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The Gut Microbiota in Multiple Sclerosis: An Overview of Clinical Trials

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Abstract

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating, and degenerative disease that affects the central nervous system. A recent study showed that interaction between the immune system and the gut microbiota plays a crucial role in the development of MS. This review reports the clinical studies carried out in recent years that aimed to evaluate the composition of the microbiota in patients with relapsing–remitting MS (RR-MS). We also report what is available in the literature regarding the effectiveness of fecal microbiota transplantation and the role of the diet in restoring the intestinal bacterial population. Studies report that patients with RR-MS have a microbiota that, compared with healthy controls, has higher amounts of *Pedobacteria*, *Flavobacterium*, *Pseudomonas*, *Mycoplana*, *Acinetobacter*, *Eggerthella*, *Dorea*, *Blautia*, *Streptococcus* and *Akkermansia*. In contrast, MS patients have a microbiota with impoverished microbial populations of *Prevotella*, *Bacteroides*, *Parabacteroides*, *Haemophilus*, *Sutterella*, *Adlercreutzia*, *Coprobacillus*, *Lactobacillus*, *Clostridium*, *Anaerostipes* and *Faecalibacterium*. In conclusion, the restoration of the microbial population in patients with RR-MS appears to reduce inflammatory events and the reactivation of the immune system.

Keywords

multiple sclerosis, microbiota, fecal microbiota transplantation, diet

Introduction

Multiple sclerosis (MS) is a chronic and inflammatory demyelinating disease of the central nervous system (CNS). This autoimmune pathology affects approximately 2.5 million people[1] and it is more prevalent in females than in males[2]. The main pathological characteristic of MS is the development of inflammatory plaques, areas of focal demyelination present in both white and gray matter of the brain and spinal cord. The plaques are caused by an inflammatory process that causes the destruction of myelin and of specialized cells such as oligodendrocytes with consequent neuronal loss. The demyelination is caused by an altered selectivity of the blood–brain barrier (BBB) that allows a wide range of immune cells to infiltrate the CNS. The lymphocytes that recognize the myelin antigen ($CD4^+$ or $CD8^+$ T cells) cross the BBB, triggering a cascade of events that leads to the formation of an inflammatory demyelinating lesion[3]. Several studies developed on animal models of MS, such as experimental autoimmune encephalomyelitis (EAE), demonstrate the central contribution of $CD4^+$ T lymphocytes in the pathogenesis of the disease[4]. In particular, $CD4^+$ T helper 1 (Th1) and T helper 17 (Th17) lymphocytes are the most involved in the initiation of the disease. Th1 are responsible for the production of interferon-γ (INF-γ) that promotes the activation of macrophages and the release of their enzymes. Furthermore, INF-γ induces the production of reactive oxygen and nitrogen intermediates that damage the surrounding tissues. Th1 are also responsible for the production of interleukin 12 (IL-12), which stimulates the production of INF-γ, and tumor necrosis factor (TNF-α) that contributes to tissue damage during chronic inflammation. Th17 cells are responsible for the production of the cytokines IL-17, IL-21 and IL-22 involved in the development of inflammation. Both Th1 and Th2 $CD4^+$ T lymphocytes targeted to CNS self-antigens are implicated in the pathogenesis of MS[5]. In addition to $CD4^+$ T and $CD8^+$ T cells, MS

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involves a variety of other immune cells, including macrophages, natural killer cells and microglial cells[6]. The crosstalk between all these cells and the cytokines they produce contributes to perpetuating the inflammatory environment within the CNS.

MS has several clinical variants, among which the most frequent is relapsing–remitting MS (RR-MS) (representing 85% of cases). This pathology is characterized by periods of exacerbation (relapsing) followed by substantial remission (remitting)[7]. Myelin-specific $CD8^+$ T cells are more represented in patients with RR-MS than in other forms of MS[8].

Today disease-modifying therapies (DMTs) are used to reduce the incidence, prevent and/or reduce the number of relapses and delay the progression of RR-MS[9]. The DMTs used include injectable drugs such as interferon β-1a (IFN β-1a), interferon β-1b (IFN β-1b), peginterferon β-1a and glatiramer acetate, and intravenous therapies such as natalizumab. Fingolimod, teriflunomide and dimethyl fumarate (DMF) are used as oral agents. All these drugs, although using different mechanisms of action, modulate or suppress the immune and inflammatory processes caused by MS. Specifically, interferons-β (IFN-β) promote the production of anti-inflammatory cytokines, inhibit T-cell proliferation and reduce inflammatory cell migration through the BBB[10]. Glatiramer acetate, a drug composed of synthetic peptides, diverts the immune system from attacking myelin by mimicking the protein sequences[11]. Natalizumab is a monoclonal antibody (mAb) responsible for the inhibition of leukocyte migration in the CNS, thus attenuating inflammatory processes[12]. Some oral agents have been approved for the treatment of RR-MS and represent an evolution in the treatment of RR-MS, primarily because of their easy administration. Teriflunomide and fingolimod are responsible for reducing the proliferation of stimulating T and B lymphocytes, with a consequent decrease in the number of active cells available to migrate into the CNS[13,14]. DMF activates the transcription pathway of the nuclear factor Nrf2 (Nuclear factor erythroid 2-related factor 2) protecting neuronal cells through reducing oxidative stress[15]. For severe forms of MS or in the presence of rapid neurological deterioration, off-label treatment with immunosuppressants such as cyclophosphamide or mitoxantrone is used[16].

Genetic and environmental factors play a key role in the development of MS[17,18]. Recently, the commensal microbiota, considered as a new environmental risk factor, has generated considerable attention in immune-mediated diseases such as MS. The gut microbiota is defined as the set of microorganisms such as bacteria (mainly), archaea, fungi, eukaryotes and viruses that reside in the mucous membranes of the human intestine. The gut microbiota appears to play an important role in the pathogenesis of MS. It appears to be involved in modulating the host's immune system, alters the integrity and function of the BBB, triggers autoimmune demyelination, and interacts directly with different cell types present in the CNS[19].

The gut microbiota undergoes constant changes during the life of the individual. It is influenced by numerous factors such as diet, drugs (especially antibiotics), and stress. Moreover, at the intestinal level, the gut microbiota promotes an anti-inflammatory and protective environment which is capable of inhibiting the growth of pathogenic microorganisms that are the cause of various diseases. Hence, through the diet, it is possible to vary the composition of the bacterial microflora. Evidence suggests that a Western diet rich in fats and refined sugars and poor in fiber can modify the intestinal bacterial flora, thus promoting the onset of a chronic inflammatory state. On the contrary, nutrients such as omega-3 polyunsaturated fatty acids, fiber, and vitamin D3 are able to modify the intestinal bacterial flora with beneficial effects on the organism, fostering the proliferation of bacterial microorganisms able to produce substances with anti-inflammatory action. Therefore, both in terms of metabolism and intestinal microflora, the diet could be an effective means to counteract inflammation in both RR-MS and progressive primary MS (PP-MS).

In addition, in recent years a further therapeutic intervention that has received widespread attention is fecal microbiota transplantation (FMT). FMT is a treatment that consists of the transplantation of microbes from one human intestine to another through fecal matter, in order to restore the intestinal micro-ecological balance. This therapeutic intervention could offer a treatment for a wide range of diseases including MS, and it could also be useful in understanding the role of the microbiota in many diseases.

The purpose of this review is to describe the role of the gut microbiota in MS and to provide an overview of clinical studies.

Gut Microbiota and Multiple Sclerosis

The gut microbiota is considered a microbial "organ," and its alteration could lead to the inflammatory activation of the immune system[20]. In fact, commensal microorganisms can promote both regulatory (Th2) and inflammatory responses (Th1 o Th17)[21–23]. The development of MS is influenced by both genetic and environmental factors. Recent studies have shown that alteration of the commensal microbiota acts as a pathogenic environmental risk factor. It directs adaptive and innate immune responses to the inflammatory profiles characteristic of many diseases such as MS[19,24,25].

Kadowaki et al.[26] conducted an investigation concerning the involvement of the gut microbiota in MS. The researchers observed that the gut microbiota influenced the interaction between T-cell C-C chemokine receptor type 9 (CCR9) and its ligand chemokine (CCL25). This interaction plays a role in T-cell development and in immunity in the small intestine epithelium. A decrease of the CCR9 functionality was observed in patients with RR-MS and secondary progressive MS (SP-MS). $CCR9^+$ expressed by T cells is upregulated by $CD4^+$ T cells. The blockade of the CCR9–CCL25 interaction determines a reduction of $CCR9^+$ $CD4^+$ T cells

in peripheral blood. Indeed, in this study, these cells display reduced frequency in patients with SP-MS. In order to determine whether $CCR9^+$ memory T (Tm) cells are affected by the gut microbiota, $CD4^+$ Tm cells were analyzed in the peripheral blood of C57BL/6G mice maintained in specific pathogen-free (SPF) condition and in C57BL/6 N germ-free mice. $CCR9^+$ Tm cell frequency decreased in germ-free mice. SPF mice were subsequently treated with a mixture of antibiotics capable of modulating their intestinal microbiota. Antibiotic treatment induced increased $CCR9^+$ Tm cell in SPF mice. These results confirmed the influence of the gut microbiota in $CCR9^+$ Tm cells. In addition, to further understand the involvement of $CCR9^+$ Tm cells in MS, EAE was induced in wild-type mice and subsequently treated with short-term antibiotic therapies. The antibiotic treatment induced an increase in $CCR9^+$ Tm cells and led to a significant reduction in EAE severity. This result shows that an alteration of the gut–systemic immune axis caused by dysbiosis may be involved in the pathogenesis of MS. In conclusion, these data suggest that $CCR9^+$ Tm cells can be considered a potential diagnostic marker for the treatment of MS.

The study conducted by Cosorich et al[27]. compared the microbiota of patients with RR-MS with that of healthy subjects. 16 S ribosomal RNA sequencing of microbiota isolated from small intestinal tissues showed an increase in phylum Firmicutes and a decrease in phylum Bacteroidetes in patients with MS in the relapse phase compared with healthy subjects, and compared with patients with MS in the remittent phase. In addition, a decrease in *Prevotella* and an increase in *Streptococcus mitis* (*S. mitis*) and *Streptococcus oralis* were found. *Prevotella* produces the anti-inflammatory metabolite propionate[28]; other studies demonstrate this reduction in patients with MS compared with healthy subjects[29–31]. A reduced level of *Prevotella* in patients with RR-MS is linked to the expansion of Th17 cells and disease activity. Even *S. mitis* is able to induce the differentiation of Th17 cells, and it is involved in the cell-mediated tissue damage of autoimmunity[32]. Therefore, this study demonstrates that the microbiota is directly involved in the pathogenesis of MS through regulating the expansion of Th17 at the intestinal level[33].

Miyake et al.[29] observed a reduction of the genus *Clostridium* (Clostridia cluster XIV and IV) in patients with RR-MS compared with healthy subjects. The reduction of this microbial population determines a decrease in the production of short-chain fatty acids. In this way, *Clostridium* are responsible for the production of regulatory T cells (Treg) in peripheral compartments and the increase of the anti-inflammatory cytokine IL-10[34,35]. In the study conducted by Chen et al.[31], Firmicutes (genera such as *Blautia* and *Dorea*) and Bacteroidetes (*Pedobacteria* and *Flavobacterium*) showed higher presence in patients with MS compared with healthy controls. Instead, Bacteroidetes genera such as *Parabacteroides*, *Bacteroides* and *Prevotella* showed lower presence. Bacteroidetes commensals are responsible for the production of Lipid 654, a Toll-like receptor ligand (TLR)-2. Levels of Lipid 654 were significantly reduced in serum samples from MS patients compared with healthy subjects and compared with patients with Alzheimer's disease. Some researchers have hypothesized that Lipid 654 is involved in the activation and regulation of immune responses, maintaining a certain level of TLR-2 and IFN-β signaling[36].

Along with the decrease in the genera of Bacteroidetes, the reduction of *Adlercreutzia* in patients with RR-MS was also observed. *Adlercreutzia* could influence anti-inflammatory responses, as a result of their connection to phytoestrogen metabolism. Phytoestrogens are molecules of plant origin, with a chemical structure and a biological activity similar to estrogen. The main sources of these compounds are legumes (particularly soybeans), many fruits, whole grains and other vegetables. Bacteria like *Aldercreutzia*, through β-glucosidase, are responsible for the conversion of phytoestrogens into monomers. In patients with RR-MS, a reduction in *Adlercreutzia* reduces the capacity for conversion of phytoestrogens. In this way, a decrease of this bacterium determines an increase of oxidative stress and inflammatory cytokines, such as chemo-attracting proteins-1 and interleukin-6, which are normally elevated in MS[37]. Therefore, an increase of *Adlercreutzia* in RR-MS patients could determine the reduction of the inflammatory state and consequently a delay in the progression of the disease.

In a study conducted by Jangi et al.[30], an increase of *Methanobrevibacter* (Euryarchaeota phylum) and of *Akkermansia* (phylum Verrucomicrobia) and a decrease of *Butyricimonas* were observed in patients with MS. *Methanobrevibacter* is involved in inflammatory processes as a result of its ability to recruit inflammatory cells and human dendritic cells[38]. *Methanobrevibacter* is distributed in the intestinal mucosa in correspondence with the lymphoid areas[39], therefore it appears to be associated with various inflammatory bowel diseases. Furthermore, in a pilot study conducted in pediatric MS patients, it was observed that colonization with *Methanobrevibacter* resulted in a shorter time to relapse[40], thus demonstrating an interaction between the gut microbiota and MS. *Akkermansia* is involved in the transformation of mucin into short-chain fatty acids, which can mediate immune-regulatory effects[41]. Moreover, *Akkermansia* can show pro-inflammatory activity, related to its ability to degrade mucus. This mechanism causes damage to the intestinal barrier and increased exposure of the resident immune cells to microbial antigens[42]. The pro-inflammatory action of *Akkermansia* may also be related to the upregulation of genes involved in the presentation of antigen, in B- and T-cell receptor signaling and in the activation of complement and coagulation cascades[41]. The genus *Butyricimonas* produces butyrate, a short-chain fatty acid that induces regulatory T cells[43]. Numerous autoimmune and inflammatory diseases, including inflammatory bowel disease, showed a reduction in butyrate producers[44]. The reduction of butyrate in the colon can disrupt the barrier function and promote inflammation. Cekanaviciute et al[45].

Table 1. Differences in Some Microbial Population in MS Patients versus Healthy Individuals. The Table Shows the Increased (↑) or Reduced (↓) Bacterial Genera in the Microbiota Gut of MS Patients.

Phylum of Bacteria	Genus	Microbiota of MS patients versus healthy individuals
Bacteroidetes	*Pedobacteria*	↑
	Flavobacterium	↑
	Prevotella	↓
	Bacteroides	↓
	Parabacteroides	↓
Proteobacteria	*Pseudomonas*	↑
	Mycoplana	↑
	Acinetobacter	↑
	Haemophilus	↓
	Sutterella	↓
Actinobacteria	*Eggerthella*	↑
	Adlercreutzia	↓
	Collinsella	↓
Firmicutes	*Dorea*	↑
	Blautia	↑
	Streptococcus	↑
	Coprobacillus	↓
	Lactobacillus	↓
	Clostridium	↓
	Anaerostipes	↓
	Faecalibacterium	↓
Verrucomicrobia	*Akkermansia*	↑

highlighted a decrease in the abundance of *Parabacteroides distasonis* in RR-MS patients compared with healthy controls. These data suggest that *Parabacteroides* could be a beneficial commensal organism and could play a protective role in RR-MS.

Moreover, some studies report increased levels of Proteobacteria[30]. A greater abundance of Proteobacteria has also been reported in other autoimmune diseases such as inflammatory bowel disease[46]. An increase in this bacterial population in more autoimmune diseases suggests that Proteobacteria can contribute to autoimmune diseases by promoting pro-inflammatory responses.

All these studies confirm a characteristic dysbiosis of the intestine is constantly present during the clinical course of MS[47] (see Table 1). Therefore, this suggests a connection between the exacerbation of the disease and commensal microbiota[31].

Diet and Gut Microbiota in Multiple Sclerosis

The etiology of MS depends on heterogeneous factors, both genetic and environmental, that determine the risk of disease. Eating habits, lifestyle and some environmental factors can influence the course of this disease. Today, the existence of a direct link between diet, intestinal microbiota and MS is the subject of many studies (see Table 2). The diet is able to influence the composition of the intestinal bacterial flora and indirectly favor the development of autoimmune inflammatory diseases such as MS[51]. Obesity is a risk factor, and recently numerous studies have shown that this increases the risk of developing MS especially in children and adolescents. An important study conducted by Turnbaugh et al.[52] shows how the intestinal microbiota changes between obese subjects and normal-weight subjects. The results of this study showed that the microbiota of obese patients was enriched in Firmicutes and Actinobacteria, a situation that normally occurs also in patients with MS. Moreover, as in MS patients, compared with subjects with normal weight, obese individuals had a reduced presence of Bacteroidetes. This phylum is responsible for the production of short-chain fatty acids capable of counteracting inflammation through the protective expansion of regulatory T cells[35]. Therefore, their reduction in obese subjects, as in patients with MS, leads to a greater predisposition to inflammatory processes. In conclusion, an altered gut microbiota shows a greater presence of pathogenic Firmicutes compared with Bacteroidetes, leading to the breakdown of that microbial balance between microbiota and host. This condition favors slight endotoxemia, which contributes to chronic and systemic inflammation of the intestine and increases the risk of immune-mediated diseases such as MS[53,54].

In a meta-analysis study conducted by Liu et al.[55], including two cohort and three case-control studies, the relationship between obesity and the increased risk in MS was evaluated. The results confirmed that overweight and obesity are factors that can alter intestinal microbiota and promote neuroinflammation, increasing the risk of MS. Furthermore, in this study, the authors observed a statistically significant association between obesity and increased risk of MS in females. On the other hand, no significant associations were observed between obesity and MS in adolescence and in males. Ouchi et al.[56] saw that the chronic inflammation characteristic of MS may be linked to body fat because adipocytes are able to secrete TNF-α, IL-6, and leptin. Moreover, in obese subjects, higher concentrations of leptin in adipose tissue are related to body fat percentage increase[57]. Leptin is a pleiotropic cytokine that serves to regulate appetite, and has an impact on the activation and migration of neutrophils, macrophages, and monocytes[58,59]. An increase in leptin in the early stages of MS leads to a reduction in regulatory T cells[60] and an increase in effector T cells, shifting the phenotype toward a pro-inflammatory response, typical of autoimmune responses[61].

Another interesting fact is the relationship between the percentage of body fat and the levels of 25-hydroxyvitamin D3 [25-(OH)-D3], which is the biologically inactive storage form of vitamin D. In a study (approved by the institutional review boards of the Harvard School of Public Health and the Walter Reed Army Institute of Research) conducted by Munger et al.[48], it was observed that obese subjects often have low levels of this metabolite and therefore are more exposed to the risk of MS. Specifically, among the examined

Table 2. Several Studies Approved by Ethics Committees (https://www.ncbi.nlm.nih.gov/pubmed/). The studies describe the role of diet in patients with MS and how eating habits, associated with supplemental integration and therapies, can modify the interaction between microbiota and MS.

Study Design	Approval of Ethics Committees	Aim of the Study	Patients	Results	Ref.
A prospective study	Approved by institutional review boards of the Harvard School of Public Health and the Walter Reed Army Institute of Research	The purpose of this study was to assess whether 25-(OH)-D3 levels are associated with MS risk.	515 MS patients	515 potential MS cases were identified. Among them, 237 had definite MS and 78 probable MS. Higher levels of 25-(OH)-D3 were found in the examined white population, resulting in a significant reduction in the risk of MS. In contrast, in the black and Hispanic population, lower values of 25-(OH)-D3 were found compared with whites, and no significant associations were found between vitamin D and MS risk. However, it appears that levels of 25-(OH)-D3 in late adolescence are particularly important before the age of 20 because they may confer protection from MS.	[48]
A randomized study	Approved by San Carlo Hospital, Potenza, Basilicata, Italy (study approval n. 132)	The focus of this study was to evaluate how a semi-vegetarian diet, associated with vitamin D and other supplements (fish oil, lipoic acid, omega-3 polyunsaturated fatty acids, resveratrol and multivitamin complex), can improve physical and inflammatory status in patients with RR-MS and PP-MS.	43 MS patients (aged between 22 and 52 years): PP-MS ($n = 10$) RR-MS ($n = 33$)	An increase in the ratio of eicosapentanoic acid + docosaexaenoic acid / arachidonic acid (n-3 / n-6 polyunsaturated fatty acids) was observed following fish oil supplementation after 3 months of treatment. A greater concentration of [25-(OH)-D3] in patients with RR-MS treated with vitamin D and other supplements than in patients who were not given either vitamin D or supplements was observed. The anti-inflammatory efficacy of combination therapy, vitamin D, diet and food supplements, showed an improvement in the inflammatory state.	[49]
A randomized pilot study	Approved by Human Research Protection Office (HRPO) at Washington University in St. Louis (study approval number: 20150105)	The aim of this study was to demonstrate how IF can improve the course of MS by causing less inflammation, demyelination and axonal damage. Furthermore, the effect of diet on the gut microbiota was evaluated.	16 patients with MS Aged 18 to 60 years old	IF led to an increase in intestinal bacterial richness, particularly in the bacterial families Lactobacillaceae, Bacteroidaceae and Prevotellaceae in an EAE animal model and in patients with RR-MS. Data obtained in the EAE animal model show that IF causes a reduction in white blood cells, inflammatory cytokines and leptin, resulting in an increase in circulating levels of cortisol and adiponectin. The IF resulted in a reduction of IL-17-producing T cells and an increase in the number of regulatory T cells in the lamina propria of the intestine, thus modulating systemic immune responses. In patients with RR-MS, an improvement in the disability status scale of the disease was observed. A significant reduction in leptin levels was observed at day 15, and an increase in adiponectin levels.	[50]

MS: Multiple Sclerosis; RR-MS: Relapsing–Remitting Multiple Sclerosis; PP-MS: Progressive Primary Multiple Sclerosis; 25-(OH)-D3: 25-hydroxyvitamin D3; IF: Intermittent Fasting; EAE: experimental autoimmune encephalomyelitis.

white population, the risk of MS decreased significantly with increasing levels of 25-(OH)-D. In contrast, in the black and Hispanic population, who had lower levels of 25-(OH)-D than whites, no significant associations were found between vitamin D and MS risk. In addition, it appears that the levels of 25-(OH)-D in late adolescence are particularly important before the age of 20 because they can confer protection against MS.

Vitamin D deficiency is a potential risk factor for MS[62]. The identification of these risk factors as potential targets in therapies can provide an opportunity to improve current treatments used in MS. It is known that the distribution of MS prevalence depends on latitude, associated with both sunlight intensity and vitamin D serum levels[63]. Vitamin D is a powerful immunomodulatory molecule and plays a role in several immune processes both in the innate and adaptive immune systems[64]. It also shows direct and indirect effects on T cells[65,66]. It has been observed that the risk to develop MS is correlated to 25-(OH)-D serum levels[48]. Large interventional randomized clinical trials in the United States and Europe are evaluating vitamin D supplementation to reduce the risk of relapse in MS[67–69].

The dietary study conducted by Riccio et al.[49] shows that a dietary intervention may be particularly important for patients with PP-MS, and may also be relevant as an adjunctive treatment in patients with RR-MS treated with IFN- β. Some patients were subjected to a low-calorie dietary regimen in which vitamin D was given, while other patients were provided with food supplements such as fish oil, lipoic acid, and a multivitamin complex in addition to the vitamin. The results of this study show that a nutritional intervention based on a semi-vegetarian and low-calorie diet and the administration of vitamin D3 can contribute to reducing chronic inflammation, which is a common feature of MS. In particular, it was observed that fish oil supplementation was sufficient to increase the eicosapentanoic acid + docosaexaenoic acid / arachidonic acid ratio (n-3 / n-6 polyunsaturated fatty acids) to an almost satisfactory level already after 3 months of treatment. These results suggest that a correct dietary regimen could be sufficient to increase the eicosapentanoic acid + docosaexaenoic acid/arachidonic acid ratio, potentially improving the patients' inflammatory status. As far as vitamin D3 is concerned, it is now known that it is also an immunomodulatory and anti-inflammatory agent, and it is established that patients with MS and other chronic inflammatory diseases have a low level of vitamin D3[70]. The results showed a greater concentration of [25-(OH)-D3] in patients with RR-MS treated with vitamin D and other supplements than in patients who were not given either vitamin D or supplements. In this study, the anti-inflammatory efficacy of combination therapy, vitamin D, diet, and food supplements produced an improvement in the inflammatory state.

In addition, vitamin D seems to be able to inhibit IFN-γ production. Moreover, it is involved in the regulation of gastrointestinal homeostasis by activating innate immune responses and inducing regulatory T cells. Vitamin D is also involved in maintaining a healthy composition of the gut microbiota and promotes the integrity of epithelial cells[71]. Vitamin D is considered a powerful antioxidant, counteracting the formation of free radicals via nitric oxide synthase and gamma-glutamyl transpeptidase. Finally, it also appears to be involved in brain development by regulating the level of neurotrophic factors[72]. Therefore, the administration of vitamin D3 can improve the course of the disease.

Furthermore, MS is seen more frequently in Western countries, and differences in diet could contribute to this geographical distribution. Several studies on the effect of the Westernized diet have been conducted on mouse models with EAE. It has been observed that a high-fat content diet in mice determines changes in the gut microbiota, with a consequent increase in plasma free fatty acids that induces oxidative stress and pro-inflammatory responses, causing a greater severity of the disease[73,74].

Cignarella et al.[50] have conducted a study concerning the effects of the intermittent fasting (IF) on animal model of EAE, and in patients with MS. IF changed the gut microbiota in the EAE animal model, with a consequent increase in the richness and enrichment of the bacteria of the Lactobacillaceae, Bacteroidaceae, and Prevotellaceae families. The richness of the gut microbiota was correlated with the levels of leptin, and involved an increase of the formation of ketones and of the metabolism of glutathione, improving the antioxidant pathways. Furthermore, IF resulted in a reduction of IL-17-producing T cells and an increase in the number of regulatory T cells in the lamina propria of the intestine, thus modulating systemic immune responses. This condition resulted in less inflammation, demyelination, and axonal damage.

Based on results obtained using IF in EAE murine models, a small randomized controlled pilot trial was undertaken to examine the effects of IF on clinical and laboratory measures in patients with RR-MS. After 15 days of IF, the patients showed an improvement in the disability status scale of the disease. Furthermore, a significant reduction in leptin levels was observed at day 15, and an increase in adiponectin levels. Stool samples were collected in MS patients, in the control group, and in IF groups at baseline and after 15 days of IF. Similar changes were observed in the four phyla considered in comparison with changes in the relative abundance of phyla in mice and in patients with RR-MS subjected to IF. Therefore, IF has potent immunomodulatory effects mediated in part by the intestinal microbiome.

In conclusion, bacterial diversity favors a healthy microbiota. All these studies show that diet could be a useful tool to improve the course of MS, by modifying the gut microbiota.

Fecal Microbiota Transplantation

The intestinal microbiota plays an important role in intestinal homeostasis and in the modulation of the host immune system[75]. Therefore, specific changes in the composition of the intestinal microbiota, called dysbiosis, are linked to various diseases, including autoimmune conditions such as MS[76]. FMT is a procedure that designs the isolation and microbiological purification of the bacterial flora[77]. FMT is the transfer of fecal material containing the microbiota from a donor to a recipient. There are various methods for

the preparation of the fecal material, and the material can be used both fresh and frozen[78]. In accordance with the Amsterdam protocol, the stool sample (200–300 g) is dissolved in 500 mL saline solution, and this mixture should preferably be used within 6 h[79].

The fecal material for transplantation can be administered orally by nasogastric tube or capsules, or by an anorectal route through colonoscopy or enema[80–82]. Administration through a nasogastric tube ensures correct positioning of the tube when it reaches the duodenum, where the fecal solution will be infused. Oral administration avoids sedation but can cause discomfort in positioning the tube, and can induce nausea and vomiting[83]. Oral administration by capsules requires that fecal material is prepared together with a 10% glycerol solution before being frozen. The capsules are hypromellose, an acid-resistant material. This method is not very invasive, does not require sedation and is more practical than the others[84]. FMT by colonoscopy involves sedation of the patient and administration of fecal material in the colon. This method has the disadvantage of sedation, is invasive, and can result in adverse events (AEs) such as colon perforation. The administration of FMT by enema is less invasive than a colonoscopy, does not need to be performed in the hospital, does not require sedation, and can be repeated easily[85].

Donor selection is important for the success of FMT. The donor can be autologous or heterologous. In the case of autologous FMT the patient receives his own stools. In heterologous FMT the patient receives stools from a donor. FMT from a heterologous donor is safe and more effective than autologous FMT[86]. Heterologous donors can be chosen from the first-degree relatives of the maternal line. These donors may have an intestinal microbiota similar to that of the recipient. The donors undergo laboratory tests to exclude the presence of various diseases. The tests include a complete assessment of clinical and social risk, and stool and blood analysis. The donor must not have active infections, and must not have traveled recently, to avoid contracting epidemic diarrheal diseases. In addition, it is important that there is no evidence of gastrointestinal diseases such as irritable bowel syndrome, inflammatory bowel disease, chronic diarrhea or constipation, and gastrointestinal neoplasia. Furthermore, the donor should not present symptoms of allergies, metabolic syndrome, systemic autoimmune diseases, or atopic diseases. Furthermore, donors also complete a particularly important questionnaire to identify the risks for diseases and conditions for which the laboratory tests are not sufficiently sensitive to detect infectious agents, and for which the tests are not able to identify first-stage infections. Therefore, all donors who have had high-risk sexual behavior, who have used illicit drugs, or who have used antibiotics in the 3 months prior to the donation will be excluded. In addition, the donor must have performed stool and blood tests to identify pathogens such as *Clostridium difficile*, *Helicobacter pylori*, *Salmonella*, and *Shigella*, in addition to those for syphilis, HIV, and hepatitis A, B, C[79].

The recipient must be appropriately prepared to receive the FMT. Although there are currently no guidelines for recipients, it is recommended to perform pre-FMT tests for viral hepatitis, syphilis, and HIV, in order to determine if the fecal sample can be implicated. In addition, the recipient must provide written consent before this treatment, as there is a risk of AEs or an allergic reaction to anesthetics of which the patient was not aware. Furthermore, the recipient must stop any antibiotic therapy some days before the administration of FMT. The day before the treatment the patient undergoes a standard intestinal purge. Usually, an hour before the procedure the patient is pretreated with bowel motility inhibitors to preserve the transplant for at least 4 h[87]. The transplant is followed by a period of monitoring of the patient's condition of varying duration. The transplant is successful if the clinical conditions of the recipient are clearly improved and if the parameters sought by laboratory diagnosis fall within the normal range. Most clinical experience has focused on the use of FMT in patients with *Clostridium difficile* infection (CDI) or other gastrointestinal disorders. FMT records a good level of success in the treatment of CDI, especially in cases where there is resistance to antibiotic treatment[88,89]. In contrast, there are few applications of FMT in MS. Borody et al.[90] in their study evaluated the efficacy of FMT for constipation in MS patients. The results showed that the three patients after FMT showed a recovery of gastric motility. In addition, an improvement in the neurological conditions and urinary functions of the patients was also observed. After 15 years of FMT in one patient, magnetic resonance imaging revealed the arrest of MS progression. The FMT procedure is safe, well tolerated and effective. Generally, the AEs that occur are mild or moderate and resolve spontaneously. The most commonly occurring AEs are transient diarrhea, abdominal cramps, vomiting, bloating, and constipation. However, although less frequent, serious adverse events (SAEs) such as pathogen infections, perforation, and sepsis can be attributed to FMT. Among these surely the most devastating SAEs is death, which has occurred in some patients undergoing FMT. Of these deaths, only one was certainly related to FMT; it is thought to have been caused by aspiration during colonoscopy sedation[91]. Two other deaths were associated with infections that could be the result of FMT procedures or the underlying immunocompromised state. In particular, one patient underwent peritoneal dialysis for end-stage renal disease and was seriously ill at the time of transplantation; on the third day after the procedure, the patient developed peritonitis and died shortly after[92]. The second death probably related to FMT was a patient who died 13 days after FMT secondary to progressive pneumonia, for which he was treated with antibiotics before and after FMT[91]. With the exception of the three patients described above, the other cases of death do not seem to be related to the FMT procedure. The microbiota can be considered as a special organ, therefore, the FMT can be considered an organ transplant that does not show the problem of immunological rejection[90].

The Role of Microbiota in Multiple Sclerosis: Clinical Trials

In the last decades, several preclinical studies have suggested how the intestinal microbiota plays a key role in the treatment of neurodegenerative diseases. The aim of this work is to provide an overview of clinical trials present on https://clinicaltrials.gov (see Table 3) related and present on PubMed that evaluate the role of the intestinal microbiota in MS.

Clinical Trials for Evaluating the Importance of Diet in Relapsing–Remitting Multiple Sclerosis

In recent years, a series of experimental evidence shows a possible involvement of nutrition and microbiota in the onset and development of MS. This hypothesis, if confirmed by further studies, could pave the way for new and more specific treatments. For this reason, the aim of these trials is to understand how an adequate diet can influence the microbiota and the course of the disease.

A pilot study NCT02411838 was conducted to evaluate the effects of IF in patients with MS compared with standard therapies during recovery from a relapse of MS (acute phase) and for 6 months later (chronic phase). For the study, 16 patients with MS were enrolled and were divided into two groups: a control group and an experimental group. Patients belonging to the control group underwent steroid treatment (standard therapy for significant relapses of MS) for a total of 10 days. The patients included in the experimental group, in addition to following the same steroid regimen of the control group, were also subjected to a caloric restriction regimen through fasting every other day for 15 days. The 15th day corresponds with the end of the acute phase of the study, therefore, patients could discontinue the study at this point. All patients provided blood and stool samples before starting the steroids (visit at baseline/day 1) and at day 15. During the visits, the blood was taken in the morning between 9 and 11 am after a 12-h fast for patients in the control group and after 1 day of IF for patients in the experimental group. The primary outcome of the study was the measurement of serum levels of adipokines (leptin, adiponectin, and resistin), pro-inflammatory cytokines (IL-6, TNF-alpha), and cortisol, after 2 weeks, and 3 and 6 months. In addition, flow cytometry analyses on fresh blood and in vitro assays were performed to determine T regulatory cell number and to evaluate their function in vitro. The secondary outcome was the observation of patients' responses to treatment by assessing the general physical and neurological conditions and the quality of life. The effect of the diet on the gut microbiota was also evaluated. The results of this study were published by Cignarella et al.[50]. Their analyses found that chronic IF produces low concentrations of white blood cells, reduced inflammatory cytokines and leptin, and increased circulating levels of cortisol and adiponectin. In particular, a reduction of IL-17-producing T cells and an increase in regulatory T cells in the gut was observed. Furthermore, IF led to an increase in intestinal bacterial wealth, in particular of the families of bacteria *Lactobacillaceae*, *Bacteroidaceae*, and *Prevotellaceae*. In conclusion, the results show that the IF could be a better and more physiological option for its powerful immunomodulatory effects that are at least partially mediated by the gut microbiota.

This research has laid the foundation for a larger and longer-lasting randomized clinical trial NCT03539094, currently ongoing. This trial will aim to test the effects of IF for 12 weeks in individuals with RR-MS. Two dietary regimens will be compared: IF and the standard Western diet, randomly assigned to 60 patients (18 years and older) in a ratio of 1:1. Patients undergoing an intermittent diet for 2 days a week will limit their diet by consuming fewer calories, be allowed to drink water and drinks without calories, and eat fresh, steamed, or roasted non-starchy vegetables. Patients will complete a clinical and laboratory evaluation before starting the diet. The main purpose is to measure leptin in the peripheral blood at week 12. After 12 weeks of treatment the metabolic and inflammatory profile, the anthropometric measures, and the composition of the gut microbiota will also be evaluated. The final data collection and the results are expected by August 2020. The results of this study will allow us to understand the effects of diet on immune system cells and intestinal microbiota in patients with RR-MS. Potentially it will be possible to understand if a restriction of the diet can improve the symptoms for people with MS.

Clinical Trials for Evaluating the Change in Relapsing–Remitting Multiple Sclerosis

Much evidence shows that in the intestine of people with RR-MS, especially during the phases that precede the reactivation of the disease, an alteration of the gut microbiota is observed along with a corresponding proliferation of T lymphocytes, considered fundamental in the development of the pathology. The expected results from these trials will help to understand the role of the microbiota in the evolution of the disease.

The prospective clinical study NCT03262870 will aim to understand the role of the gut microbiota in the modification of the course of MS. In the trial, 40 patients with MS will be recruited according to the MS diagnostic criteria, classified into two types of MS: RR-MS (group 1) and PP-MS, SP-MS and progressive relapsing MS (PR-MS) (group 2). Although the completion of the clinical trial was scheduled for January 2019, no results are available yet for this study.

The ongoing clinical trial NCT02580435 aims to understand the role of the microbiome in MS. It intends to enroll 520 subjects (aged 18–65) and will offer the possibility of identifying multiple subgroups of patients with the aim of detecting the signatures of microbiomes. The entire study will last for 5 years. A first primary outcome will be to

Table 3. Clinical Trials: The Role of Microbiota in Multiple Sclerosis. The table describes the clinical trials recorded on https://clinicaltrials.gov/, which assess the gut microbiota in MS, and how this can be modified through various therapeutic interventions such as diet, fecal transplantation and DMT.

Identifier	Study Title	Status	Subjects	Conditions	Treatment	Inclusion Criteria	Exclusion Criteria
Clinical trials for evaluating the importance of diet in Relapsing–Remitting Multiple Sclerosis							
NCT02411838	Calorie Restriction in Multiple Sclerosis Patients	Recruiting	16 participants (aged 18 to 60)	MS	IF	• Participants must be diagnosed with relapsing MS. • Participants must be 18 - 60 years old. • Participants will need to be experiencing a relapse as identified by their neurologist. • Participants must have a body mass index (BMI) of 23 or higher. • Participants must not have other ongoing diseases in other systems.	• History of any chronic disease process (excluding MS) that could interfere with interpretation of results. • Use of insulin pumps or insulin injections for diabetes. • Use of drugs like Warfarin or Coumadin that need to monitor the intake of vegetables containing high levels of vitamin K. • Patients that are required by a physician to follow a special diet or food restriction. • Alcoholism, psychiatric problems, life situations that would interfere with study participation and compliance.
NCT03539094	Intermittent Fasting in Multiple Sclerosis	Recruiting	60 participants (aged 18 and older)	RR-MS	IF	• Diagnosis of RR-MS (2010 Mc Donald criteria). • EDSS <6.0 and disease duration $\leq$15 years. • On an injectable therapy for MS, glatiramer acetate (GA) or beta-interferon (beta-IFN) for at least 3 months prior to the study and with no anticipated changes of the medication for a duration of 12 weeks. • Age $\geq$18 years. • BMI >22 and <35 kg/m^2 with stable weight in the 3 months prior to screening.	• History of any chronic disease process (excluding MS) that could interfere with interpretation of results. • Diagnosis in the past of an eating disorder. • Relapsing at the time of enrollment. • On corticosteroid treatment in the past month. Nasal corticosteroid treatments are allowed. • Diagnosis of diabetes. • History of food allergies or food intolerance that would interfere with the study. • History of antibiotic treatment within the past 3 months prior to enrollment. • Use of anticoagulant drugs that need to monitor their intake of vegetables containing high levels of vitamin K. • Currently on a special diet and not willing to stop at least 1 month prior to enrollment. • Currently taking omega 3/fish oil supplements and not willing to stop administration 1 month prior to enrollment. • Currently pregnant or plan to become pregnant within 6 months. • Current tobacco or e-cigarette smoker.

(continued)

Table 3. (continued)

Identifier	Study Title	Status	Subjects	Conditions	Treatment	Inclusion Criteria	Exclusion Criteria
Clinical trials for evaluating the change in Relapsing–Remitting Multiple Sclerosis							
NCT03262870	Gut Microbiota and Multiple Sclerosis	Not yet recruiting	40 participants (aged 25 to 40)	MS	–	• The study will include 40 cases of MS according to diagnostic criteria of multiple sclerosis they classified into two types of MS Relapsing–remitting MS (group 1), and Primary progressive MS, Secondary progressive MS, and Progressive relapsing MS (group 2). • Each patient was submitted to the following: Expanded disability status scale score between 1 and 6 and functional system score; demographic and clinical data.	The general exclusion criteria included prior surgeries, any patients or controls currently taking antibiotics or probiotic supplements, or having a known history of the disease with a disease such, rheumatoid arthritis, type-1 diabetes, and IBD, were also excluded from the study. Microbial DNA was extracted from fecal material of each sample.
NCT02580435	Deciphering the Role of the Gut Microbiota in Multiple Sclerosis	Not yet recruiting	520 participants (aged 18 to 65)	MS	–	• Diagnosis of RIS, CIS, RR-MS, PP-MS. • Signed written informed consent.	• Pregnancy. • Lactation. • Severe cognitive decline.
NCT03797937	Microbiome Benchmarking to Identify Perturbations in Multiple Sclerosis II	Recruiting	100 participants (aged 18 to 55)	RR-MS	–	• Diagnosis of MS (as defined by the 2010 McDonald criteria). • Occurrence of symptoms no longer than 5 years before baseline. • Aged 18–55. • Willingness to participate in the study and to sign the informed consent.	• Current treatment with monoclonal antibodies. • Treatment with high doses of systemic steroids 2 months before baseline. • Use of antibiotics 3 months before baseline.
Clinical trials for evaluating the efficacy of Fecal Microbiota Transplantation in Relapsing–Remitting Multiple Sclerosis							
NCT03594487	Fecal Microbiota Transplantation (FMT) of FMP30 in Relapsing–Remitting Multiple Sclerosis	1b phase Recruiting	30 participants (aged 18 to 60)	RR-MS	FMT of FMP30 Donor Stool	• Diagnosis of relapsing-remitting multiple sclerosis (MS) by International Panel McDonald Criteria (2010) incorporating 2017 revisions which reclassify select high-risk Clinically Isolated Syndromes under 2010 criteria as RR-MS under 2017 criteria, and Lublin criteria (2014). • Recent documented MS disease activity. • Expanded Disability Status Scale (EDSS) less than or equal to 6.0; EDSS 5.5 or less if MS disease duration is greater than 15 years. • Must have positive serology for Epstein-Barr Virus (EBV) (IgG anti-EBNA positive) at screening, indicating prior exposure. • No prior MS disease-modifying therapy or a washout period of 12 weeks for subjects on glatiramer acetate or interferon-beta therapy. • At least 4 weeks from baseline since last use of IV or oral glucocorticoids Protocol: MS-BIOME Study. • Agree to maintain a stable diet during the course of the study.	• Prior use of fingolimod, dimethyl fumarate, teriflunomide, natalizumab, alemtuzumab, mitoxantrone, cyclophosphamide, rituximab, ocrelizumab, daclizumab, methotrexate, azathioprine. mycophenolate mofetil, cyclosporine, leflunomide or induction chemotherapy. • No use of diuretics like furosemide (Lasix) 1 week before the first dose oral antibiotics. The use of hydrochlorothiazide (HCTZ) for hypertension at a dose <50 mg/day is allowable. • Progressive MS by Lublin criteria (2014). • No oral or IV antibiotics within 8 weeks of screening and 12 weeks of scheduled of the planned FMT procedure if in the FMT arm or first stool collection if in control arm. • Hypersensitivity or allergy to study antibiotics, conscious sedation medications or bowel preparation.

(continued)

Table 3. (continued)

Identifier	Study Title	Status	Subjects	Conditions	Treatment	Inclusion Criteria	Exclusion Criteria
						• Premenopausal women and women <12 months after the onset of menopause must have a negative serum pregnancy test unless they have undergone surgical sterilization. • Female subjects of childbearing potential who are sexually active with a non-sterilized male partner must agree to use a highly effective method of contraception; non-sterilized male subjects who are sexually active with a female partner of childbearing potential must agree to use a highly effective method of contraception. • Not actively participating in another interventional MS clinical trial.	• Contraindication to study procedures including MRI, anesthesia, colonoscopy, phlebotomy. • History of inflammatory bowel disease. Active symptomatic *C. difficile* infection. • Active gastrointestinal condition being investigated; history of known or suspected toxic megacolon and/or known small bowel ileus, major gastrointestinal surgery within 3 months before enrollment; or history of total colectomy or bariatric surgery. • History of malignancy including no concurrent induction chemotherapy, radiation therapy or biological treatment for active malignancy. • Pregnant or lactating women or intention of getting pregnant during the trial period. • Active infection including untreated latent or active tuberculosis, HIV, hepatitis, syphilis or other major active infection. • Known immunodeficiency. • Any condition that in the opinion of the study PI could jeopardize the safety of the subject, would make it unlikely for the subject to complete the study or could confound the results of the study. • Unable or unwilling to comply with study protocol requirements.
NCT03183869	Fecal Microbial Transplantation in Relapsing Multiple Sclerosis Patients	2 phase Recruiting	40 participants (aged 18 and older)	RR-MS	FMT	• Have a confirmed diagnosis of relapsing MS defined by the 2010 Revised McDonald Criteria for the Diagnosis of Multiple Sclerosis. • Any disease duration will be accepted. • Have a baseline EDSS $\leq$7.0. • Older than 18 years of age. • Be able to attend all clinic appointments without interruption. • Patients must be able to understand English sufficiently well to understand and comply with the clinic and medication schedules and procedures. • Be willing and able to give written informed consent. • Negative blood pregnancy test at screening.	• Pregnancy or breastfeeding. • Current or recent [in the last 90 days] exposure to high dose corticosteroids. • Ongoing use of antibiotics. • Standard of care exclusions for MRI scans. • Presence of a chronic intestinal disease e.g. Celiac, malabsorption, colonic tumor • Inability to provide informed written consent. • Immunosuppression from transplantation, HIV, cancer chemotherapy or ongoing use of any immunosuppressive agents. • Concomitant inflammatory diseases. • Pregnant women. • Any contraindications for MRI. Participants are to be screened by a

(continued)

Table 3. (continued)

Identifier	Study Title	Status	Subjects	Conditions	Treatment	Inclusion Criteria	Exclusion Criteria
							CMRTO (The College of Medical Radiation Technologists of Ontario)-certified MRI Technologist in order to determine the MRI compatibility or exclusion of implantable/external devices according to the manufacturer's safety guidelines. The deviCes include cerebral aneurysm clips, neuro-stimulator, mechanical heart valves, cardiac stents, IUDs (intrauterine device), vena cava filters, shunts, embolization coils, cochlear implants, non-removable prosthesis/artificial limbs. Contraindications are pacemaker of defibrillator, shrapnel/metallic fragments, previous brain surgery, seizure, severe claustrophobia, weight or body index that will prevent a successful MRI study.
Clinical trials for evaluating the efficacy of Dimethyl Fumarate in Relapsing-Remitting Multiple Sclerosis							
NCT02471560	Tecfidera and the Gut Microbiota	4 phase Completed	37 participants (aged 18 and older)	RR-MS	DMT	• Have a confirmed diagnosis of RR-MS and satisfy the therapeutic indication as described in the local label. • Female subjects of childbearing potential who are not surgically sterile must practice effective contraception according to the summary of product characteristics (SPC) during their participation in the study and be willing and able to continue contraception for 30 days after their last dose of study treatment.	• Diagnosis of primary progressive, secondary progressive or progressive relapsing MS. • Antibiotic treatment in the last month prior to study entry. • Scheduled alteration of diet, including the use of probiotics.
NCT02736279	Impact of Tecfidera on Gut Microbita	Recruiting	25 participants (aged 18 and older)	RR-MS	DMT	• Confirmed diagnosis of a relapsing form of multiple sclerosis by McDonald criteria. • Age 18 years or older. • Able to provide informed consent. • Treatment naïve to DMF, Fumaderm or other fumarate containing compound. • Neurologically stable within 4 weeks prior to screening. • Stable gross diet composition type (Western, vegetarian with dairy, vegan, gluten-limited, Paleo) within 12 weeks of screening visit. • Able to complete study specific questionnaires and demographic information via HIPAA compliant secure internet based portal.	• Treatment with immunosuppressive therapies (other than steroids) within 12 months of screening, experimental or FDA-approved cell trafficking modulators, experimental immune cell vaccines, or stem cell therapy. • GI diagnostic or therapeutic procedure within 24 weeks of screening visit, or at any time during participation in the study. • Steroid therapy (oral or intravenous) within 4 weeks of screening visit. • Chronic use of a proton pump inhibitor therapy within 3 months of screening. • Chronic use of laxatives other than Colace within 6 months of screening. • Intravenous antimicrobial therapy within 24 weeks of screening.

(continued)

Table 3. (continued)

Identifier	Study Title	Status	Subjects	Conditions	Treatment	Inclusion Criteria	Exclusion Criteria
						• No oral antibiotics within 4 weeks of screening. • Able to abide by safety surveillance monitoring and management as part of standard of care. • Able and willing to comply with the protocol requirements for the duration of the study.	• Oral antimicrobial therapy within 4 weeks of screening. • Dental procedure within 4 weeks of screening visit. • Total parenteral nutrition (TPN) within 12 months of screening. • History of Crohn's disease, ulcerative colitis, biliary cirrhosis, celiac disease, chronic pancreatitis, gastric lap-band procedure, gastric or colon cancer, bowel resection, colitis within past 6 months. • Women who are pregnant, breastfeeding, planning pregnancy, or potentially fertile and not willing to abide by effective contraception while being treated with DMF.
NCT03092544	Investigating Indirect Mechanism of Neuroprotection of Tecfidera® (Dimethyl Fumarate) in RR-MS and Progressive Patients	4 phase Active not recruiting	54 participants (aged 18 to 65)	MS	DMT	Inclusion (MS Population Only): • Male or female subject, age 18-65 (inclusive) at the time of informed consent, with a confirmed diagnosis of multiple sclerosis and meeting multiple sclerosis patient population as specified in section 4/ study design/multiple sclerosis population of this protocol. • Subject has the ability to understand the purpose and risks of the study and provide signed and dated informed consent and authorization to use protected health information (PHI) in accordance with national and local subject privacy regulations. • Expanded Disability Status Scale (EDSS) score between 0 and 7, inclusive, at the screen. Inclusion (Normal Control Volunteer Only): • Subject has the ability to understand the purpose and risks of the study and provide signed and dated informed consent and authorization to use protected health information (PHI) in accordance with national and local subject privacy regulations.	• Current smoker. • History of or current clinically relevant gastrointestinal bleeding or other gastrointestinal diseases or processes that may interfere with the analysis of stool samples per protocol. • Any sign of chronic active infection except for those requiring topical medication for treatment, or screening laboratory evidence consistent with a significant chronic active acute infection requiring systemic treatment. This must be resolved before treatment may commence. • Pregnant females; breastfeeding females. • Known Positive HIV antibody, hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb), or hepatitis C antibody tests indicative of present or prior infection. • Any abnormal hematology values or clinical chemistry values judged by the Investigator or Sponsor as clinically significant. • Positive purified protein derivative (PPD) skin test or positive quantiferon (QTFN) at screen visit or know history of active tuberculosis not adequately treated. • Any malignancy within 5 years, except for basal or squamous cell skin lesions which have been surgically excised, with no evidence of metastasis.

(continued)

Table 3. (continued)

Identifier	Study Title	Status	Subjects	Conditions	Treatment	Inclusion Criteria	Exclusion Criteria
							• Any clinical, CSF or MRI evidence for Progressive multifocal leukoencephalopathy (PML), from historical MRI or results of the screen MRI. • Other severe acute or chronic medical or psychiatric condition or laboratory abnormality. • Subjects participating in or expecting to participate in other interventional clinical trials. • Inability or unwillingness to record online dietary questionnaire information as required by protocol. • History of drug or alcohol abuse (as defined by the investigator) within 2 years prior to screen. • History of chronic urinary tract infections, irritable bowel syndrome, inflammatory bowel disease, diabetes mellitus or vascular disease as determined by history and investigator decision (normal control population only). • Evidence or history of autoimmune disease including the diagnosis of MS or MS symptomatology (normal control population only). • Any malignancy within 5 years, except for basal or squamous cell skin lesions which have been surgically excised, with no evidence of metastasis (normal control population only).

MS: Multiple Sclerosis; RR-MS: Relapsing–Remitting Multiple Sclerosis; FMT: Fecal Microbial Transplantation; DMF: Dimethyl Fumarate; IF: Intermittent Fasting; MRI: Magnetic Resonance Imaging.

observe how the expression of the gut microbiome composition changes between MS patients and healthy controls. This endpoint will be evaluated using a cohort of untreated initial MS patients (up to 12 months from onset) not treated with immunomodulatory drugs or steroids for at least 3 months and a cohort of healthy controls (obtained from the Weizmann DataBank). The secondary outcome will be the comparison between patients suffering from MS with a similar time from the diagnosis but with different severity of the disease. The cohort profile will be determined by obtaining from each patient clinical data (including the form of consent; drugs; the annual rate of recurrence), blood tests (including a complete blood count, complete biochemistry, lipid profile, cholesterol profile), and the gut microbiota profile obtained from stool samples, by shotgun metagenomic sequencing and 16 S rRNA profiling. The third step of the study will perform data analysis and algorithmic development. Investigators for this study will develop algorithms useful for deciphering the role of microbiota in MS. These algorithms will allow classification of the severity of the disease, prediction of relapse risk, differentiation of the phenotypes of the disease, and identification of patients responding to the treatment. In the last step, the investigators will perform univariate and multivariate analyses of the data obtained. The results of this predictive study could reveal important and still unknown aspects of MS and also identify new therapeutic avenues that target the microbiota. The final data collection and the results of this study are expected by the end of the year 2020.

The non-randomized interventional clinical trial NCT03797937 will evaluate the association between gut microbiota and the inflammatory disease activity in early onset RR-MS. Furthermore, the investigators will try to understand if patients with active RR-MS possess a more inflammatory gut microbiota than patients with no-active RR-MS, and compared with healthy individuals. Specifically, the researchers will assess whether the temporal variability of the intestinal microbiota is related to the inflammatory activity of the disease in MS, or on the contrary, if changes in the gut microbiota are predictors of future inflammatory activity in MS. Moreover, they will try to understand if the characteristics of the intestinal microbiota are predictive of the course of the disease after 2 years. For the study, 100 participants with RR-MS (aged between 18 and 55) will be recruited, and will be divided into two experimental groups: a control group, consisting of healthy individuals, and an experimental group composed of patients with RR-MS who will undergo magnetic resonance imaging (MRI). The main outcome will be to evaluate the clinical evidence for the active disease over the 3 years, reporting the time to first relapse (after baseline) for all patients. The secondary outcome will be the radiological highlights of the active disease. The final data collection and the results of this study are expected by the end of the year 2021.

Clinical Trials for Evaluating the Efficacy of Fecal Microbiota Transplantation in Relapsing–Remitting Multiple Sclerosis

FMT consists of the transfer of fecal material from healthy donors to the recipients' gastrointestinal tract, and is effective for the treatment of diseases associated with the microbiota alterations such as MS. FMT-based therapies, during intestinal inflammatory states, stimulate the immune response of the host's intestinal mucosa through the secretion of specific cytokines. Therefore, the restoration of a normobiosis represents the first step to simultaneously trigger different pathways to resolve the inflammatory state. The manipulation of the gut microbiota, therefore, is to be considered a therapeutic option in the treatment of various disorders such as RR-MS. The expected results from these studies will help in understanding how fecal transplantation can improve the state of the disease.

The ongoing NCT03594487 is a prospective, open-label phase 1b study. The purpose of this trial will be to evaluate the feasibility, safety, tolerability and effects on the immune function of the FMT of FMP30 (about 12.5 g of filtered donor stool homogenized in 30 mL of normal saline solution, obtained from the non-profit stool OpenBiome) in RR-MS. To date, 30 patients (aged 18–60) with RR-MS have been enrolled, and will be divided into two arms: interventional FMT treatment arm and observational control arm. Patients enrolled at the screening must not show positive for Epstein-Barr Virus (EBV) (positive anti-EBNA IgG). In addition, they must not have undergone any previous MS-modifying therapy. In the case of subjects treated with glatiramer acetate or interferon-beta therapy, a 12-week washout period should be performed. During the study, the participants will have to follow a stable diet. Patients in the experimental group, in order to pre-condition the intestine for FMT of FMP30 donor stool and to facilitate the engraftment of the microbiome, will undergo an oral antibiotic regimen for 5 days. Through colonoscopy, patients will receive three doses of FMP30 engrafts. This arm of the study will last 52 weeks: after 4 weeks of screening, the active phase of the study will continue for 12 weeks post-FMT followed by 36 weeks of safety follow-up. Patients who are part of the comparative observation group will have been prior subjected to the admissible MS therapies; moreover, immunological measurements of serum and feces will be performed. The study for the observational control arm will last for 12 weeks. The primary outcome will be assessing the change in the fecal microbiota in subjects who complete the study, during the reference period of 2 weeks, 4 weeks, 8 weeks, and 12 weeks compared with baseline, to verify the effectiveness of the treatment. To assess the safety and tolerability of the treatment, emerging AEs up to week 12 will be observed. The secondary outcome will be to evaluate the immune response by observing the induction of regulatory T cells or Th2 and/or reduction of Th1 or Th17 cells, by measuring $CD20^+$ B and $CD19^+$ B plasma cells, and by measuring

serum immunoglobulin levels. The final data collection and the results are expected by the end of the year 2019.

The phase 2 clinical study NCT03183869, still in the recruitment phase, is an interventional study. The aim of this trial will be to evaluate the effect that FMT will determine on peripheral blood cytokines in patients with relapsing MS. For the study, 40 patients (18 years and older) with a diagnosis of RR-MS according to the Mc Donald criteria will be enrolled, and they will undergo FMT via enema. Patients will be randomized 1:1 in two groups. One group will receive FMT every month for the first 6 months (early intervention group); the other group will be a control group during the first 6 months, then it will receive the FMT for the last 6 months of the study (late intervention group). Prior to treatment and throughout the study, both groups will undergo stool, urine, and blood collection to study the microbial profile and cytokine levels as well as blood DNA bacteria and to assess intestinal permeability. The effects of FMT on intestinal permeability will be evaluated through measurements of lactulose and mannitol levels in urine. At visits 1, 4, and 7 patients from both groups will receive a lactulose solution and urine will be collected overnight and the following morning. Finally, an assessment of the clinical safety of the treatment will be performed by means of a neurological examination, and MRI at months 1, 6, and 12 will verify the clinical picture of the disease. The results of this study will be needed to guide the future use of FMT by observing the efficacy, safety profile, and mechanism of action.

Clinical Trials for Evaluating the Efficacy of Dimethyl Fumarate in Relapsing–Remitting Multiple Sclerosis

Recently it has been observed that DMTs used for the treatment of MS can influence the composition of the intestinal microbiota. In fact, the study conducted by Cantarel et al.[93] showed that treatment with glatiramer acetate led to a modification of the gut microbiota in patients with RR-MS. Therefore, the aim of these trials will be to evaluate how DMF can alter the intestinal microbiota in patients with RR-MS. DMT is an oral agent approved for the treatment of RR-MS. This treatment activates the transcription pathway of the nuclear factors Nrf2, which in MS patients induces upregulation of Nrf2-dependent antioxidant genes, executing its cytoprotective and neuroprotective effects[15]. Furthermore, together with its metabolite monomethyl fumarate, it also produces a significant reduction in the activation of the cells of the immune system and the subsequent release of pro-inflammatory cytokines in response to inflammatory stimuli.

The completed phase 4 clinical trial NCT02471560 aimed to evaluate the effect of DMF (Tecfidera®) on the intestinal microbiota of patients with RR-MM. For the study, 37 subjects (18 years and older) were enrolled, and were divided into two experimental groups: an experimental group of patients undergoing treatment with DMF and an active comparison group was subjected to injectable MS DMTs. DMF was administered at a dose of 120 mg twice a day orally for 7 days, followed by a recommended dose of 240 mg daily for 12 weeks. Safety results show that SAEs occurred in 3.70% of patients treated with DMF and in 11.11% of patients treated with MS-DMT. No SAEs occurred in 55.56% of patients in the DMF group and in 88.89% of the MS-DMT group. The primary outcome was to observe changes in the composition of the intestinal microbiota in pre-treatment participants compared with patients treated with DMF at day 1, week 2, and week 12. The secondary objectives of this study were to identify the differences in the composition of the intestinal microbiota among patients who develop or not adverse gastrointestinal (GI) events, both pre and post DMF treatment. In addition, it was evaluated how and if the incidence of gastrointestinal GI AEs influenced the intestinal microbiota. The microbial profile of patients treated with DMF was significantly altered, and GI symptoms after 2 weeks of treatment compared with baseline worsened. It was also observed that patients with severe GI disturbances showed a reduction in Actinobacteria, also linked to a reduction in *Bifidobacterium*. After 12 weeks of treatment with DMF, patients had a greater abundance of Firmicutes represented mainly by *Faecalibacterium*. As reported by the results, the GI disturbances caused by the treatment are linked to a variation of the microbiota[94].

The double-blinded, prospective, single-center pilot clinical trial NCT02736279, now in the recruitment phase, plans to enroll 25 participants (18 years and older) with RR-MS. The purpose of this study will be to observe the change in the gut microbiota, and the onset and severity of GI disorders during the first 6 months of treatment with DMF. The primary outcome will be to observe the change in the diversity and abundance of bacterial and archaeal species in the intestinal microbiota after treatment with DMF. The microbiota profile will be determined by sequencing 16 S ribosomal RNA extracted from patients' stool samples. The first stool sample will be collected before the initial dose of DMF, with subsequent collections at certain time intervals during the study (weeks 4, 8, 12, and 24). The secondary outcome will be to evaluate the onset of GI side effects after 12 and 24 weeks of treatment and to observe the change in the profile of the microbiota following the onset of these disorders. Furthermore, the study will also assess how DMF treatment can influence patients' behavior and mood. Final data collection for the measurement of the primary outcome is expected by June 2020.

The ongoing phase 4 NCT03092544 non-randomized clinical trial will aim to identify the types of bacteria that reside in the intestines of healthy individuals compared with those found in individuals with MS (especially RR-MS and SP-MS) and will evaluate the effects of treatment with DMF. For the study, 54 subjects (aged 18–65) will be recruited and divided into five experimental groups: two active comparator groups and three no-intervention groups. Recruited subjects will be required to complete an online dietary questionnaire required by the protocol. Patients in the active

comparator groups will be treated with DMF for 6 months; one group will be patients aged 18–55 with RR-MS; the second group will consist of patients aged 25–65 with a diagnosis of SP-MS. Patients included in the three intervention groups will not be treated with DMF. One group will include patients with a diagnosis of SP-MS (aged 25–65), one group will consist of a cohort of mean age 38, and one group of a cohort of mean age 58. Primary outcomes will aim to assess changes in neuronal bioenergetics from baseline after 6 months of treatment using plasma and cerebrospinal samples from patients with RR-MS treated with DMF. The change in levels of neurofilament, ceramide sphingosine, and other lipid species in cerebrospinal samples collected from patients with RR-MS before and after 6 months of therapy, and changes in microbiota composition following treatment with DMF at 6 months of therapy, will also be observed. Although the completion of the clinical trial was scheduled for December 2018, no results are available yet for this study.

Clinical Trials Approved by Local Ethics Committees

In this subsection, clinical studies published in the indexed journals are described. These manuscripts report the results obtained from the analysis of gut microbiota of patients with MS as compared with healthy subjects.

Miyake et al.[29] conducted a study to understand the role of indigenous gut microbiota in the pathogenesis of MS. Therefore, the intestinal microbiota of patients with MS was compared with that of healthy subjects. This study was approved by The National Center of Neurology and Psychiatry Ethics Committee, the Hospital Ethics Committee at Juntendo University Hospital, the Human Research Ethics Committee of Azabu University, and the Research Ethics Committee of the University of Tokyo. In addition, 50 volunteers (aged 27.2 $\pm$ 9.2 years) were recruited as healthy controls at the University of Azabu. Individuals undergoing antibiotic therapy were excluded from the study. For all patients, stool samples were collected; specifically, in patients with RR-MS, fecal samples were collected during the remission phase. A 16 S ribosomal RNA (rRNA) gene analysis was performed on the fecal samples collected during the study, using a pyrosequencing method. The results obtained showed a significantly higher inter-individual variability in the intestinal microbiota of subjects with RR-MS compared with healthy subjects. Both groups of studies presented a microbiota characterized by Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Comparative analysis showed that patients with RR-MS showed an abundance of species belonging to Actinobacteria, while species belonging to Bacteroidetes and Firmicutes were more abundant in healthy subjects. The genus-level analysis identified 21 species that showed significant changes in relative abundance between patients and controls. Among these 21 species, in patients with RR-MS, a depletion of species belonged to Clostridia clusters XIVa and IV was observed. These clusters are made up of highly diversified bacterial species, many of which are responsible for the production of short-chain fatty acids such as butyrate. Butyrate is responsible for stimulating the anti-inflammatory response, therefore it is conceivable that the reduction of these Clostridia species may be associated with the pathogenesis of MS. Moreover, compared with healthy individuals, in patients with RR-MS, five other less abundant species have been identified and classified in three genera: Bacteroides (*B. stercoris*, *B. coprocola*, and *B. coprophilus*), Prevotella (*P. copri*), and *Sutterella* (*S. wadsworthensis*). In contrast, the species *Streptococcus thermophilus* and *Eggerthella lenta* were significantly increased in patients with RR-MS compared with the controls. In conclusion, given the results obtained, the authors confirm the existence of a significant association between the altered intestinal microbiota and the pathogenesis of MS. Therefore, these results may be important for the future development of new preventive or therapeutic strategies for MS.

Chen et al[31]. conducted a study designed to assess how the microbiota of MS patients varies compared with healthy subjects. This study was approved by Mayo Clinic Institutional Review Board. Sixty-two patients with RR-MS (between 18 and 80 years) and 36 healthy individuals including an age and sex cohort with no known symptoms of disease were recruited. All subjects undergoing antibiotic therapy, probiotic supplements, or with autoimmune disease were excluded from the study. All individuals provided stool samples, but for analysis, only 31 samples were included. Patients with RR-MS were defined as having active disease if the stool sample was collected within 1 month of relapse; otherwise, they were considered as being in the remission phase. Stool samples were used to determine profiles typical of intestinal microbial populations that were generated using the sequencing of the hypervariable marker of the V3-V5 region of the 16 S ribosomal RNA gene. The identification of the bacterial taxa expressed differentially in the disease state was performed by means of abundance analysis and the use of prediction models. Data obtained from the analysis of species richness (α-diversity) showed no differences between patients with total RR-MS and healthy controls; however, a decrease in species richness was observed in RR-MS patients with active disease compared with patients in remission and controls. Detailed analyses of fecal microbiomes revealed that patients had a distinct microbial community profile that included Firmicutes (58.6%), Bacteroidetes (40.4%), Proteobacteria (0.7%), Actinobacteria (0.1%), and a rare bacterial phyla tail (0.2%). Within the phylum Actinobacteria, the genera *Adlercreutzia* and *Collinsella* were less abundant in patients with RR-MS than in controls. The genera *Bacteroides*, *Pedobacter*, and *Flavobacterium* showed a greater abundance in patients with RR-MS compared with controls; on the contrary, Parabacteroides were less represented. Other genera of Firmicutes such as *Blautia* and *Dorea* were found to be increased in patients with RR-MS; Erysipelotrichaceae, Lachnospiraceae,

Veillonellaceae and the genera *Lactobacillus* and *Coprobacillus* were less represented in patients with RR-MS than in healthy controls.

Among the Proteobacteria, *Pseudomonas* and *Mycoplana* were more abundant in patients with RR-MS, while *Haemophilus* was more represented in healthy subjects. The results of the predictive study also confirmed that some genera such as *Adlercreutzia*, *Pedobacter*, *Pseudomonas*, *Coprobacillus*, *Dorea*, *Flavobacterium*, *Parabacteroides*, *Mycoplana*, *Haemophilus*, *Blautia*, and *Collinsella* were predictive of disease status, indicating the robustness of both analyses. Therefore, the results of this study confirmed the hypothesis that a microbial dysbiosis can be related to MS.

Conclusion

The development of MS is influenced by genetic and environmental factors. Commensal microbiota is one of the environmental risk factors related to the development of the MS.

The microbiota is mainly represented by the phyla Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria. A dysbiosis of the intestine has been recognized as a constant feature during the clinical course of MS.

As observed in several studies, MS patients, compared with healthy controls, show a numerical decrease in the gut microbiota of genera *Parabacteroides*, *Bacteroides stercoris*, *Bacteroides coprocola*, *Bacteroides coprophilus*, and *Provotella copri* (phylum Bacteroidetes). Furthermore, a reduction of some families belonging to the order of the Clostridia (phylum Firmicutes), of the genera *Sutturella* and *Haemophilus* (Phylum Proteobacteria), *Aldercreutzia* and *Collinella* (phylum Actinobacteria), was observed. On the contrary, an increase was recorded in the following genera: *Blautista*, *Dorea*, and *Streptococcus thermophilus* (phylum Firmicutes), *Pedobacteria* and *Flavobacterium* (phylum Bacteroidetes), *Pseudomonas* and *Mycoplana* (phylum Proteobacteria) and *Eggerthella lenta* (phylum Actinobacteria). This dysbiosis in patients with MS has a pro-inflammatory and regulatory effect in human T lymphocytes.

An interesting aspect suggests that MS patients, treated with FMT and under modified diet, show the normalization of some of these microbial populations. FMT and modified diet change the gut microbiota, favoring the development of "good" microorganisms such as *Lactobacilli*, *Bacteroides*, and *Prevotella* that show anti-inflammatory action.

Through these therapeutic interventions, that modify the gut microbiota so as to favor the development of "good" microorganisms with anti-inflammatory action, such as *Lactobacilli*, *Bacteroides*, and *Prevotella*, it is possible to consider reducing the onset of clinical relapses of disease in patients suffering from MS. Therefore, a healthy and balanced diet plays a protective role in the course of the disease, also through a modification of the composition of the intestinal microbiota. It is therefore important to modify the quality of the fats introduced by limiting saturated fats and hydrogenated fats and preferring the consumption of mono and polyunsaturated lipids. Furthermore, the correct intake of vitamins, mineral salts, and antioxidants must be guaranteed, especially in fruit and vegetables. Among vitamins, the introduction of vitamin D seems to be very important, as people with MS are often deficient. It is also important to prevent overweight, which can make disability worse.

The expected results, supported by further research, will pave the way to the development of better and more effective future therapies that would allow treatment of an often devastating disease such as MS with low-tech therapies such as fecal transplants or/and diet.

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