disease duration, disability level (e.g., the Expanded Disability Status Scale (EDSS) score), lifestyle factors, as available (e.g., BMI, diet-related metrics). Medication use, including drugs used to treat MS, e.g., the immunomodulatory drug (IMDs), captured as therapeutic class or generic name (e.g., interferon-beta, glatiramer acetate) and IMD treatment status [treated/untreated/naïve (as defined by the study authors)]. Systemic corticosteroid around the time of sample collection (yes, no). Other medication use, including antibiotics.
4. *Microbiota sampling and quantification information:* body site sampled (e.g., stool, mucosa biopsy of small intestines, etc.), number of samples collected, number of samples excluded, DNA isolation kit, microbiota quantification instrument, sequence molecule/ region, primer sequence, method of generating OTUs ('Operational Taxonomic Units'; species-like taxonomy), and OTU statistical normalization method.
5. *Microbiota analysis results:* Total OTUs, differential abundance statistical method, taxa-level [phylum, genus, species (or individual OTUs)] differences between cases and control, number OTUs differentially abundant before and after multiple-testing adjustment, potential confounding factors considered in the study design and/or adjusted statistically in the analyses, diversity metric(s) considered, diversity comparisons between groups, e.g., MS vs. controls, and when available, untreated MS vs controls and treated MS vs untreated MS.

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Self reported use of vitamin D supplements is associated with higher physical quality of life scores in multiple sclerosis

Steve Simpson-Yap [1], Pia Jelinek [2], Tracey Weiland [3], Nupur Nag [3], Sandra Neate [3], George Jelinek [3]

Abstract

Background: Sun exposure and vitamin D, including intake and serum levels, have been associated with reduced risk of MS onset and less progression and may affect quality of life (QoL). We investigated the prospective relationship of these factors with QoL from baseline to 2.5 years' follow-up, in an international cohort of people with MS.

Methods: Data derive from the HOLISM international cohort. Sun exposure and vitamin D supplement use were queried at both timepoints. QoL was assessed by MSQOL-54, estimating physical and mental health QoL composite scores. Characteristics of QoL were assessed by linear regression, adjusted for age, sex, socioeconomic status, treated comorbidity number, MS type, disability, clinically significant fatigue, prescription antidepressant medication use, and ongoing relapse symptoms, and baseline QoL score, as appropriate, estimating adjusted coefficients (aβ).

Results: At 2.5-year review, QoL scores were higher among those reporting taking vitamin D supplements (physical: aβ=3.58, 95%CI=1.35-5.80; mental: aβ=3.08, 95%CI=0.72-5.44), particularly average daily dose over 5,000IU/d. Baseline-reported vitamin D supplementation was associated with greater increase in physical (aβ=1.02, 95%CI=0.22-1.81), but not mental QoL (aβ=0.11, 95%CI=-1.00-1.23). Sun exposure was cross-sectionally associated with higher QoL scores at follow-up but was not associated with change in QoL.

Conclusions: Self-reported vitamin D supplement use was cross-sectionally associated with higher physical and mental QoL, but prospectively only with increased physical QoL.

Efficacy of Diet on Fatigue and Quality of Life in Multiple Sclerosis: A Systematic Review and Network Meta-analysis of Randomized Trials

Linda Snetselaar [1], Joshua J Cheek [2], Sara Shuger Fox [2], Heather S Healy [3], Marin L Schweizer [4], Wei Bao [5], John Kamholz [6], Tyler J Titcomb [7] [8]

Abstract

Background: Emerging evidence suggests a role for diet in multiple sclerosis (MS) care; however, due to methodological issues and heterogeneity of dietary interventions in preliminary trials, the current state of evidence does not support dietary recommendations for MS.

Objective: To assess the efficacy of different dietary approaches on MS-related fatigue and quality of life (QoL) through a systematic review of the literature and network meta-analysis (NMA).

Methods: Electronic database searches were performed in May 2021. Inclusion criteria were: 1) randomized trial with a dietary intervention; 2) adults with definitive MS based on McDonald criteria; 3) patient-reported outcomes for fatigue and/or QoL; and 4) minimum intervention period of 4 weeks. For each outcome, standardized mean differences (SMDs) were calculated and included in random effects NMA to determine the pooled effect of each dietary intervention relative to each of the other dietary interventions. The protocol was registered at PROSPERO (CRD42021262648).

Results: Twelve trials comparing 8 dietary interventions (low-fat, Mediterranean, ketogenic, anti-inflammatory, Paleolithic, fasting, calorie restriction, and control [usual diet]), enrolling 608 participants, were included in the primary analysis. The Paleolithic (SMD: -1.27; 95% CI: -1.81, -0.74), low-fat (SMD: -0.90; 95% CI: -1.39, -0.42), and Mediterranean (SMD: -0.89; 95% CI: -1.15, -0.64) diets showed greater reductions in fatigue compared to control. The Paleolithic (SMD: 1.01; 95% CI 0.40, 1.63) and Mediterranean (SMD: 0.47; 95% CI 0.08, 0.86) diets showed greater improvements in physical QoL compared to control. For improving mental QoL, the Paleolithic (SMD: 0.81; 95% CI 0.26, 1.37) and Mediterranean (SMD: 0.36; 95% CI 0.06, 0.65) diets were more effective compared to control. However, the NutriGRADE credibility of evidence for all

direct comparisons is very low due to most of the included trials having high or moderate risk of bias, small sample sizes, and the limited number of studies included in this NMA.

Discussion: Several dietary interventions may reduce MS-related fatigue and improve physical and mental QoL; however, due to the limitations of this NMA, which are driven by the low quality of the included trials, these findings must be confirmed in high quality, randomized, controlled trials.

MSJ Experimental Translational Clinical

Original Research Article

Impact of the Swank and Wahls elimination dietary interventions on fatigue and quality of life in relapsing-remitting multiple sclerosis: The WAVES randomized parallel-arm clinical trial

Multiple Sclerosis Journal–Experimental, Translational and Clinical

July–September 2021, 1–11

DOI: 10.1177/20552173211035399

Terry L Wahls*, Tyler J Titcomb*, Babita Bisht, Patrick Ten Eyck, Linda M Rubenstein, Lucas J Carr, Warren G Darling, Karin F Hoth, John Kamholz and Linda G Snetselaar

Abstract

Objective: To compare the effect of the modified Paleolithic elimination (Wahls) and low-saturated fat (Swank) diets in relapsing-remitting MS (RRMS).

Methods: Individuals (n = 87) with RRMS were randomized to the Swank or Wahls diets in a parallel group clinical trial consisting of four timepoints: 1) run-in, 2) baseline, 3) 12-weeks, and 4) 24-weeks.

Results: 77 participants completed 12 weeks and 72 completed 24 weeks. The 12-week change from baseline in fatigue was -0.94 ± 0.18 (FSS) and -9.87 ± 1.93 (MFIS; both $p < 0.0001$) for Swank, and -0.71 ± 0.24 (FSS; $p = 0.004$) and -14.41 ± 2.22 (MFIS; $p \leq 0.0001$) for Wahls. Physical MSQoL scores improved by 6.04 ± 2.18 ($p = 0.006$) for Swank and by 14.5 ± 2.63 ($p < 0.0001$) for Wahls. Mental MSQoL scores improved by 11.3 ± at 2.79 ($p < 0.0001$) for Wahls while the Swank did not change (3.85 ± 2.63; $p = 0.14$). Neither group showed significant changes in 6-minute walking distance at 12 weeks. All outcomes were maintained or further improved at 24 weeks.

Conclusions: Both diets were associated with clinically meaningful within-group reductions in fatigue and improvements in QoL.

Trial Registration: Clinicaltrials.gov Identifier: NCT02914964

Keywords: Multiple sclerosis, fatigue, quality of life, 6-minute walk test, low-saturated fat diet, paleolithic diet

Date received: 30 May 2021; accepted: 8 July 2021

Introduction

Multiple sclerosis (MS) is a chronic immune-mediated neuroinflammatory condition that affects nearly one million people in the United States.[1] Fatigue is one of the most common and debilitating symptoms,[2] and is associated with increased disability and reduced quality of life (QoL).[3] Pharmacological treatment options for MS-related fatigue have limited efficacy[4]; thus, many individuals with MS seek non-pharmacologic therapies to reduce their fatigue burden.

Despite a lack of consistent evidence for any specific therapeutic diet for MS,[5] surveys observe that half of individuals with MS report implementing dietary modifications.[6,7] Due to the lack of evidence demonstrating diet intervention-related reduced disease activity[5] and the limited role of the neurologist in providing dietary recommendations,[8] people newly diagnosed with MS receive little dietary advice[9] which forces this information to be sought from internet sources that are often not evidence-based.[10] Two dietary approaches with supporting preliminary evidence are the low-saturated fat diet developed by Dr. Swank and the modified Paleolithic diet developed by Dr. Wahls.[5]

Based on epidemiological evidence that regions with higher saturated fat intake experience higher incidences of MS,[11] Swank began recommending his

*Joint first authors.

Correspondence to:
Terry L Wahls,
Terry L Wahls, Department of Internal Medicine, University of Iowa, 200 Hawkins Dr., SE 636 GH, Iowa City, IA 52242, USA.
Terry-Wahls@uiowa.edu

Terry L Wahls*, Department of Internal Medicine, University of Iowa, Iowa City, IA, USA

Tyler J Titcomb*, Department of Internal Medicine, University of Iowa, Iowa City, IA, USA
Department of Epidemiology, University of Iowa, Iowa City, IA, USA

Babita Bisht, Department of Internal Medicine, University of Iowa, Iowa City, IA, USA

Patrick Ten Eyck, Institute for Clinical and Translational Science, University of Iowa, Iowa City, IA, USA

Linda M Rubenstein, Department of Epidemiology, University of Iowa, Iowa City, IA, USA

Lucas J Carr, **Warren G Darling**, Department of Health and Human Physiology, University of Iowa, Iowa City, IA, USA

Karin F Hoth, Department of Psychiatry, University of Iowa, Iowa City, IA, USA

John Kamholz, Department of Neurology, University of Iowa, Iowa City, IA, USA

Linda G Snetselaar, Department of Epidemiology, University of Iowa, Iowa City, IA, USA

patients consume a low saturated fat diet,[12] and followed them for up to 50 years.[13] His studies observed that patients who consumed the least amount of saturated fat were less likely to have exacerbations, more likely to continue to ambulate, and had reduced risk of mortality.[14,15] In recent years, randomized controlled trials using plant-based and omega-3 supplemented low-fat diets have demonstrated favorable outcomes on fatigue and QoL among individuals with RRMS.[16,17]

Wahls believed that a Paleolithic-based diet that eliminated specific dietary antigens (gluten, casein, and lectins) and maximized micronutrient density could optimize health and prevent disease progression. In a single-arm study using this dietary approach as part of a multimodal intervention, improvements in fatigue, QoL, and gait, were observed in half of a cohort with progressive MS.[18,19] Due to the multimodal intervention in that study it was not possible to determine the effect attributable to the diet; however, two small follow-up randomized, controlled trials comparing the modified Paleolithic diet to usual diet demonstrated favorable outcomes for fatigue and QoL among individuals with progressive or RRMS.[20,21]

Both the low-saturated fat and modified Paleolithic diets are popular among individuals with MS[6]; however, due to a lack of data from high-quality randomized controlled trials, it is not possible for either of these diets to be recommended in clinical practice. The objective of this study was to assess and compare the effect of the Swank and Wahls diets on perceived fatigue and QoL in individuals with RRMS.

Participants and methods

Study design and participants

This 36-week, randomized, parallel-group, single-blinded trial was conducted at the University of Iowa Prevention Intervention Center, with participant recruitment taking place from August 2016 to May 2019 and follow-up from February 2017 to January 2020. The trial protocol[22] was approved by the University of Iowa Institutional Review Board. Written and informed consent was obtained from all participants, and data safety and management of the trial was monitored by a National Multiple Sclerosis Society (NMSS) data safety monitoring board. This trial followed the Consolidated Standards of Reporting Trials (CONSORT) reporting guidelines.[23]

Participants were recruited from within a 500-mile radius of Iowa City, Iowa. The research team worked with local NMSS support groups, regional MS centers, the North American Research Committee on Multiple Sclerosis, the University of Iowa Hospitals & Clinics Department of Neurology, the Iowa City VA Health Care System neurology clinic, the Swank Foundation, terrywahls.com, and other organizations to recruit study participants.

Participants aged 18-70 years were eligible for enrollment if they had: 1) neurologist-confirmed RRMS based on the 2010 McDonald criteria,[24] 2) moderate to severe fatigue (FSS $\geq$ 4.0), 3) an ability to walk 25 feet with unilateral or no support, 4) were not pregnant or planning on becoming pregnant, and 5) were willing to comply with all aspects of the study intervention and assessments. Major exclusion criteria included: 1) MS-relapse or change in disease modifying drug use within the previous 12 weeks, 2) change in medication to manage MS symptoms, 3) low body weight (BMI $<$ 19 kg/m^2), 4) severe mental impairment, 5) self-reported adverse reactions to gluten-containing foods, 6) diagnosed conditions including eating disorders, severe psychiatric disorders, celiac disease, kidney stones, heart failure, angina, or liver cirrhosis, and 7) insulin, warfarin, radiation, or chemotherapy use. An exhaustive list of inclusion and exclusion criteria can be found in Supplemental Table 1 or the published protocol.[22] The study consisted of four study visits for data collection: 1) run-in, 2) baseline, 3) 12-weeks, and 4) 24-weeks. Eligible participants were enrolled in a 12-week run-in phase for observation prior to treatment randomization at baseline. Participants who did not complete all study procedures during the run-in phase were excluded from the study.

Randomization and masking

Eligible participants were randomized 1:1 to follow either the modified Paleolithic elimination (Wahls) or low-saturated fat (Swank) diet at baseline. Two randomization tables were used to achieve similar mean FSS values at baseline—one for moderate perceived fatigue (FSS $\geq$ 4 and $<$ 5.5) and the other for high perceived fatigue (FSS $\geq$ 5.5). Password-protected randomization tables were accessible only by the intervention registered dietitians (RDs). Study participants, intervention RDs, and the statistician overseeing randomization and data management were not masked to treatment randomization. The study principal investigator, co-investigators, study coordinator, data analysis statistician, and outcome assessors were masked to treatment

randomization. All analyses were performed by the masked statistician (PTE) with groups coded "X" and "Y" and then decoded by the unmasked statistician (LMR).

Procedures

Following a 12-week run-in period for observation of usual diet and stability of pre-intervention outcomes, participants were randomized to either the Wahls or the Swank diets for 24 weeks. During the first 12 weeks post-randomization, participants received two in-person and five telephone-based nutrition counseling sessions from an intervention RD. Participants also received personalized emails with feedback on their diet checklists every 4 weeks. At week 12 of the intervention period, in-person and telephone counseling sessions were discontinued, but participants were allowed to contact the intervention RD at any time for additional support.

Intervention diets

The Swank diet restricts saturated fat to $\leq$ 15 g per day and provides 20-50 g (4-10 teaspoons) unsaturated fat per day and four servings each of grains, whole preferred, and fruits and vegetables (FV; Supplemental Table 2). The Wahls diet recommends 6-9 servings of FV and provides 6-12 ounces meat per day according to gender. It excludes all grain, legumes, eggs, and dairy (except for clarified butter or ghee). Nightshade vegetables were also excluded in the Wahls group during the first 12-week period from baseline and then the intervention RDs provided guidance to reintroduce nightshades during the second 12-week period on the diet. A review of the published research and potential mechanisms of both diets can be found elsewhere.[25] Participants in both groups were instructed to follow their assigned diet *ad libitum* and were given the following daily supplement regimen: 1 teaspoon cod liver oil, 1,000 µg methyl-B_{12}, 1,000 µg methylfolate, a multivitamin without iron, and 5,000 IU vitamin D_3, the latter of which was adjusted based on serum levels with a target range of 40 to 80 ng/mL.[22]

Outcomes

The primary endpoint of the trial was the change in perceived fatigue from baseline to 12 weeks as assessed by the Fatigue Severity Scale (FSS). Secondary endpoints included perceived fatigue assessed by the Modified Fatigue Impact Scale (MFIS), mental and physical QoL assessed by the Multiple Sclerosis Quality of Life-54 (MSQoL-54), and the 6-minute walk test (6MWT) and an additional 12-week follow-up period. Clinically meaningful changes were defined as 0.45 for FSS,[26] 4.0 for MFIS,[26] 5.0 points for both mental and physical MSQoL-54,[27] and 6% change for 6MWT.[28] Adherence to diet specific food components (i.e., grams of gluten for the Wahls group and grams of saturated fat for the Swank group) was monitored using three-day weighed food records collected on three consecutive days including one weekend day in the week prior to each study visit and were analyzed at the University of Minnesota Nutrition Coordinating Center using Nutrition Data System for Research software.

Statistical analysis

The trial was powered to detect a group mean difference of 0.7 ± 0.9 for FSS within and between groups. With two-sided significance set at $\alpha = 0.05$ and power of 85%, we determined the required sample size to be 34 participants per group. Assuming 25% attrition based on our previous experience, we aimed to recruit 43 participants per group.

Descriptive statistics were calculated for every variable at enrollment using frequencies, means $\pm$ standard error (SE), and medians (interquartile range). Outliers were checked for accuracy and possible data entry errors. Distributions of continuous variables were evaluated for normality by graphical observation. Generalized linear mixed models[29] were used to test the interacting effects of diet and time on outcome measures while accounting for repeated measures for each participant. Other potentially important variables (age, sex, BMI, smoking status, alcohol consumption, walking assistance, years since MS diagnosis, disease modifying drug use, baseline vitamin D, baseline six-minute walk distance) were considered for inclusion in each model to assess their relationship with the outcome and whether they modified the estimates for the diet and time interaction. For each outcome, the model with the smallest Akaike information criterion (AIC)[30] was deemed to have the optimal predictor set. Point estimates, 95% confidence intervals, and p-values of the group mean changes in outcome measures over visits were generated for each optimal model. For primary data analysis, data from all participants completing 12- and 24-week assessments were included in an intention-to-treat analysis. A secondary per-protocol analysis was used to examine the participants who successfully met study compliance requirements for their assigned diet intervention. Participants were included in the per-protocol analysis if weighed food records indicated that they consumed within 20% of recommendations for key diet components ($\leq$ 0.2 grams of gluten or $\leq$ 18

grams of saturated fat for the Wahls or Swank diets, respectively) on a minimum of two of three food records at 12 weeks. All analyses were conducted with two-sided tests ($\alpha = 0.05$) using SAS software (version 9.4, SAS Institute, Inc.).

Results

A total of 95 participants enrolled in the 12-week run-in period, of whom 8 were excluded for not providing complete study logs during the run-in period (Figure 1). Of the remaining 87 randomized participants, 43 were randomized to the Wahls diet and 44 to the Swank diet. There were no significant group differences at baseline (Table 1) and baseline outcome values were largely stable compared to run-in values (Table 2). A total of 77 participants (39 Wahls and 38 Swank) completed the primary endpoint at 12 weeks and 72 (35 Wahls and 37 Swank) completed follow-up at 24 weeks. No serious adverse events were reported. Diet adherence was high for both groups. At 12 weeks, 86.8% (33 of 38) of the Swank group and 79.5% (31 of 39) of the Wahls group participants were considered adherent to the respective study diets and were included in the secondary per-protocol analysis. At 24 weeks, diet adherence was 81.1% (30 of 37) for the Swank group and 74.3% (26 of 35) for the Wahls group.

Significant mean reductions from baseline were observed in the primary outcome of FSS at both 12 and 24 weeks for the Swank (-0.94 ± 0.18 and -1.01 ± 0.24, respectively; $p \leq 0.0001$ for both) and Wahls (-0.71 ± 0.24 and -1.31 ± 0.29, respectively; $p \leq 0.004$ for both) groups, which exceeded the clinically meaningful threshold, defined as 0.45 FSS points,[26] at all time points (Figure 2(a)). There were no differences in the magnitude of 12- and 24-week FSS changes between groups. In addition, both

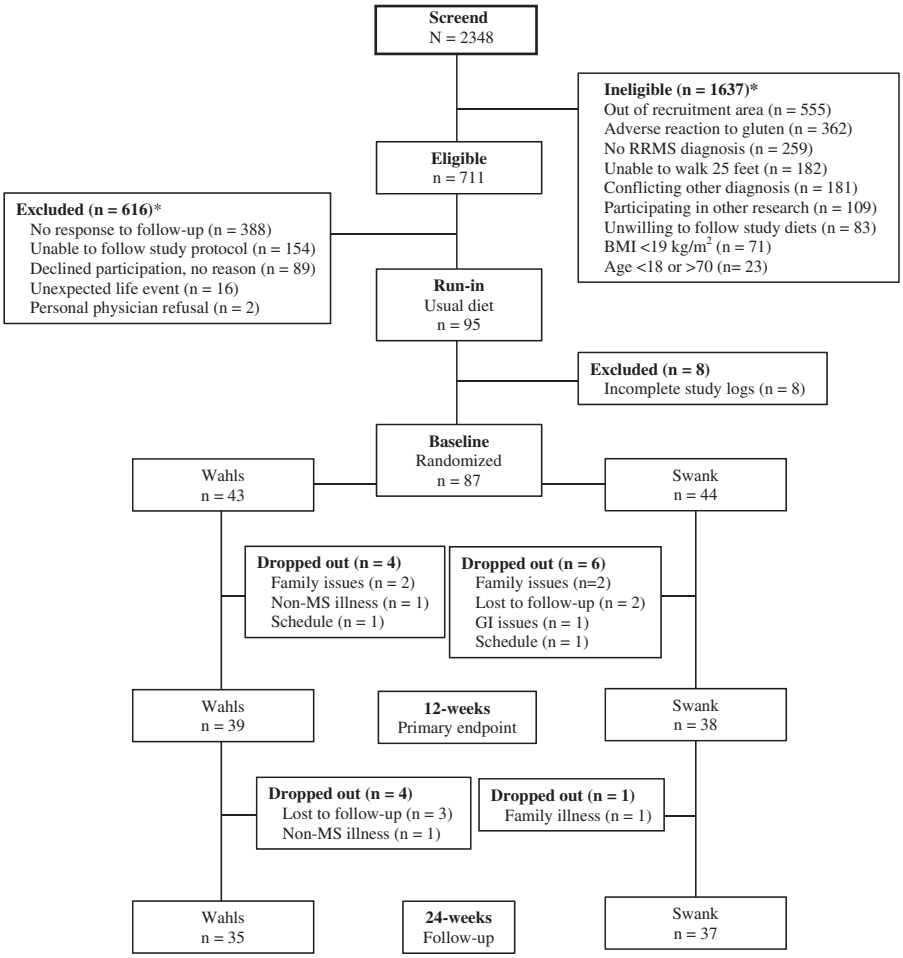

Figure 1. CONSORT diagram of study recruitment and participant flow. Reasons for ineligibility or exclusion may not add up to value of ineligible or excluded because some participants were found ineligible or were excluded for multiple reasons.

Table 1. Baseline characteristics of participants who completed a 12-week intervention of the Swank or Wahls diets.[a]

Characteristics	Swank	Wahls	p-value[b]
N	38	39	–
Age (years)	46.9 ± 1.7	46.4 ± 1.5	0.84
Gender (female)	35 (92.1)	32 (82.1)	0.31
MS duration (years)	12.1 ± 1.6	9.3 ± 1.0	0.14
Disease modifying drug use			0.83
None	13	10	
Oral	11	11	
Injectable	10	12	
Infused	4	6	
Race (Caucasian)	36 (94.7)	38 (97.4)	0.99
Education			0.32
High school	0 (0.0)	3 (7.7)	–
Some college	12 (31.6)	10 (25.6)	–
4-year degree	11 (28.9)	8 (20.5)	–
Advanced degree	15 (39.5)	18 (46.2)	–
Smoking status			0.13
Never	29 (76.3)	23 (59.0)	–
Former	3 (7.9)	2 (5.1)	–
Current	6 (15.8)	14 (35.9)	–
Alcohol drinks per month[c]			0.99
None	6 (15.8)	7 (17.9)	–
Within recommendations	29 (76.3)	29 (74.4)	–
Above recommendations	3 (7.9)	3 (7.7)	–
BMI (kg/m^2)	27.6 ± 0.94	30.2 ± 1.3	0.11
6-minute walk distance (meters)	481 ± 16.3	459 ± 10.3	0.40
Walking assistive device used (y)	5 (13.2)	4 (10.3)	0.73
Serum vitamin D (nmol/L)	47.9 ± 3.9	50.9 ± 3.2	0.55
Fatigue Severity Score[d]	5.3 ± 0.2	5.2 ± 0.2	0.62

[a]Data are shown as mean ± SEM or N (%). There were no significant differences in baseline values between groups.
[b]Significance determined by Fisher's exact test or generalized linear models.
[c]Alcohol recommendations defined as ≤ 1 standard drink per day for females and ≤ 2 standard drinks per day for males.
[d]Participants were randomized at baseline based on run-in FSS values.

groups had significant reductions in MFIS, the 12- and 24-week mean differences for the Swank group were -9.87 ± 1.93 and -10.5 ± 2.46, respectively ($p \leq 0.0001$ for both) and for the Wahls group were -14.4 ± 2.22 and -19.1 ± 2.66, respectively ($p \leq 0.0001$ for both), which exceeded the clinically meaningful threshold, defined as 4.0 MFIS points,[26] at all time points (Figure 2(b)). The Wahls group experienced a 24-week MFIS mean reduction that was significantly greater than the Swank group ($p = 0.02$).

Significant improvements occurred in both the mental and physical MSQoL-54 subscales for each group. Among the Swank group, the 12-week mental MSQoL-54 mean difference (3.85 ± 2.63; $p = 0.14$) did not achieve clinical significance defined as 5.0 MSQoL points,[27] though the 24-week difference (5.87 ± 2.65; $p = 0.03$) did achieve clinical significance (Figure 2(c)). The Wahls group had a clinically meaningful mean difference in mental MSQoL-54 scores at both 12 and 24 weeks (11.3 ± 2.79 and 14.0 ± 3.15, respectively; $p \leq 0.0001$ for both), which were significantly greater than the 12- and 24-week differences experienced by the Swank group (both time periods $p = 0.05$). The 12- and 24-week mean differences in physical MSQoL-54 scores were clinically meaningful for both the Swank (6.04 ± 2.18 and 9.25 ± 2.12, respectively; $p \leq 0.006$ for both) and the Wahls (14.5 ± 2.63

Table 2. Fatigue and QoL values among participants with RRMS assigned to the Swank or Wahls dietary interventions.[a]

Outcome (range)	Study visit Run-in	Baseline	12 Weeks	24 Weeks
Swank				
FSS (1–9)	5.45 ± 0.15	5.32 ± 0.18	$4.39 \pm 0.22^{***}$	$4.32 \pm 0.25^{***}$
MFIS total (0–84)	43.6 ± 2.50	40.7 ± 2.40	$30.8 \pm 2.35^{***}$	$30.2 \pm 2.63^{***}$
MFIS physical (0–36)	19.3 ± 1.26	18.9 ± 1.36	$13.8 \pm 1.29^{***}$	$14.7 \pm 1.40^{***}$
MFIS cognitive (0–40)	$20.2 \pm 1.40^{**}$	17.6 ± 1.37	$14.0 \pm 1.28^{**}$	$13.5 \pm 1.37^{**}$
MFIS psychosocial (0–8)	4.08 ± 0.37	4.18 ± 0.40	$3.05 \pm 0.31^{***}$	$3.03 \pm 0.34^{***}$
MSQoL-54 mental (0–100)	$63.2 \pm 3.11^{*}$	67.7 ± 2.90	71.6 ± 2.93	$73.6 \pm 2.81^{*}$
MSQoL-54 physical (0–100)	53.2 ± 2.85	55.6 ± 3.01	$61.7 \pm 3.27^{**}$	$64.9 \pm 3.15^{***}$
6MWT (meters)	460 ± 19.1	481 ± 16.3	485 ± 15.7	491 ± 16.6
Wahls				
FSS (1–9)	5.36 ± 0.15	5.19 ± 0.20	$4.47 \pm 0.22^{**}$	$3.87 \pm 0.27^{***}$
MFIS total (0–84)	45.4 ± 2.33	45.6 ± 1.99	$31.2 \pm 2.72^{***}$	$26.5 \pm 3.00^{***}$
MFIS physical (0–36)	20.5 ± 1.05	20.6 ± 0.98	$13.7 \pm 1.23^{***}$	$11.3 \pm 1.17^{***}$
MFIS cognitive (0–40)	20.4 ± 1.47	20.4 ± 1.24	$14.5 \pm 1.58^{***}$	$12.8 \pm 1.73^{***}$
MFIS psychosocial (0–8)	4.56 ± 0.31	4.59 ± 0.31	$2.92 \pm 0.37^{***}$	$2.37 \pm 0.35^{***}$
MSQoL-54 mental (0–100)	60.0 ± 3.33	62.3 ± 3.49	$73.7 \pm 3.43^{***}$	$76.3 \pm 3.59^{***}$
MSQoL-54 physical (0–100)	54.3 ± 2.89	53.8 ± 3.05	$68.2 \pm 3.02^{***}$	$71.0 \pm 3.20^{***}$
6MWT (meters)	468 ± 19.3	459 ± 10.3	468 ± 20.3	$495 \pm 18.7^{**}$

FSS: Fatigue Severity Scale; MFIS: Modified Fatigue Impact Scale; MSQoL-54: Multiple Sclerosis Quality of Life-54 Scale; 6MWT: 6-minute walk test.
[a]All values mean ± SEM.
*indicates statistical significance compared to baseline values ($P \leq 0.05$), **indicates statistical significance compared to baseline values ($P \leq 0.01$), ***indicates statistical significance compared to baseline values ($P \leq 0.001$).

and 17.24 ± 2.84, respectively; $p \leq 0.0001$ for both) groups, which exceeded clinically meaningful threshold at all time points (Figure 2(d)). The Wahls group had 12- and 24-week physical MSQoL-54 mean improvements that were significantly greater than those experienced by the Swank group (both time periods $p \leq 0.05$).

Neither group experienced significant improvements in distance walked during the 6MWT at 12 weeks (Table 2). The 12-week mean differences were 3.7 ± 5.5 meters for the Swank group ($p = 0.50$) and 9.3 ± 7.8 meters for the Wahls group ($p = 0.23$). By 24 weeks, the mean difference from baseline for the Swank group did not change (10.0 ± 6.7 meters; $p = 0.13$), whereas the Wahls group walked 36.6 ± 13.5 meters further compared to baseline distance ($p = 0.007$) and achieved clinical significance defined as 6% change[28] (Figure 2(e)). After removal of non-adherent participants in the secondary per-protocol analysis, the 24-week mean difference within each group was significant at 18.6 ± 5.7 meters for the Swank group ($p = 0.001$) and 39.9 ± 16.3 meters for the Wahls group ($p = 0.02$; Supplemental Figure 1).

Significant within-group 12- and 24-week mean improvements were observed in all outcomes assessed for the Wahls group and nearly all outcomes for the Swank group (Table 2). In the primary analysis, 47.4% (mental) and 50.0% (physical) of participants in the Swank group experienced clinically meaningful improvements in QoL while 68.4% (mental) and 60.5% (physical) of participants in the Wahls group experienced clinically meaningful improvements in QoL ($p > 0.05$ for both; Table 3). However, after removal of non-adherent participants in the secondary per-protocol analysis, the percentage of participants at 12 weeks who achieved clinically meaningful improvements in the mental MSQoL-54 subscale was higher in the Wahls group (74.2%) compared to the Swank group (45.5%; $p = 0.02$). In addition, in the primary analysis the proportion of participants at 12 weeks in the Swank group that experienced clinically meaningful reductions in fatigue was 59.5% (FSS) and

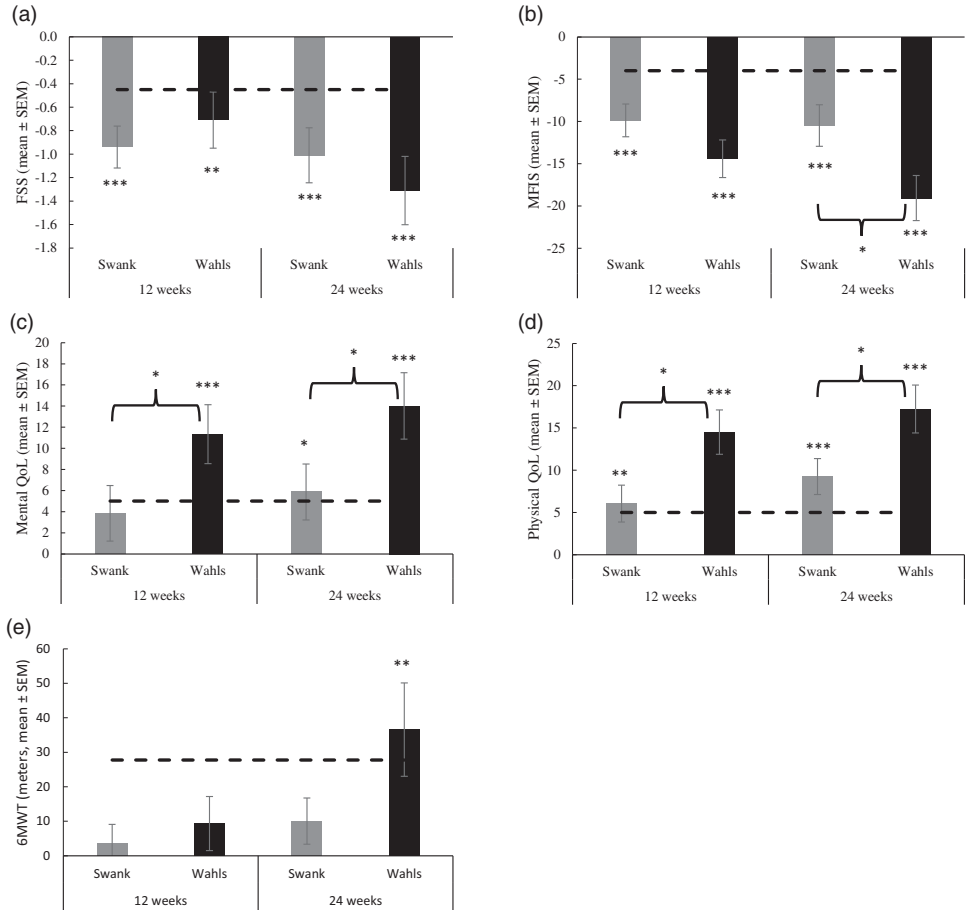

Figure 2. Change from baseline for (A) Fatigue Severity Scale (FSS), (B) total Modified Fatigue Impact Scale (MFIS), (C) Multiple Sclerosis Quality of Life-54 (MSQoL-54) mental, (D) MSQoL-54 physical, and (E) 6-minute walk test among study participants with RRMS randomized to either the Swank diet (grey bars) or the Wahls elimination diet (black bars) for 24 weeks. The dashed lines represent thresholds of clinically meaningful changes. Statistical significance determined by generalized linear mixed models, statistical significance is represented by * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

73.7% (MFIS) compared to 56.8% (FSS) and 76.3% (MFIS) of participants in the Wahls group ($p > 0.05$ for both). No individuals in the Swank group and 5.4% of individuals in the Wahls group achieved clinically meaningful improvements in the 6MWT at 12 weeks ($p = 0.49$). For all outcomes, the proportion of participants that experienced clinically meaningful improvements was lower at 24 weeks compared to respective values at 12 weeks. Additional results of the secondary per-protocol analysis can be found in Supplemental Table 3.

Discussion

The findings from this trial confirm those of preliminary trials that the Wahls and Swank diets are associated with significant reductions in fatigue and improvements in QoL among RRMS participants. In both diet groups, between 1/2 to 3/4 of participants reported clinically meaningful reductions in fatigue, depending on the scale used, at 12 weeks and were maintained by most individuals at 24 weeks despite receiving no active RD support in the final 12 weeks. In the Wahls group, the 12-week change in FSS scores (-0.71 ± 0.24) is approximately half the magnitude compared to findings from the 12-week preliminary trial[20]; however, the 24-week change in FSS scores in this study (-1.31 ± 0.29) is similar to the preliminary findings. Similarly, the 12- and 24-week FSS differences among the Swank group (-0.94 ± 0.18 and -1.01 ± 0.24, respectively) confirm the three- and six-month changes observed in the plant-based low-fat diet preliminary trial.[17] Furthermore, FSS changes in both groups are similar to those observed in pharmacological interventions for MS-related fatigue,[4] suggesting significant

Table 3. Percent (95% CI) of participants with clinically meaningful improvements in fatigue and MSQoL scores at 12 and 24 weeks compared to baseline.

		Primary intention-to-treat analysis			Secondary per-protocol analysis		
Outcome	Threshold[a]	Swank	Wahls	p-value[b]	Swank	Wahls	p-value[b]
FSS	0.45[26]						
12 weeks		59.5 (43.6, 75.3)	56.8 (40.8, 72.7)	0.81	62.5 (43.7, 78.9)	56.7 (37.4, 74.5)	0.80
24 weeks		45.9 (29.9, 62.0)	48.6 (32.5, 64.8)	0.82	50.0 (32.7, 67.3)	46.7 (28.8, 64.5)	0.79
MFIS	4.0[26]						
12 weeks		73.7 (59.7, 87.7)	76.3 (62.8, 89.8)	0.91	72.7 (54.5, 86.7)	80.0 (61.4, 92.3)	0.56
24 weeks		55.3 (39.5, 71.1)	68.4 (53.6, 83.2)	0.24	54.5 (37.6, 71.5)	70.0 (53.6, 86.4)	0.21
MSQoL-54 mental	5.0[27]						
12 weeks		47.4 (31.5, 63.2)	68.4 (53.6, 83.2)	0.06	45.5 (28.1, 63.7)	74.2 (55.4, 88.1)	0.02
24 weeks		36.8 (21.5, 52.2)	52.6 (36.8, 68.5)	0.17	36.4 (20.0, 52.8)	58.1 (40.7, 75.4)	0.08
MSQoL-54 physical	5.0[27]						
12 weeks		50.0 (34.1, 65.9)	60.5 (45.0, 76.1)	0.36	48.5 (30.8, 66.5)	61.3 (42.2, 78.2)	0.33
24 weeks		42.1 (26.4, 57.8)	50.0 (34.1, 65.9)	0.49	39.4 (22.7, 56.1)	54.8 (37.3, 72.4)	0.22
6MWT	6%[28]						
12 weeks		0.0 (0.0, 0.0)	5.4 (0.7, 18.2)	0.49	0.0 (0.0, 0.0)	6.9 (0.9, 22.8)	0.22
24 weeks		0.0 (0.0, 0.0)	3.1 (0.1, 16.2)	0.48	0.0 (0.0, 0.0)	4.0 (0.1, 20.3)	0.45

FSS: Fatigue Severity Scale; MFIS: Modified Fatigue Impact Scale; MSQoL-54: Multiple Sclerosis Quality of Life-54 Scale; NA: not applicable; 6MWT: 6-minute walk test.
[a]Thershold value corresponds to clinically meaningful change in outcome.
[b]Between-group significance determined by Pearson's chi-square test.

benefit of these two dietary interventions for MS-related fatigue.

Given fatigue is inversely associated with QoL among individuals with MS,[3] it is not surprising that significant improvements in QoL were also observed in this study. The 12-week change in mean values from baseline in both mental and physical MSQoL-54 scores among the Wahls group (11.3 ± 2.79 and 14.5 ± 2.63, respectively) confirm preliminary findings.[20] The Swank group experienced greater improvement in the physical compared to the mental MSQoL-54 subscale, which corresponds well to preliminary findings.[16,17] These findings demonstrate that adoption of the Swank or Wahls diets is associated with reduced fatigue and improved QoL for up to 24 weeks.

High diet adherence was observed in both groups at 12 and 24 weeks, demonstrating that these dietary approaches can be adopted with RD support and then maintained without support. The mechanism by which diet affects MS-related fatigue and QoL is not known; however, results from preliminary trials suggest that modulation of inflammation or oxidative stress is likely.[17,20] In addition, two trials show that dietary modification improves the mass and diversity of the gut microbiota in people with MS.[31,32] Evidence suggests that people with MS have reduced mass and diversity of their gut microbiota compared to healthy controls,[31] which likely promotes inflammation.[33] There are currently no studies investigating changes in inflammatory profiles or gut microbiota following adoption of the Swank or Wahls diets; however, both diets are rich in fiber and plant-derived phytochemicals that are known to be beneficial to the gut microbiota[34] and modulate neuroinflammation.[35]

Due to the significant and consistent improvements observed in both groups in this study, it is important to consider how these two diets are similar rather than how they differ. Both diets include recommendations for high intake of fruits, vegetables, and unsaturated fats and for limited intake of highly processed foods. These recommendations are consistent with diet quality indexes that are associated with reduced symptom burden in observational studies.[36]

The consistency of run-in and baseline values, similar conclusions from the primary and secondary analyses, and the use of a blinded statistician are strengths of this study. This study is limited by the short duration of the intervention, the use of participant-reported data for primary and secondary outcomes, lack of racial/ethnic diversity in the study participants, the lack of a usual diet comparison group, and that dietary adherence was not evaluated

with a biomarker. Due to these limitations and the wide range of exclusion criteria for this study, the generalizability of the observed findings is limited to fatigued individuals with RRMS.

The observation that both dietary approaches in this study are associated with significant reductions in fatigue and improvements in QoL greatly benefits the MS community in that it allows for patient preference for either diet and suggests that the benefits of dietary approaches are due to underlying mechanisms rather than unique characteristics of specific diets. This study was unable to show significant improvements in the 6-minute walk test at 12-weeks; however, the Wahls group in the primary analysis and both groups in the secondary analysis showed significant increases in meters walked during the 6MWT at 24-weeks. It is possible that walking endurance takes longer to develop than participant-reported outcomes such as fatigue and QoL with dietary interventions. The results from this study provide justification for future randomized controlled trials with larger sample sizes and longer duration to evaluate changes in brain MRI-evaluated disease activity and exploration of underlying mechanisms by which diet may affect the MS disease course.

Authors' contribution

The authors' responsibilities were as follows: TJT cleaned data and wrote the first draft of the manuscript; PTE performed all blinded statistical analyses; LR managed unblinded data and confirmed statistical analyses; BB, LC, WD, KH, and JK trained staff, assisted in data acquisition and interpretation, and oversaw study procedures; JK was the study team neurologist who confirmed diagnoses; TW and LS designed the study and oversaw all study procedures. All authors have read and approve the final version of the manuscript.

Acknowledgments

The authors would like to acknowledge the following contributors: Mary Ehlinger for her role in study recruitment and as the primary study coordinator; Karen Smith for her role in nutrition data acquisition and as an intervention dietitian; Lisa Brooks for her role as an intervention dietitian; Michaella Edwards for her role in study coordination and data acquisition; Zaidoon Al-Share for his role in data acquisition; and the students who supported recruitment and data acquisition. The authors also thank Kristina Greiner for her editing assistance.

Conflict of Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Terry Wahls personally follows and promotes the Wahls™ diet. She has equity interest in the following companies: Terry Wahls LLC; TZ Press LLC; The Wahls Institute, PLC; FBB Biomed Inc; and the website http://www.terrywahls.com. She also owns the copyright to the books *Minding My Mitochondria* (2nd edition) and *The Wahls Protocol*, *The Wahls Protocol Cooking for Life*, and the trademarks The Wahls Protocol® and Wahls™ diet, Wahls Paleo™ diet, and Wahls Paleo Plus™ diets (the Wahls elimination diet is not trademarked). She has completed grant funding from the National Multiple Sclerosis Society for the Dietary Approaches to Treating Multiple Sclerosis Related Fatigue Study. She has financial relationships with BioCeuticals, MCG Health LLC, Genova Diagnostics, and the Institute for Functional Medicine. She receives royalty payments from Penguin Random House. Dr. Wahls has conflict of interest management plans in place with the University of Iowa and the Iowa City VA Health Care System. All other authors report no personal or financial conflicts of interest in this work.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported in part by the National Multiple Sclerosis Society grant RG-1506-04312 (TW), the Institute for Clinical and Translational Science (ICTS) at the University of Iowa, and University of Iowa institutional funds. The ICTS is supported by the National Institutes of Health Clinical and Translational Science Award program (grant UL1TR002537). TJT is a research trainee of the University of Iowa Fraternal Order of Eagles Diabetes Research Center (T32DK112751-01) and is supported by the Carter Chapman Shreve Family Foundation and the Carter Chapman Shreve Fellowship Fund for diet and lifestyle research conducted by the Wahls Research team at the University of Iowa. In-kind support was provided by the University of Iowa College of Public Health Preventive Intervention Center.

ORCID iD

Tyler J Titcomb https://orcid.org/0000-0002-8162-4768

Supplemental material

Supplementary material for this article is available online.

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European Journal of Clinical Nutrition www.nature.com/ejcn

ARTICLE

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Nutrition in acute and chronic diseases

Causal association of genetically determined circulating vitamin D metabolites and calcium with multiple sclerosis in participants of European descent

Yan Zhang[1], Haijie Liu[2], Haihua Zhang[3], Zhifa Han[4], Tao Wang[5], Longcai Wang[6] and Guiyou Liu[3,5,7,8]✉

BACKGROUND: Vitamin D is an important regulator of calcium. Mendelian randomization (MR) studies exclusively focused on the circulating total 25-hydroxyvitamin D (25(OH)D) as a biomarker of vitamin D status, and have found the causal association between 25(OH)D and the risk of multiple sclerosis (MS). However, it currently remains unclear about the causal association of the 25(OH)D subtypes including 25(OH)D3 and C3-epi-25(OH)D3, as well as calcium with the risk of MS.
METHODS: We performed a two-sample MR study to evaluate the causal association of circulating total 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3, and calcium with the risk of MS using large-scale genome-wide association studies (GWAS) datasets from total 25(OH)D ($n = 417,580$), 25(OH)D3 ($n = 40,562$), C3-epi-25(OH)D3 ($n = 40,562$), calcium ($n = 305,349$), and MS (14,802 MS and 26,703 controls). We selected five MR methods including inverse-variance weighted (IVW), simple median, weighted median, MR-Egger, MR-PRESSO (Mendelian Randomization Pleiotropy Residual Sum and Outlier), and contamination mixture method.
RESULTS: IVW showed that the genetically increased circulating 25(OH)D level (OR = 0.81, 95% CI: 0.70–0.94, $P = 4.00E-03$), circulating 25(OH)D3 level (OR = 0.85, 95% CI: 0.76–0.95, $P = 5.00E-03$), and circulating C3-epi-25(OH)D3 level (OR = 0.85, 95% CI: 0.74–0.98, $P = 2.30E-02$) were causally associated with reduced risk of MS. However, IVW showed no causal association between circulating calcium level and the risk of MS with OR = 2.85, 95% CI: 0.42–19.53, $P = 2.85E-01$.
CONCLUSIONS: Our current findings together with evidence from other MR studies support the use of vitamin D but not calcium supplementation for the prevention of MS.

European Journal of Clinical Nutrition; https://doi.org/10.1038/s41430-023-01260-4

INTRODUCTION

Multiple sclerosis (MS) is a neuroinflammatory and neurodegenerative disease in the central nervous system [1]. Vitamin D deficiency is a candidate risk factor for kinds of adverse health outcomes. Observational studies have showed that vitamin D deficiency is associated with increased MS risk [2, 3], and high circulating levels of vitamin D could reduce the risk of MS [4, 5]. Until recently, Mendelian randomization (MR) studies and genetic risk scores (GRS) further supported the causal association between circulating vitamin D levels and the risk of MS [6–14]. Mokry and colleagues selected four genetic variants (rs2282679, rs12785878, rs10741657 and rs6013897) as the instrumental variables [7]. Rhead and colleagues selected three genetic variants (rs2282679, rs2060793, and rs3829251) as the instrumental variables [8]. Gianfrancesco and colleagues constructed the GRS using 3 genetic variants including rs2282679, rs2060793, and rs3829251 [9]. Jacobs and colleagues conducted the MR analysis using 6 genetic variants including rs10741657, rs12785878, rs17216707, rs3755967, rs7979805 and rs8018720 [10]. Harroud and colleagues selected 88 independent genetic variants in their MR study [11]. Wang and colleagues selected 20 most effective and independent genetic variants [13]. Vandebergh and colleagues selected 5, 6, 70, and 104 genetic variants from four circulating vitamin D GWAS datasets [14]. Importantly, all these MR studies and GRS show that genetically lowered circulating vitamin D level is causally associated with increased risk of MS [6–14].

However, these studies evaluating the causal association between circulating vitamin D levels and the risk of MS exclusively focused on the total 25-hydroxyvitamin D (25(OH)

[1]Department of Pathology, The Affiliated Hospital of Weifang Medical University, Weifang 261053, China. [2]Department of Neurology, Xuanwu Hospital, Capital Medical University, Beijing 100053, China. [3]Beijing Institute of Brain Disorders, Laboratory of Brain Disorders, Ministry of Science and Technology, Collaborative Innovation Center for Brain Disorders, Capital Medical University, Beijing 100069, China. [4]State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China. [5]Chinese Institute for Brain Research, Beijing, China. [6]Department of Anesthesiology, The Affiliated Hospital of Weifang Medical University, Weifang 261053, China. [7]Key Laboratory of Cerebral Microcirculation in Universities of Shandong; Department of Neurology, Second Affiliated Hospital; Shandong First Medical University & Shandong Academy of Medical Sciences, Taian 271000 Shandong, China. [8]Beijing Key Laboratory of Hypoxia Translational Medicine, National Engineering Laboratory of Internet Medical Diagnosis and Treatment Technology, Xuanwu Hospital, Capital Medical University, Beijing 100053, China. ✉email: liuguiyou1981@163.com

Received: 5 May 2022 Revised: 22 December 2022 Accepted: 5 January 2023
Published online: 12 January 2023

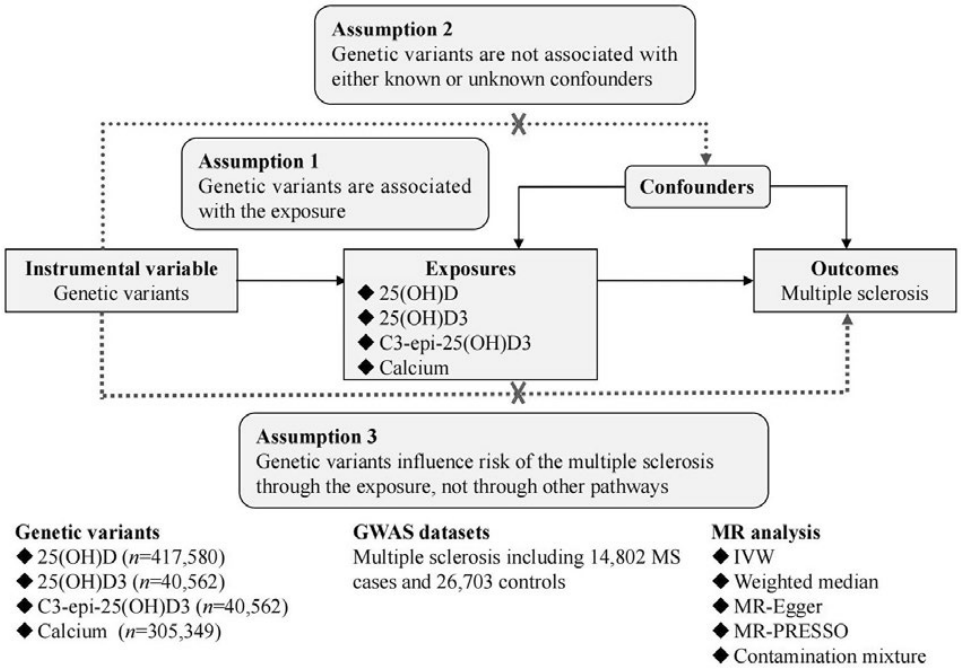

Fig. 1 The flow chart about the MR study design. GWAS Genome-wide association studies, IVW Inverse-variance weighted.

D) as a biomarker of vitamin D status [6–13]. In fact, the total 25(OH)D is the combination of 25(OH)D3, C3-epi-25(OH)D3 (epimeric form of 25(OH)D3), and 25(OH)D2 [15]. 25(OH)D3 is the major component of total 25(OH)D [15]. The European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct case-cohort study identified the mean concentrations (SD) of total 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3, and 25(OH)D2 to be 41.1 (17.2), 40.7 (17.3), 2.13 (1.31), and 8.16 (6.52) nmol/L, respectively [15]. Until now, it remains unclear about the causal association of circulating 25(OH)D3 or C3-epi-25(OH)D3 with the risk of MS.

Meanwhile, vitamin D is an important regulator of serum calcium, and work together with serum calcium in many biological processes [16, 17]. It is recommended that the daily calcium intake is 1000 to 1200 mg [18]. However, diet calcium intake could not achieve this amount. Hence, vitamin and mineral supplements have a large worldwide market, especially in North America and Europe [19]. Evidence shows that calcium supplements are very common in 43% of people (about 70% of older women) in the United States [18, 19]. A population-based cohort study in 475,255 UK Biobank participants (median age 58 years, 55.8% women) indicated that 33,437 (7.04%) participants reported taking calcium supplements; 19,089 (4.02%) vitamin D; and 10,007 (2.11%) both [20]. Importantly, MR study indicated that high genetically determined lifelong circulating 25(OH)D levels were associated with higher calcium levels [21]. However, it currently remains unclear about the causal association between circulating calcium level and the risk of MS.

Until now, large-scale genome-wide association studies (GWAS) have been conducted to identify novel genetic variant associated with circulating total 25(OH)D [22], 25(OH)D3 [23], C3-epi-25(OH)D3 [23], calcium [24], and MS [25]. Therefore, the publicly available GWAS datasets provide strong support to determine the causal association of circulating vitamin D metabolites and calcium with the risk of MS. Here, we performed a MR study to investigate the causal association of circulating total 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3, and calcium with the risk of MS using multiple large-scale GWAS datasets.

METHODS

Study design

This study is based on large-scale GWAS summary datasets from circulating total 25(OH)D [22], 25(OH)D3 [23], C3-epi-25(OH)D3 [23], calcium [24], and MS [25]. All participants have provided informed consent in the original studies [22–25]. MR is based on three principal assumptions: (1) genetic variants as the instrumental variables should be significantly associated with the exposure (such as circulating calcium levels) (assumption 1) [26]; (2) genetic variants should not be associated with the confounders of an outcome (MS) (assumption 2) [26]; (3) genetic variants should affect the risk of the outcome (MS) only through the exposure (circulating calcium levels) (assumption 3) [26]. Both the assumption 2 and 3 are defined as the independence from pleiotropy [26]. We provide a flowchart about our MR study design, as described in Fig. 1.

Circulating 25(OH)D genetic variants

Revez and colleagues performed a large-scale GWAS in 417,580 European UK Biobank participants, and identified 143 independent loci including 20 indels and 123 SNPs associated with total 25(OH)D at the genome-wide significance threshold ($P < 5.00E-08$) [22]. Total 25(OH)D including 25(OH)D3 and 25(OH)D2 was measured using chemiluminescence immunoassay [22]. Revez and colleagues applied a rank-based inverse-normal transformation to the 25(OH)D levels by adjusting for some key covariates including age at time of assessment, sex, assessment month, assessment centre, supplement-intake information, genotyping batch and the first 40 ancestry PCs [22]. The median, mean and interquartile range for 25OHD concentration are 47.9, 49.6, 33.5–63.2 nmol/L [22]. Here, we selected the 123 SNPs as the potential instrumental variables, and provided their detailed information in Supplementary Table 1.

Circulating 25(OH)D3 and C3-epi-25(OH)D3 genetic variants

Zheng and colleagues conducted a meta-analysis of GWAS for 25(OH)D3 and C3-epi-25(OH)D3 in 40,562 participants of European descent including EPIC–InterAct study, EPIC-Norfolk study, EPIC-CVD study, Ely study, and the SUNLIGHT consortium [23]. The 25(OH)D3 and C3-epi-25(OH)D3 metabolites were measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS) [23]. Zheng and colleagues identified 7 genetic variants associated with 25(OH)D3 and 3 genetic variants associated with C3-epi-25(OH)D3 at the genome-wide significance threshold ($P < 5.00E-08$) [23]. Zheng and colleagues calculated the standardised residuals of natural-log transformed 25(OH)D metabolites by adjusting for age, sex, BMI, season of

Table 1. Sample information about the MS GWAS datasets in IMSGC discovery stage.

GWAS dataset	Case #	Control #	% males	Genotyping platform
GeneMSA DU	219	221	39.09%	HumanHap550
GeneMSA SW	239	190	29.84%	HumanHap550
GeneMSA US	437	402	31.94%	HumanHap550
IMSGC	790	1677	43.13%	Affymetrix 500 K
BWH/MIGEN	821	2705	50.68%	Affy 6.0
ANZ	1582	1949	33.42%	Illumina Infinium Hap370CNVarray
Berkeley	544	513	18.53%	Omni Express Exome
Rotterdam	459	1938	41.05%	Ilumina 610 K
UK	1851	5163	45.04%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
CE	2226	2034	41.48%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
Medi	940	1293	46.75%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
Nordic	1960	2011	26.39%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
US	1374	2373	42.19%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
AUS	782	2084	46.23%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
FINLAND	578	2150	44.46%	Cases: Human660-Quad chip; Controls: Human1.2M-Duo chip
All	14802	26703		

blood collection, and study centre [23]. Here, we selected these 7 and 3 genetic variants including 8 unique genetic variants as the potential instrumental variables, and provided their detailed information in Supplementary Table 2. Meanwhile, 7 of these 8 unique genetic variants were also significantly associated with total 25(OH)D as provided in Supplementary Table 3, which indicated that these variants were not specific for 25(OH)D metabolites.

Circulating calcium genetic variants

A large-scale GWAS using 305,349 individuals from the UK Biobank had identified 208 independent variants including 32 insertion–deletion mutations (indels), and 176 single nucleotide polymorphisms (SNPs) associated with serum calcium levels at the genome-wide significance threshold ($P < 5.00E-08$) [24]. These 208 variants could explain 5.8% of the total variance of total serum calcium [24]. Here, we selected these 176 SNPs as the potential instrumental variables, and provided their detailed information in Supplementary Table 4.

MS GWAS dataset

The MS GWAS dataset is from a large-scale meta-analysis of MS GWAS datasets from the International Multiple Sclerosis Genetics Consortium (IMSGC) including 47,429 MS and 68,374 controls [25]. However, only the MS GWAS dataset from the IMSGC discovery stage is publicly available [25]. In brief, IMSGC discovery stage consisted of 14,802 MS cases and 26,703 controls from 15 MS GWAS datasets including GeneMSA DU, GeneMSA SW, GeneMSA US, IMSGC, BWH/MIGEN, ANZ, Berkeley, Rotterdam, UK, CE, Medi, Nordic, US, AUS, and FINLAND [25]. More detailed information about the MS GWAS dataset in IMSGC discovery stage is provided in Table 1.

Establishing the Wald estimator

For each genetic variant G_j ($j = 1, \ldots, k$), we assume that its beta coefficient $\hat{\beta}_{Xj}$ and standard error $se(\hat{\beta}_{Xj})$ correspond to the associations of each genetic variant with circulating total 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3, and calcium. We then aligned the effect alleles of circulating total 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3, and calcium genetic variants in MS GWAS dataset, and extracted the summary results including the odds ratios (ORs) and the P values. We further translated the ORs and the P values to the beta coefficients and their standard errors corresponding to the associations of each genetic variant G_j ($j = 1, \ldots, k$) with MS ($\hat{\beta}_{Yj}$, and $se(\hat{\beta}_{Yj})$). For a given genetic variant, the causal effect of circulating total 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3, and calcium on MS can be estimated by a Wald estimator $\hat{\theta}_j \frac{\hat{\beta}_{Yj}}{\hat{\beta}_{Xj}}$ and its approximate variance $v_j \frac{se(\hat{\beta}_{Yj})^2}{\hat{\beta}_{Xj}^2}$ [27].

MR analysis

We selected five methods to conduct the MR analysis including inverse-variance weighted (IVW) [28], weighted median [28], MR-Egger [27], MR-PRESSO (Mendelian Randomization Pleiotropy RESidual Sum and Outlier) [29], and contamination mixture method [30]. IVW is a main and popular MR approach, it assumes that there is no horizontal pleiotropy and all genetic variants are valid instrumental variables, and then combines the single Wald estimates into an overall estimate [28, 30]. MR-Egger could test the directional pleiotropy using MR-Egger intercept test, and correct for the presence of pleiotropy [27]. If the intercept is 0, MR-Egger is the same as IVW [27]. Median-based methods consist of simple median and weighted median [28]. The simple median assumes that at least 50% of genetic variants are valid instrumental variables [28]. The weighted median assumes that at least 50% of the weight is from the valid instrumental variables [28, 31]. Importantly, both simple median and weighted median provide a consistent estimate of causal effect [28]. MR-PRESSO consists of three functions including (1) evaluating the horizontal pleiotropy (MR-PRESSO global test); (2) correcting for the horizontal pleiotropy via outlier removal (MR-PRESSO outlier test); (3) testing the significant distortion in the causal estimates before and after outlier removal (MR-PRESSO distortion test) [29]. Contamination mixture method is newly developed method for MR analysis with hundreds of genetic variants [30]. It performs MR robustly and efficiently even in the presence of invalid instrumental variables [30].

The OR and 95% confidence interval (CI) of MS corresponds to per unit increase in rank-based inverse normal transformed 25OHD levels, per unit increase in natural-log transformed 25(OH)D metabolites (25(OH)D3 and C3-epi-25(OH)D3), and per 1 standard deviation (SD) increase in calcium. All the statistical tests were completed using three R Packages 'MendelianRandomization' and 'MR-PRESSO', respectively [29, 32]. The threshold of statistically significant association is a Bonferroni corrected significance $P < 0.05/4 = 0.0125$. The threshold of suggestive association is $P < 0.05$.

Power analysis

The proportion of exposure variance (R^2) explained by the instrumental variables can be estimated as the following formula

$$R^2 = \sum_{i=1}^{K} \frac{\beta_i^2}{\beta_i^2 + N^* se(\beta_i)^2}$$

Where β_i is the effect size (beta coefficient) for SNP_i, se (β_i) is the standard error for SNP_i, K is the number of genetic variants, and N is the sample size [33]. The strength of the instrumental variables was evaluated using the

Table 2. MR results about the causal association of circulating vitamin D metabolites and calcium with the risk of MS.

Exposure	Method	OR	95% CI	*P*-value
Circulating 25(OH)D	Weighted median	0.84	0.71–1.00	5.10E-02
	IVW	0.81	0.70–0.94	4.00E-03
	MR-Egger	0.82	0.67–0.99	4.30E-02
	MR-PRESSO Raw	0.81	0.70–0.94	5.34E-03
	MR-PRESSO Outlier-corrected	0.82	0.72–0.94	3.97E-03
	Contamination mixture method	0.79	0.69–0.90	5.32E-04
Circulating 25(OH)D3	Weighted median	0.86	0.76–0.97	1.80E-02
	IVW	0.85	0.76–0.95	5.00E-03
	MR-Egger	0.93	0.77–1.14	4.85E-01
	MR-PRESSO Raw	0.85	0.76–0.95	3.60E-02
	Contamination mixture method	0.84	0.48–0.97	2.92E-02
Circulating C3-epi-25(OH)D3	Weighted median	0.86	0.74–1.01	6.10E-02
	IVW	0.85	0.74–0.98	2.30E-02
	MR-Egger	1.14	0.48–2.70	7.63E-01
	Contamination mixture method	0.85	0.59–1.05	6.55E-02
Circulating calcium	Weighted median	0.70	0.07–7.33	7.63E-01
	IVW	2.85	0.42–19.53	2.85E-01
	MR-Egger	1.31	0.04–45.42	8.81E-01
	MR-PRESSO Raw	2.86	0.42–19.54	2.87E-01
	MR-PRESSO Outlier-corrected	2.38	0.44–12.76	3.14E-01
	Contamination mixture method	3.29	0.45–19.69	3.08E-01

OR Odds ratio, *CI* Confidence interval, *IVW* Inverse-variance weighted meta-analysis, *MR-PRESSO* Mendelian Randomization Pleiotropy RESidual Sum and Outlier.

first-stage F-statistic [34, 35].

$$F = \frac{R^2(N-1-k)}{(1-R^2)k}$$

A common threshold is $F > 10$ to avoid bias in MR studies [36]. Using the proportion of variance of the R^2, the total number of individuals in the analysis, and the proportion of cases in the study, we calculated the statistical power using the web-based tool mRnd, and a two-sided type-I error rate 0.05 [37].

RESULTS

Causal association between circulating 25(OH)D and MS

Using 123 circulating 25(OH)D genetic variants, we successfully extracted their corresponding summary statistics in MS GWAS dataset for 106 genetic variants. 17 genetic variants are not available in MS GWAS dataset, most of which have the minor allele frequency < 5%. Therefore, our MR analysis is based on these 106 genetic variants. For ambiguous palindromic variants (i.e., with alleles either A/T or C/G), we selected their proxy variants using HaploReg v4.1 based on linkage disequilibrium information from 1000 Genomes Project (CEU) with $r^2 >= 0.8$ [38]. IVW showed that the genetically increased circulating 25(OH)D level was causally associated with reduced risk of MS (OR = 0.81, 95% CI: 0.70–0.94, $P = 4.00E-03$). Interestingly, weighted median, MR-Egger, MR-PRESSO and contamination mixture method further supported the causal association of circulating 25(OH)D level with the risk of MS, and had the consistent estimates with the IVW estimate in terms of direction and magnitude, as provided in Table 2. MR-Egger intercept test did not identify evidence of pleiotropy with intercept = 0, $P = 0.91$. MR-PRESSO Global Test showed evidence of pleiotropy with $P = 1.00E-03$, and MR-PRESSO Outlier-corrected test still supported these above findings, as provided in Table 2. The individual causal estimates about the causal association of circulating 25(OH)D levels with the risk of MS using IVW method are provided in Fig. 2.

Causal association between circulating 25(OH)D3 and MS

Of the selected 7 circulating 25(OH)D3 genetic variants, 6 genetic variants excluding rs116970203 are available in MS GWAS dataset. rs116970203 is a rare genetic variant with minor allele frequency 2% [23]. For rs116970203, we identified its proxy variants including rs117621176, rs117361591, rs117913124 using HaploReg v4.1 linkage disequilibrium information from 1000 Genomes Project (CEU) with $r^2 >= 0.8$ [38]. However, these proxies are not available in MS GWAS dataset. Therefore, our MR analysis is based on these 6 genetic variants. IVW showed that the genetically increased circulating 25(OH)D3 level was causally associated with reduced risk of MS (OR = 0.85, 95% CI: 0.76–0.95, $P = 5.00E-03$). Interestingly, weighted median, MR-PRESSO and contamination mixture method further supported the causal association of circulating 25(OH)D3 level with the risk of MS, and had the consistent estimates with the IVW estimate in terms of direction and magnitude, as provided in Table 2. Meanwhile, we identified no evidence of pleiotropy using MR-Egger intercept test (intercept = −0.016, $P = 0.247$) and MR-PRESSO Global Test ($P = 0.186$). The individual causal estimates about the causal association of circulating 25(OH)D3 levels with the risk of MS using IVW method are provided in Fig. 3.

Causal association between circulating C3-epi-25(OH)D3 and MS

Using 3 circulating C3-epi-25(OH)D3 genetic variants, we extracted their corresponding summary statistics in MS GWAS dataset. IVW showed suggestive causal association between genetically increased circulating C3-epi-25(OH)D3 level and reduced risk of MS (OR = 0.85, 95% CI: 0.74–0.98, $P = 2.30E-02$). Meanwhile, the estimates from weighted median and contamination mixture

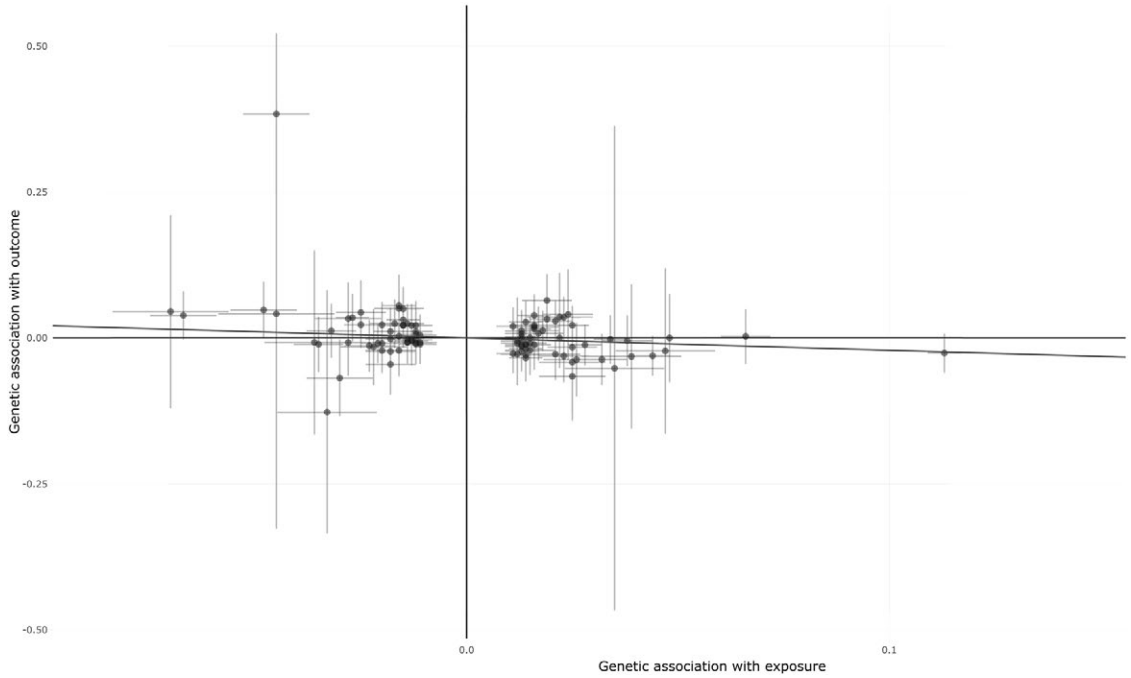

Fig. 2 Individual estimates about the causal effect of circulating 25(OH)D on MS using IVW method. The x-axis shows the single nucleotide polymorphism (SNP) effect, and standard error on circulating 25(OH)D, and the y-axis shows the SNP effect, and standard error on MS. IVW, Inverse-variance weighted.

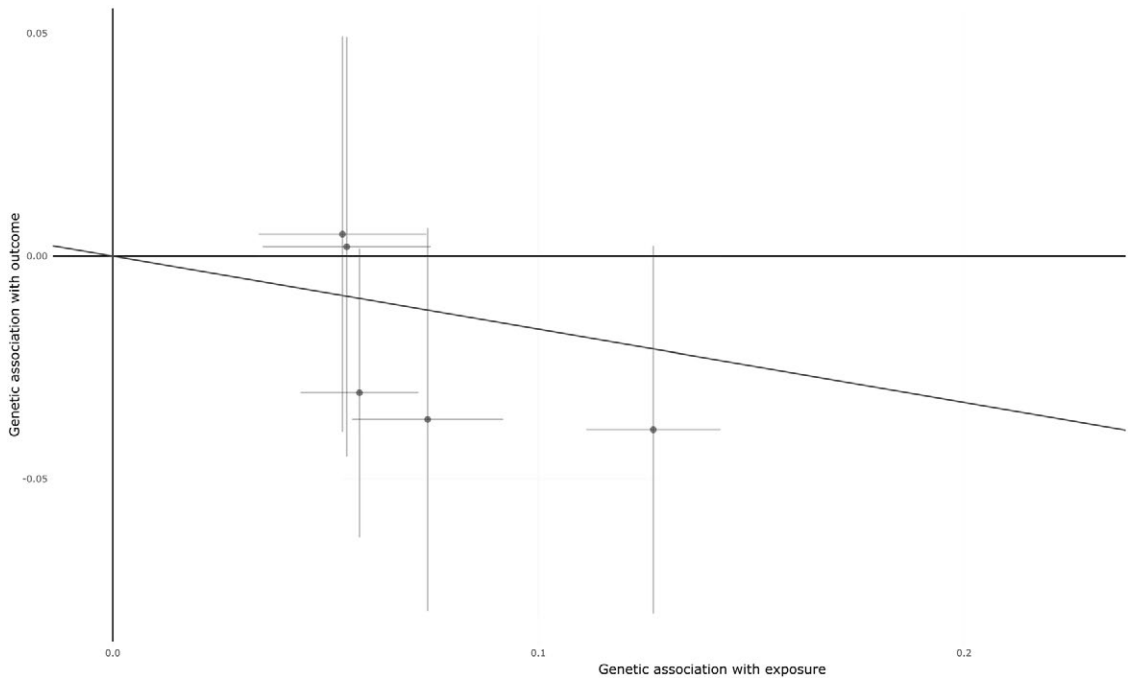

Fig. 3 Individual estimates about the causal effect of circulating 25(OH)D3 on MS using IVW method. The x-axis shows the single nucleotide polymorphism (SNP) effect, and standard error on circulating 25(OH)D3, and the y-axis shows the SNP effect, and standard error on MS. IVW, Inverse-variance weighted.

method were consistent with the IVW estimate in terms of direction and magnitude, although lack of significant association, as provided in Table 2. MR-Egger intercept test indicated no evidence of pleiotropy with intercept $= -0.050$, $P = 0.501$. Supplementary Fig. 1 provides the individual estimates about the causal effect of circulating C3-epi-25(OH)D3 on MS using IVW method.

Causal association between circulating calcium and MS

Of the 176 circulating calcium genetic variants, we extracted the summary statistics for 149 common genetic variants in MS GWAS dataset. IVW showed no causal association between circulating calcium level and the risk of MS with OR = 2.85, 95% CI: 0.42–19.53, $P = 2.85E\text{-}01$. Meanwhile, weighted median, MR-Egger,

Table 3. Power analysis for the causal association of circulating vitamin D metabolites and calcium with the risk of MS.

Exposure	Sample Size	Number of instrument	R^2	*F*	Detectable OR with 80% power
Circulating 25(OH)D	417,580	106	4.85%	200.75	< = 0.88
Circulating 25(OH)D3	40,562	6	3.75%	263.34	< = 0.86
Circulating C3-epi-25(OH)D3	40,562	3	0.66%	89.82	< = 0.69
Circulating calcium	305,349	149	4.42%	94.72	> = 1.14

R^2 Proportion of exposure variance explained by the instrumental variables, *F* Strength of the instrumental variables was evaluated using the first-stage F-statistic.

MR-PRESSO, and contamination mixture method suggested no causal association of circulating calcium level with the risk of MS, as provided in Table 2. MR-Egger intercept test indicated no evidence of pleiotropy with intercept = 0.002, $P = 0.608$. MR-PRESSO Global Test showed evidence of pleiotropy with $P < 5E-04$. However, MR-PRESSO Outlier-corrected test still reported negative findings, as provided in Table 2. Supplementary Fig. 2 provides the individual estimates about the causal effect of serum calcium on MS using IVW method.

Power analysis

The selected genetic variants explain 4.85%, 3.75%, 0.66%, and 4.42% of the variance in circulating 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3 and calcium, respectively. Meanwhile, these genetic variants have strong strength as the instrumental variables with $F > = 89.82$. The sample size for the MS GWAS is 41505, and the proportion of MS is 0.36. Using mRnd, our MR study had 80% power to detect the risk of MS with OR < = 0.88, < = 0.86, < = 0.69, > = 1.14 per SD increase in circulating 25(OH)D, 25(OH)D3, C3-epi-25(OH)D3 and calcium, respectively. Table 3 provides the detailed power analysis results.

DISCUSSION

Until now, the causal association between circulating vitamin D level and the risk of MS has been well established [6–13]. However, these studies exclusively selected the circulating total 25(OH)D as a biomarker of vitamin D status [6–13]. It currently remains unclear about the causal association of the 25(OH)D subtypes including 25(OH)D3 and C3-epi-25(OH)D3 with the risk of MS. Here, we investigated the causal association of circulating total 25(OH)D, 25(OH)D3, and C3-epi-25(OH)D3 with the risk of MS using a MR design. We found significant or suggestive causal association of the increased total 25(OH)D, 25(OH)D3, and C3-epi-25(OH)D3 with the reduced risk of MS. Our results are consistent with previous findings that genetically increased circulating total 25(OH)D level was causally associated with increased risk of MS [6–13]. In addition to circulating total 25(OH)D level, we highlighted for the first time that genetically increased circulating 25(OH)D3 and C3-epi-25(OH)D3 were also causally associated with increased risk of MS, as provided in Table 2.

Although these interesting findings, current randomised controlled trials (RCTs) have not provided clear beneficial effects of vitamin D supplementation on MS risk [39–42]. In 2020, clinical and radiographical findings from EVIDIMS study (Efficacy of Vitamin D Supplementation in Multiple Sclerosis (NCT01440062) did not support nor disprove a therapeutic benefit of high-dose vitamin D supplementation (20,400 IU)/day low-dose (400 IU)/day cholecalciferol supplementation on MS for 18 months [43]. Interestingly, MS patients with higher 25(OH)D levels had lower T2 weighted lesion count and lower Expanded Disability Status Scale (EDSS) score [44]. Meanwhile, there are some promising results young MS patients early after initial diagnosis [40]. A meta-analysis of 12 clinical trials indicated that vitamin D supplementation might be beneficial in preventing MS relapse rates and new radiological signs [42, 45].

Until recently, Galoppin and colleagues have discussed several mechanisms by which 25(OH)D may decrease the risk for MS [46]. First, 25(OH)D has biological effects on immune cells. vitamin D involves immunomodulation by decreasing differentiation of effector T and B cells, and modulates innate immune cells including macrophages, monocytes and dendritic cells [46]. Second, 25(OH)D has biological effects on blood–brain barrier (BBB) function by reducing immune cell trafficking [46]. Third, 25(OH)D reduces microglial and astrocytic activation in the central nervous system [46].

Generally, C3-epi-25(OH)D3 accounts for a significant portion of total 25(OH)D detected in maternal and neonatal umbilical sera [47], and has very low concentration in general population [15]. Interestingly, 25(OH)D3 supplementation increases maternal-fetal C3-epi-25(OH)D3. Importantly, C3-epi-25(OH)D3 increases with increasing 25(OH)D levels and shows a high degree of tracking over time [48]. We consider that the positive association between C3-epi-25(OH)D3 and 25(OH)D may explain why a higher circulating level of C3-epi-25(OH)D3 also reduces the risk of MS in the general population.

It is reported that 25(OH)D regulates the calcium, and work together with calcium in many biological processes [16, 17, 49]. Importantly, MR study supported the causal association between circulating 25(OH)D level and calcium level [21]. Therefore, we further conducted a MR analysis to evaluate the causal association between circulating calcium level and the risk of MS. However we did not find any evidence supporting the causal association. On the one hand, our current findings indicate no beneficial effect of genetically predicted serum calcium on MS risk. On the other hand, our findings show no evidence for harmful effects of genetically predicted serum calcium effects on MS risk. these findings may further suggest that high calcium intake from diet or supplements may not contribute to reduce or increase the risk of MS. It should be pointed out that the circulating calcium level is measured in the general population including 305,349 individuals from the UK Biobank [24]. Therefore, our findings may only reflect the effects of lifelong genetically increased circulating calcium level on MS in the general population, but not in MS persons with osteoporosis.

It has been widely reported that bone mineral density (BMD) is reduced in MS patient, and MS patients have increased osteoporosis risk compared with general population [50–52]. Bazelier and colleagues conducted a population-based cohort study in UK General Practice Research Database to estimate the relative and absolute risk of fracture in patients with MS using 5565 MS patients and 33,360 population-based controls [51]. Bazelier and colleagues found 2.79-fold increased risk of hip fracture and 1.35-fold increased risk of osteoporotic fracture in MS compared with controls [51]. Bisson and colleagues analyzed the population-based administrative data from 783 MS cases and 3915 controls to compare measures of BMD in MS and controls [52]. Bisson and colleagues found that the average femoral BMD was lower in MS cases compared with matched controls [52]. The prevalence of osteoporosis across BMD sites was higher in MS (16–26%) compared with matched controls (6–15%) [52]. MS patients had 2.41-fold increased risk of osteoporosis [52].

Until recently, several studies have determined whether increased circulating calcium level could improve BMD and reduce the osteoporotic fractures in the general populations including individuals with normal calcium levels using MR design [53–56]. Cerani and colleagues performed a MR analysis using large-scale serum calcium GWAS dataset ($n = 61,079$), heel BMD GWAS dataset ($n = 426,824$), and fracture GWAS dataset (76,549 cases and 470,164 controls) [53]. They found that genetically increased circulating calcium levels in individuals with normal calcium levels could not increase heel BMD, and could not protect these individuals against fracture [53]. In 2020, Li and colleagues conducted a MR study using large-scale circulating calcium ($n = 39,400$) and whole-body BMD ($n = 66,628$) GWAS datasets [54]. They found that genetically increased circulating calcium level was associated with reduced whole-body BMD by adjusting the serum parathyroid hormone, 25(OH)D, and phosphate [54]. Qu and colleagues found that serum calcium levels were inversely associated with lumbar spine BMD ($n = 28,498$) [55]. Therefore, our finding is comparable to recent MR studies evaluating the effect of circulating calcium level on BMD, which highlighted that genetically increased circulating calcium level was not always better.

Our MR analysis may have several strengths. First, we selected large-scale circulating total 25(OH)D (417,580 individuals) [22], 25(OH)D3 (40,562 participants) [23], C3-epi-25(OH)D3 (40,562 participants) [23], calcium (305,349 individuals) [24], and MS (14,802 MS cases and 26,703 controls) [25] GWAS datasets, which may contribute to provide ample power. Second, all the individuals from these above GWAS datasets are of the European descent, which may contribute to reduce the influence population stratification [57, 58]. Third, more additional genetic variants are selected as the potential instrumental variables compared with previous studies [6–13]. Fourth, we selected two statistical methods including MR-Egger intercept test and MR-PRESSO test to conduct the pleiotropy analysis, which ensure the selected genetic variants to meet the MR assumptions. Fifth, we selected five different MR methods including IVW, weighted median, MR-Egger, MR-PRESSO, and contamination mixture method to ensure the precision of the causal estimate.

Meanwhile, our MR study may have some limitations. First, we only selected one large scale GWAS dataset for MS. Until now, an independent replication dataset with a similar sample size was not publicly available. Second, we could not completely exclude additional confounders. Until now, it is impossible to fully rule out pleiotropy [59, 60]. Some selected genetic variants as the instrumental variables may still have pleiotropic effects, as stated in an editorial regarding vitamin D and CVD risk [61]. Therefore, our current MR study may not provide absolute causality. Third, we limit our MR analysis in individuals of European ancestry. However, the causal association may vary in different ancestries. Fourth, only 3 genetic variants associated with C3-epi-25(OH)D3 at the genome-wide significance threshold ($P < 5.00E-08$) only explain 0.66% of the variance in circulating C3-epi-25(OH)D3 level. Our MR study only had 80% power to detect the risk of MS with OR $<= 0.69$ per SD increase in circulating C3-epi-25(OH)D3 level.

Fifth, Yuan and colleagues conducted a MR analysis to evaluate the causal associations of 65 possible risk factors including calcium with the risk of MS [62]. They selected 7 calcium genetic variants as the instrumental variables, and found no significant association between calcium and MS with precise confidence intervals using IVW (OR = 0.78, 95% CI: 0.33–1.83, $P = 0.564$), weighted median (OR = 0.88, 95% CI: 0.50–1.54, $P = 0.644$), MR-PRESSO (OR = 0.98, 95% CI: 0.54–1.77, $P = 0.940$) [62]. Our current findings are consistent with Yuan and colleagues, but with broad confidence intervals, as provided in Table 2. Importantly, Young and colleagues also found broad confidence intervals when they investigated the causal association of serum calcium with ECG markers of ventricular depolarization and repolarization [24]. We consider that these may be caused by the validity of the instrumental variables for calcium from the UK Biobank. Therefore, future studies are required to replicate our findings.

CONCLUSIONS

We comprehensively evaluated the causal association of genetically determined circulating vitamin D metabolites and calcium with the risk of MS in individuals of European descent. We demonstrate the causal association between genetically increased circulating vitamin D metabolites and the reduced risk of MS. Our current findings together with evidence from other MR studies support the use of vitamin D supplementation for the prevention of MS. However, we demonstrate no causal association between circulating calcium level and the risk of MS. Therefore, high calcium intake from both diet and supplements may not contribute to reduce the risk of MS.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article and its Additional files. The authors confirm that all data underlying the findings are either fully available without restriction through consortia websites, or may be made available from consortia upon request. International Multiple Sclerosis Genetics Consortium (IMSGC): https://imsgc.net/.

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ACKNOWLEDGEMENTS

We thank International Multiple Sclerosis Genetics Consortium (IMSGC) for the GWAS summary statistics.

AUTHOR CONTRIBUTIONS

GYL and YZ conceived and initiated the project. GYL and YZ analyzed the data, and wrote the first draft of the manuscript. All authors contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content and approved the final version of the manuscript.

FUNDING

This work was supported by funding from the National Natural Science Foundation of China (Grant No. 82071212, and 81901181), Beijing Natural Science Foundation (Grant No. JQ21022), the Mathematical Tianyuan Fund of the National Natural Science Foundation of China (Grant No. 12026414), and Beijing Ten Thousand Talents Project (Grant No. 2020A15). This work was also partially supported by funding from the Science and Technology Beijing One Hundred Leading Talent Training Project (Z141107001514006), the Beijing Municipal Administration of Hospitals' Mission Plan (SML20150802), the Funds of Academic Promotion Programme of Shandong First Medical University & Shandong Academy of Medical Sciences (No. 2019QL016, No. 2019PT007).

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

This article contains human participants collected by several GWAS. All participants gave informed consent in all the corresponding original studies. Here, we only used the large-scale GWAS summary datasets, and not the individual-level data. Hence, ethical approval was not sought.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41430-023-01260-4.

Correspondence and requests for materials should be addressed to Guiyou Liu.

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