Universidade de Lisboa
Faculdade de Farmácia

Role of Microbiome in Multiple Sclerosis

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Monografia orientada pela Professora Doutora Adelaide Maria Afonso Fernandes Borralho, Professora Auxiliar

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Abstract

The composition of the microbiome has demonstrated to play a role in autoimmune diseases, in particular, in neurodegenerative diseases. Multiple Sclerosis is a demyelinating, autoimmune disease whose aetiology is still not completely understood, although there are several factors that contribute to the development of the disease. The gut-brain axis is of extreme importance in the comprehension of diseases like Multiple Sclerosis and the influence that the microbiome has in disease pathogenesis has made these microorganisms of great interest. By comprehending the importance of microbial communities in the modulation of immune responses, the modulation of the microbiome could potentially be an innovative therapeutic approach to neurodegenerative diseases. Faecal matter transplantation is a procedure used to restore the microbial environment of patients with recurrent gastrointestinal infections, but its use in the treatment of Multiple Sclerosis has been of growing focus. These studies have led to more research on microbiome modulation as a therapy for neurodegenerative diseases, including symptomatic treatment and prevention of disease progression.

Keywords: Multiple Sclerosis, Microbiome, Gut Microbiota, Faecal Matter Transplantation, Autoimmune.

Resumo

A composição do microbioma já demonstrou desempenhar um importante papel em doenças autoimunes, em particular, em doenças neurodegenerativas. A Esclerose Múltipla é uma doença desmielinizante e autoimune, cuja etiologia ainda não está completamente esclarecida, embora existam vários fatores que contribuem para o desenvolvimento da doença. O eixo intestino-cérebro é de extrema importância na compreensão de doenças como a Esclerose Múltipla e na influência que o microbioma tem na patogénese da doença, tornando estes microorganismos de grande interesse. Ao compreender a importância da flora microbiana na modulação de respostas imunes, a modulação do microbioma pode ser uma abordagem terapêutica inovadora para doenças neurodegenerativas. O transplante de matéria fecal é um procedimento utilizado para restaurar o ambiente microbiano de doentes com infeções gastrointestinais recorrentes, mas a sua utilização no tratamento da Esclerose Múltipla tem tido um foco crescente. Estes estudos levaram a mais pesquisas sobre a modulação do microbioma como uma terapêutica para doenças neurodegenerativas, incluindo o tratamento sintomático e prevenção da progressão desta doença.

Palavras-chave: Esclerose Múltipla, Microbioma, Microbioma Intestinal, Transplante de Matéria Fecal, Autoimune.

List of acronyms and abbreviations

ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
BBB	Blood-brain barrier
BDNF	Brain derived neurotrophic factor
CNS	Central Nervous System
CS	Corticosteroid
CSF	Cerebrospinal fluid
DMF	Dimethyl fumarate
DMT	Disease modifying therapy
DSS	Disability Status Scale
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
FMT	Faecal microbiota transplantation
GA	Glatiramer acetate
GALT	Gut-associated lymphoid tissue
GFAP	Glial fibrillary acid protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenal axis
HRQOL	Health-related quality of life
JCV	John Cunningham virus
LPS	Lipopolysaccharide
MENA	Middle East North Africa
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
MSIF	Multiple Sclerosis International Federation
NK	Natural killer
NT-3	Neurotrophin-3

PPMS	Primary-progressive Multiple Sclerosis
PRMS	Progressive relapsing Multiple Sclerosis
RRMS	Relapsing-remitting Multiple Sclerosis
SCFA	Short-chain fatty acids
SPMS	Secondary progressive Multiple Sclerosis
TLR-2	Toll-like receptor ligand
YLDs	Years of life with disability

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1. Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) whose incidence keeps increasing worldwide. It is characterised by demyelination and axonal loss, which results in lesions in the brain and spinal cord. MS is a debilitating disorder and there is still no cure. Although its mechanisms remain unclear, there are a few factors that can contribute to the development of disease, such as genetic and environmental factors (1–3).

The microbiome has been of great interest amongst the scientific community and its role in neurodegenerative diseases has been reported. The growing evidence suggesting that the microbiome can play both protective and pathogenic roles in MS, has made this topic a new focus for the treatment of these diseases. New DNA sequencing technologies have made it possible to sequence all sorts of microorganisms, including those in the gastrointestinal tract. The study of bacterial populations in the human gut is of particular interest in autoimmune diseases, as they have shown to play an important role in the modulation of immune responses (1).

The main objective of this thesis is to showcase the role of the microbiome in MS, not only its impact in the development of the disease, but also the differences in the microbiome of patients and how the modulation of the gut microbiome could potentially be an innovative therapy for the treatment of symptoms and avoid progression of the disease.

2. Methodologies

For the development of this work, Pubmed and Google Scholar databases were used for the research of scientific articles. The keywords used for the research were, essentially: *multiple sclerosis*, *microbiome*, *gut microbiota*, *aetiology*, *epidemiology*, *modulation*, *faecal matter transplantation*, *autoimmune* and *neurodegenerative*.

To complement the research, other websites were visited such as *"The Atlas of MS"*, to further complete epidemiological information, and *clinicaltrials.gov*, to search clinical trials regarding the use of faecal matter transplantation in MS patients.

Initially, the research included more general information regarding MS, such as its history, epidemiology, aetiological factors and pathogenesis. Then, research was narrowed to the current therapies used in MS, as well as modulation of microbiome in MS as a therapeutic approach, including the search of pre-clinical and clinical trials.

3. Multiple Sclerosis

3.1. History

The first description suggestive of MS is of Saint Lidwina of Schiedam, in the late fourteenth century, in Holland. At the age of 16, on February 2nd 1396, she fell while ice-skating, representing the first signs of weakness in her legs (4,5). At the age of 19, Saint Lidwina of Schiedam had even more difficulties walking by herself and as years went by, there were progressively swallowing difficulties. She died on April 14th of 1433, suffering from different wounds, at the age of 53 (5).

About three hundred years later, an illegitimate grandson of King George III of England and cousin of Queen Victoria, Augustus d'Este, kept a diary for 26 years where he described the progressive course of symptoms resembling what we now consider to be MS. The first symptom described by Augustus was an episode of transient visual impairment, at the age of 28, in 1822, reporting difficulties in his vision (4,6,7). However, he later developed episodes of motor symptoms and weakness of the lower limbs as well as sensibility, which slowly began to compromise his ability to walk. He died at the age of 54 (4,7).

Robert Carswell, in 1838, described the disease as a "peculiar diseased state of the cord and pons Varolii, accompanied with atrophy of the discoloured portions. Around the same time, in France, Jean Cruveilhier was making similar observations in autopsies, but he provided a clinical description of a woman who had limb weakness associated with difficulties in swallowing. He attributed the lesions to the upper portion of the spinal cord (4). After the first macroscopic illustrations of the lesions by Robert Carswell in 1838 and Cruveil-heir in 1841, early microscopic investigations by Rindfleisch, in 1863, described the perivenous distribution of the lesions associated with inflammation and demyelination (8).

Although the first description of MS dates back to the 14th century, it was Jean-Martin Charcot who made the first correlations between the clinical features of MS and the pathological changes noted post-mortem. He was the first to come up with a concise disease concept. His illustrations of MS showed focal white matter lesions and their relation to blood vessels (9,10). He described clinical symptoms and signs of illness and came up with the term "sclerose en plaques" in 1868 (11,12). Jean-Martin Charcot himself hinted that MS could be an infectious disease caused by a microorganism, but while a lot of experts relied on that idea, others, claimed that an infection could aggravate the disease, but not primarily cause it. Much interest has focussed on a potential role of Epstein-Barr virus, specifically (4).

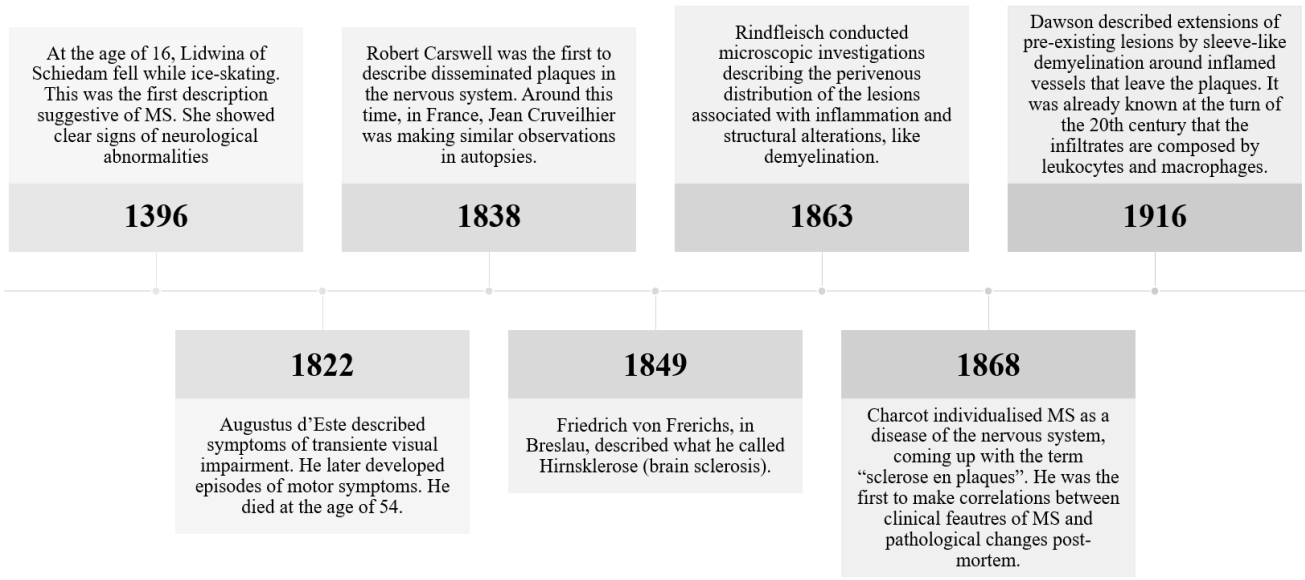

Figure 1 – Timeline of events regarding MS history. From (4–12).

3.2. Epidemiology

The cause of MS is multifactorial as both genetic and environmental factors contribute to disease risk. These factors have been associated with the heterogeneous prevalence of the disease worldwide and can lead us to a better understanding of MS and even have implications for therapeutic strategies (13).

Genetic factors support the excess occurrence in Northern Europeans relative to indigeneous populations from the same geographical location. Additionally, familial aggregation also supports the genetic effects of MS - MS is 20 to 40 times more common in first-degree relatives. On the other hand, environmental influence on MS aetiology suggests a variation in the disease's prevalence and incidence (14).

In a meta-analysis of nine reports of relapse incidence in the northern hemisphere regarding seasonal incidence of relapses and its epidemiology, an association between seasonal variation and a peak of relapses was demonstrated. The results showed that the lag between seasonal ultraviolet radiation trough levels in midwinter and subsequent peak in relapse probability was inversely associated with latitude. For example, in Melbourn (37,8º) the lag was of 5 months, whereas in Montreal (45,5º) the lag was only of 3 months, demonstrating that there was a lag of 3 months between the lowest level of UV radiation and the peak of relapse probability, for the highest latitude (15). One popular hypothesis that could partially explain the seasonal variability of relapses hints that there is an association between the seasonal incidence of relapses and the serum levels of 25-hydroxyvitamin D. An increase of 10 nmol/L in the concentration of vitamin D is connected to a reduced hazard of relapses (15).

Over the years, the gender ratio for MS has changed due to an increase in the incidence of the disease in women, being females twice as likely to live with MS as males (16). The increase of prevalence in women over time can also be suggested by their environment, secondary to a change in lifestyle (13).

A total of 2.8 million people are estimated to live with MS worldwide (35.9 per 100,000 population), as described in Figure 2. The global prevalence has been rising since 2013, according to the Atlas of MS, compiled by the Multiple Sclerosis International Federation (MSIF), with the mean age of diagnosis being 32 years. The third edition of the Atlas of MS collects epidemiological data from 115 countries, which is representative of 87% of the world's population, with Africa being the continent with only 56% of its population represented. These epidemiological differences could be from the lack of capacity of these countries to perform diagnosis and from different diagnosis criteria. The major findings of the epidemiology survey are as follows: the estimated number of people with MS in 2020 is 30% higher when compared to 2013; recognition of paediatric-onset MS has substantially increased with more than 30,000 cases of MS diagnosed in people under 18 years old reported by 47 countries, whereas, in 2013, 7,000 cases were reported by 34 countries (16,17).

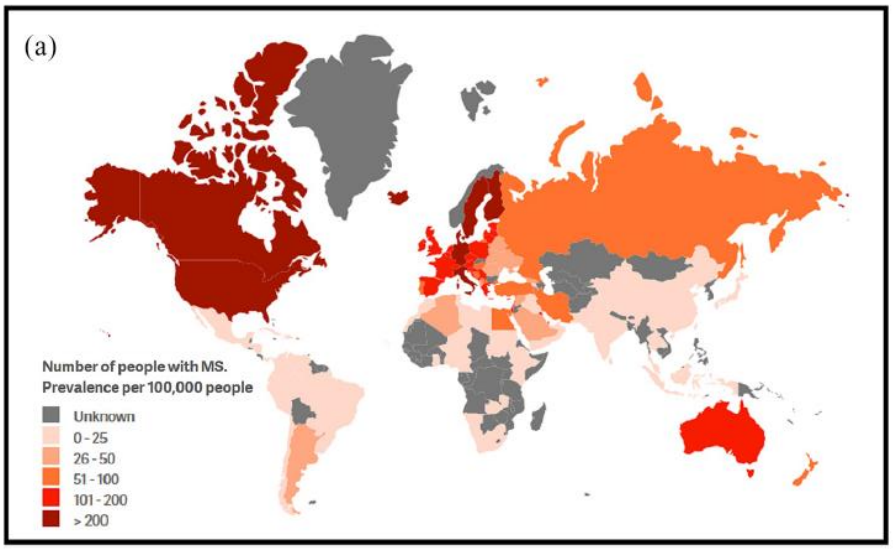

Figure 2 - MS prevalence population worldwide. From (17).

In Europe, mean rates are higher in northern countries, which can be associated with better accuracy in survey methodology - nationwide investigations and the use of registry systems. However, there is a certain extent of prevalence heterogeneity within countries, such as Sardinia (Italy), Scotland (UK) or southern Norway (18). In the Nordic region, the highest annual incidence rate was found in Sweden while the lowest one was in Iceland. As for the Iberian Peninsula, a Portuguese study used the capture-recapture method (CRM) to correct the age-specific prevalence of MS obtained from two data sources. In Portugal, prevalence of MS was around 40/100,000 at the time of the study, however, prevalence increased to 56,2/100,000 specifically (17,19–21). There are still some European regions where MS epidemiology is not

well documented. However, it is safe to say that an increasing prevalence is observed, most likely due to increasing survival and an increasing incidence. As far as prevalence goes, epidemiological studies suggest a decreasing north-to-south gradient across Europe and it tends to be lower in Portugal, Serbia, Kosovo, Albania, Turkey and Russia. Incidence is reported to be increasing over time, but mortality rates are generally decreasing, which increases survival time after onset up to 45 years. This leads to an increased burden of the disease because of the greater number of years of life with disability (YLDs) (18,19). The United States of America and Canada are classified as a high-risk area for MS, with prevalence rates around 288 and 250 per 100,000, respectively. However, regional variations in prevalence remain largely unexplained and still raises questions regarding genetic and environmental effects (22,23). As for Latin America, it has classically been considered a low MS prevalence region and local genetic and environmental factors could explain the low frequency (24). When it comes to MS epidemiology in Asia and Oceania, the mean prevalence and incidence in both continents were 37.89/100,000 and 2.4/100,000, respectively (25). On the other hand, the MS epidemiology is still unknown in a lot of African countries. The Middle East North Africa (MENA) region is classified in the low-to-moderate MS prevalence zone. Its prevalence rates are much lower than those in Southern Europe but much higher than sub-Saharan Africa. The MSIF recorded a 10% increase in global prevalence of MS from 30 to 33/100,000 from 2008 to 2013 (26).

4. Aetiology

Despite the fact that the aetiology of MS remains unclear, it is safe to say that the cause of MS is multifactorial and includes genetic predisposition combined with environmental factors, such as infectious agents, vitamin deficiencies and smoking (27).

MS genetic susceptibility has been associated to the human leukocyte antigen (HLA), in particular to the HLA-DRB1 locus, with the HLA-DR15 haplotype (DRB1*1501-DQA1*0102-DQB1*0602-DRB5*0101. In Northern Europeans, MS has been linked to haplotypes containing HLA-DRB1*1501, dominating MS risk in Caucasians, which can explain some epidemiological differences, as it will be discussed later (28,29).

Several studies have reported a strong association between HLA class II and MS susceptibility. The HLA-DRB1*17, in Sweden, was shown to be associated to MS susceptibility but to a lesser extent when compared to HLA-DRB1*15 in a 1.7 fold risk increase as compared to 3 (28,30). It has been suggested that interactions between different genes could contribute to an increase in susceptibility (31). As it was mentioned before, the gene HLA-DRB1 influences the risk of MS. It comes in over 400 different alleles and a common form in Europe, HLA-DRB1*1501, increases the risk by 3-fold. A coercive observation from a Canadian study suggests the existence of epistatic interactions between HLA-DRB1 haplotypes. On its own, HLA-DRB1*08 increases the risk of MS, but when present with HLA-DRB1*15 on the other parental haplotype, it doubles the risk associated with a single copy of HLA-DRB1*15 (28,32). It is clear that the effect of two parental haplotypes in combination,

also called the diplotype, can determine an individual's risk of MS in a phenomenon called epistasis. Epistasis of haplotypes play a very important role in determining the risk of MS from a genetic susceptibility standpoint (27,28,32). These interactions are summarised in the table below.

Table 1 – The effect of different alleles and their interactions.

Allele	Effect	Interactions
HLA-DRB1*14 HLA-DRB1*11	Protective	HLA-DRB1*14 significantly reduces the risk associated with HLA-DRB1*15 when present together. In Asia, the HLA-DRB1*14 allele is frequent, explaining, in part, why MS is rare there (32)
HLA-DRB1*10 HLA-DRB1*01		HLA-DRB1*01 and HLA-DRB1*10 have a protective effect against MS only in the presence of HLA-DRB1*15 in trans (32).
HLA-A*0201		-
HLA-A*0301	Increases risk of disease	HLA-A*0301 allele, located in the HLA class I region, was found to increase the risk of MS independently of the HLA-DRB1*15 haplotype (33).
HLA-DRB1*15		Higher risk of MS in individuals who carry both HLA-DR15 and HLA-A3, when compared to those who carry only HLA-DR15, only HLA-A3 or none of these alleles (33).

HLA-DRB1*15 is the major risk factor for MS and is regulated by epigenetic mechanisms such as DNA methylation and histone deacetylation. Moreover, major environmental risk factors, such as smoking, vitamin D deficiency and Epstein-Barr virus infection are known to exert epigenetic changes (34,35).

Epidemiological studies have demonstrated a connection between diet and incidence of MS, as there is evidence of the diet playing an important role in conditioning the inflammatory cascade, by acting on molecular pathways as well as the composition of gut microbiota. Additionally, research has shown the vitamin D deficiency can be related to relapses associated with a greater degree of disability (36,37).

Smoking is a critical environmental risk factor for MS, increasing the risk of the disease with approximately 50%. Cigarette smoke contains high concentrations of free radicals, which

have been implicated in oxidative injury to neural tissue. Exposure to it has shown to cause axonal degeneration or block axonal conduction, resulting in extensive cerebral demyelination. Apart from this, there is evidence of the influence of smoking on HLA-DRB1*1501, as smoking induces epigenetic alterations that can lead to reversible changes in gene expression (38).

There is evidence that MS can be triggered by microbial infections. One of the microorganisms that has been most strongly linked to MS is the Epstein-Barr virus (EBV). It infects 90% of the general population during the first decade of life, persisting a latent infection. Although the mechanism through EBV can develop MS is still unclear, in a cohort comprising more than 10 million young adults in the US military, people infected with EBV were 32 times more likely to develop MS than people who were EBV-seronegative. However, the risk was not increased after infection with other viruses, including the cytomegalovirus which is similarly transmitted (39,40).

5. Disease Stages

5.1. Progression of Multiple Sclerosis & Expanded Disability Status Scale

MS is a progressive disease characterised by a gradual and irreversible accumulation of neurological deficits (41). It consists of three phases: the high-risk phase, the relapsing-remitting phase and the progressive phase, and its heterogeneity in clinical, radiologic, biological and pathologic representations has been documented, making this disease with an interesting phenotypic variability (42). Progression of MS is identified retrospectively based on a history of gradual exacerbation of disability during an observation of months or years and clinical progression of the disease is related to the accumulation of neuro-axonal loss in a lifelong inflammatory CNS environment that follows a series of mechanisms. These combine compartmentalised persistent inflammation and an unbalance between damage, repair and functional reserve (41,43). The fundamental driver of clinical progression in MS is compartmentalised T and B-cell mediated inflammation. However, recent studies have suggested the importance of CNS tissue's response to lifelong inflammation injury as a critical player for pathological and clinical outcomes (43).

The Disability Status Scale (DSS) was elaborated in 1983 to assess physical disability in MS cases. It was later transformed into the Expanded Disability Status Scale (EDSS). Step increases in EDSS help to identify disability progression and must be confirmed after 3/6 months in order to distinguish true progression from reversible disability associated with a relapse or possible assessment errors. Despite including all functional systems that may be affected in MS and reflecting the clinical status as a number, which is an advantage, it still has a few limitations, such as low reliability, reliance on locomotor functions above 4.0, limited sensitivity to progression at higher scores and represents only a part of health-related quality

of life (HRQOL) (41,44,45). The EDSS functional systems consist of 8 categories - pyramidal, cerebellar, brain stem, sensory, bladder-bowel, visual (optical), cerebral (cognitive) and other functions. These are defined by categorising abnormal findings in neurological examination and one of its goals is to exclude non-MS related causes of disability. Additionally, functional systems were developed to assess the frequency, spread and severity of clinical involvement in cases of the disease based on neurological examination. According to the score of EDSS, "0" indicates a normal neurological examination with minimal disability, whereas "10" indicates death due to MS, as is represented in table 2 (44).

Table 2 – The Expanded Disability Status Scale.

1.0	No disability, minimal signs in one FS
1.5	No disability, minimal signs in more than one FS
2.0	Minimal disability in one FS
2.5	Mild disability in one FS or minimal disability in two FS
3.0	Moderate disability in one FS, or mild disability in three or four FS. No impairment to walking
3.5	Moderate disability in one FS and more than minimal disability in several others. No impairment to walking
4.0	Significant disability but self-sufficient and up and about some 12 hours a day. Able to walk without aid or rest for 500 m
4.5	Significant disability but up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance. Able to walk without aid or rest for 300 m
5.0	Disability severe enough to impair full daily activities and ability to work a full day without special provisions. Able to walk without aid or rest for 200 m
5.5	Disability severe enough to preclude full daily activities. Able to walk without aid or rest for 100 m
6.0	Requires a walking aid-cane, crutch, etc. –to walk about 100 m with or without resting
6.5	Requires two walking aids-pair of canes, crutches, etc. –to walk about 20 m without resting
7.0	Unable to walk beyond approximately 5 m even with aid. Essentially restricted to wheelchair; though wheels self in standard wheelchair and transfers alone. Up and about in wheelchair some 12 hours a day
7.5	Unable to take more than a few steps. Restricted to wheelchair and may need aid in transferring. Can wheel self but cannot carry on in standard wheelchair for a full day and may require a motorised wheelchair

8.0	Essentially restricted to bed or chair or pushed in wheelchair. May be out of bed itself much of the day. Retains many self-care functions. Generally, has effective use of arms
8.5	Essentially restricted to bed much of day. Has some effective use of arms retains some self-care functions
9.0	Confined to bed. Can still communicate and eat
9.5	Confined to bed and totally dependent. Unable to communicate effectively or eat/swallow
10.0	Death due to MS

5.2. Types of Disease

MS course varies from relapsing to remitting, where patients have periods of remission, to progressive forms. There are four clinical forms of the disease: primary-progressive MS (PPMS), secondary progressive (SPMS), relapsing-remitting MS (RRMS) and progressive relapsing (PRMS). All of them are characterised by periods of active disease interspersed with inactive periods (46). PPMS is characterised by disease progression from onset with occasional plateaus and temporary minor improvements. About 10-15% of patients with MS have a gradually increasing neurological disability, which is compatible with the definition of PPMS. People with PPMS are older at onset of the disease when compared with relapse-onset MS and a small group of patients develop PPMS with steady progression without periods of remission (46,47). On the other hand, SPMS is characterised by a steady accumulation of fixed disability after an initial relapsing remitting course. It is based on a history of gradual worsening and, although it is not clearly understood and still hard to define this phase of disease, conversion to SPMS is described by irreversible disability progression that is independent of a relapse, even though patients can still experience them (48,49). Interestingly, RRMS is the most common form of MS, which is characterised by worsening of clinical symptoms followed by periods of partial or complete recovery. It ultimately evolves into a progressive disease in 80% of patients - SPMS - with worsening and steady progression of symptoms. MS is more frequently defined by an initial relapsing-remitting phase that is followed by evolution to SPMS(41,46). Finally, PRMS is the least common form of the disease and is expressed by a progressive neurological dysfunction from onset. It was also characterised by heavy distinct acute relapses and, between relapses, there is a continuous disease progression. Consequently, patients with this form of MS accumulate disability from incomplete recovery of these acute relapses and exacerbations, in addition to gradual deterioration (50). More recently, the International Advisory Committee on Clinical Trials of MS conducted a re-examination of MS disease phenotypes, based on disease activity. With that being said, the category of PRMS was suggested to be eliminated, since these patients would be classified as PPMS patients, since they present both disease activity and disease progression (51).

6. Pathogenesis of MS

6.1. The immune-mediated inflammatory response

MS is described by the presence of inflammatory demyelinating lesions in the central nervous system. Chronic neuroinflammation and neurodegeneration lead to myelin damage that leads to blocking of nerve impulse conduction, resulting in neurological deficit giving rise to a variety of clinical symptoms. Inflammatory infiltrates contain mostly T-cells with a dominance of MHC class I restricted CD8+ cells. Plasma cells and B-cells are also present, but in much lower numbers, when compared to T-cells. There is also a deep expression of MHC molecules: class I MHC molecules are present on inflammatory cells, glial cells and neurons, whereas class II MHC molecules are present on microglial and macrophages (52–54).

Although inflammation is present in all MS stages, as long as the disease is active, it is more noticeable in acute or relapsing MS when compared to the progressive stage. However, throughout the years, it has been shown that active lesions are present not only in white matter, but also in grey matter and cortical regions. These cortical lesions suggest a perivascular inflammation as well as dispersion of inflammatory cells into the cortical parenchyma. As far as disease stages go, in an early stage, inflammation is associated with blood brain barrier leakage, hinting that an infiltration of inflammatory cells from blood circulation to the brain occurs, whereas with disease chronicity, the inflammatory process becomes trapped within the CNS compartment. Besides this, it is important to mention that tissue injury mechanisms can differ within different subsets of patients, involving macrophages, antibodies and T-cells, as well as different susceptibilities by the target tissue and another important mechanism of tissue injury is oxidative stress initiated by activated macrophages and microglia. This leads to mitochondrial injury which is prominent in active MS lesions (52).

Because MS is an inflammatory demyelinating disease, there is a strong immune-mediated inflammatory response associated, as it was mentioned before. Besides inflammatory infiltrates containing T-cells, B-cells, plasma cells, activated microglia and/or macrophages, the inflammatory process is also linked to expression of adhesion molecules, chemokines, cytokines, which, again, suggest a T-cell mediated inflammatory process that drives disease and tissue injury. Additionally, HLA-related genetic predisposition associated with microbial infection with molecular mimicry towards myelin structures leads to cross-reactive immune responses, which, consequently, leads to autoimmunity to myelin structures(52–54). As activated T-cells enter the CNS through the blood brain barrier, clonal expansion leads to T-cell reactivation by myelin antigens, release of mediators, recruitment of other immune cells from the bloodstream, that, subsequently, cause the release of proteases, glutamate and free radicals, responsible for destruction of myelin and axonal damage. The T-cells mediate an inflammatory response that is conducted by an autoimmune process, characterised by a Th1-type bias in MS, such as interferon-gamma, IL-12, IL-18, osteopontin, proinflammatory cytokines, whereas factors associated with Th2-type or Th3-type responses were shown to be beneficial in MS (54–56).

6.2. Adaptive immune response

The adaptive immune system comprises the immune cells and its mechanisms that respond to infections or tumours in an antigen-specific manner, such as B-cells and T-cells with antigen-specific receptors on their surfaces (57). T helper type 1 (Th1) cells are the major T-cells associated with MS secreting interferon-gamma, but Th17 CD4+ T-cells have also been linked to the disease (58). Interferon-gamma is present in MS lesions, as well as macrophages, the main responding cells in Th1-mediated immunity (57).

Cellular immunity

CD4+ and CD8+ T-cells have been isolated from MS lesions and, in the CNS, they were derived from clonal expansion, suggesting that there is an antigen-specific T-cell response that contributes to the disease process and, even though these T-cells can have autoantigens as a target, they could possibly be long lived memory T-cells against various neurotropic viruses that reside in the CNS and expand because of specific cytokines. However, autoreactive T-cell activity in the peripheral immune compartment is associated with a possible pathogenic event in this disease. The inflammatory autoimmune pathogenesis of the disease starts with activation of CNS antigen-specific CD4+ T-cells in the periphery. The activation of antigen specific T-cells and their differentiation in T-helper cells can be triggered in lymphoid tissue associated with the human gut or bronchial system. The microbiome, especially gut microbiome, could possibly provide antigenic and adjuvant signals to the differentiation of T-cells. The identification of these anatomical areas as places where autoreactive T-cells are primed could lead to new opportunities to investigate the role of environmental factors that influence the human microbiome, in the immune response in MS (56,57).

Humoral immunity

In most MS patients, B-cells, plasma cells and plasmablasts are present in lesions, meninges and the cerebrospinal fluid (CSF) and these B-cells and plasma cells present in the CNS are supported by cytokines and survival factors produced by glial cells. Moreover, the humoral immune response is most likely related to the presence of IgG, mainly consisting of IgG1 and IgG3 isotypes that, when bound to their target epitopes, activate the complement cascade and identify cells for phagocytosis. Gene sequencing and proteomic analyses suggest that part of the IgGs present in the CSF are secreted by clonotypic B-cells present in both CSF and blood (57). Furthermore, studies on B-cells as well as plasma cells present in the meninges and CSF have shown an association between the presence of these cells, disease activity and clinical outcome. The first evidence that the disease is immune mediated emerged in 1942, as patients showed oligoclonal immunoglobulin (Ig) production in the CSF. Although the role of B-cells is still up to debate, rather than production of pathogenic antibodies, these cells are also drivers of inflammatory activity, as they have regulatory functions and participate in antigen presentation. In MS particularly, B-cells are observed within the CNS, in the perivascular infiltrates and meninges. Furthermore, ectopic B-cell follicles are suggested to be connected to infection by EBV, as a substantial fraction of the infiltrating B-cells was found to be infected with the virus and their presence is also associated with grey matter lesions (56–58).

6.3. Innate immune response

Dendritic cells

Dendritic cells are important in promoting pro-inflammatory T-cell responses, by presenting antigens to T-cells, in MS and can also activate NK cell-mediated cytotoxicity. In MS patients, dendritic cells have an activated phenotype with an increased expression of activation markers, as well as a profound secretion of pro-inflammatory cytokines (57–59).

Macrophages/microglial cells

Many cell types are involved in innate immune reactions in the nervous system, such as astrocytes, dendritic cells, mast cells and natural killer cells, but macrophages and microglia are the most prominent innate cells that contribute to pathological alterations in MS and are located in MS lesions in both relapsing-remitting and progressive phases of the disease. Some studies in animal models of experimental autoimmune encephalomyelitis (EAE) have shown that accumulation of phagocytes induces axonal degeneration. Additionally, blocking the activation of these cells, as well as the release of reactive oxygen species, from oxidative stress, and nitrogen species restricts axonal damage. However, phagocytes are also linked to the tissue repair mechanism during lesion resolution. Phagocytes can have different origins: monocyte-derived phagocytes are derived from peripheral monocytes and seem to trigger demyelination, whereas microglia-derived phagocytes promote tissue recovery (57,58). Microglial cells comprise about 10-20% of glial cells, are the most common immune cells in the CNS and their activation contributes to MS and EAE by antigen presentation and secretion of pro-inflammatory cytokines. Persistent activation of these cells has been observed in progressive MS and in association with inflammation of white matter (59).

Natural killer cells

Natural killer cells play a role in effector and regulatory functions of the disease via their cytotoxic activity, mainly against viral infected cells or tumour cells, although its importance and mechanisms remain unclear. However, *in vitro*, NK cells show cytotoxic activity towards oligodendrocytes and other glial cells, like microglial cells and astrocytes. NK can also play a role in CNS repair and protection, as these cells produce neurotrophic factors like brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), as reported in mice with EAE, but a detrimental role in MS is suggested by their presence in MS lesions (58–60).

Mast cells

These cells are present in the brain parenchyma and at the blood-brain barrier and can interact with myelin. Besides this, *in vitro*, myelin proteins can stimulate degranulation of mast cells and release of proteases that can then lead to myelin basic protein degradation. Additionally, histopathological analysis has shown an accumulation of mast cells in MS plaques. The role of mast cells in MS has made these cells a potential target for therapeutic designing - the use of hydroxyzine, an anti-histaminic drug, or drugs that can block mast cell activation by myelin basic protein, such as flavonoid luteolin, could be beneficial in MS (59).

Gamma-delta T-cells

These cells are a subset of lymphocytes that recognize non-MHC restricted antigens through invariant gamma-delta T-cell receptors. They are present particularly in the intestinal epithelium rather than in peripheral blood. Although their role is still not completely understood, in MS patients, studies have reported an increase in these cells in the CSF, which was associated with high MRI disease activity. In conclusion, these cells are increased in MS patients with active or progressive disease, as they are present in MS lesions, and can contribute to MS pathology by exerting a cytotoxic effect on oligodendrocytes (59).

6.4. Astrogliosis

In MS, different stages of the disease involve different plaque types and, because of this, there are different stages of demyelinating activity. One of the few mechanisms through which these plaques are formed is astrogliosis, for example. Astrogliosis is observed in chronic inactive plaques, the most abundant in the MS brain that are sharply demarcated and show reactive gliosis, as well as partial axonal preservation. As lesions progress from acute active to chronic inactive, astrocytes produce glial fibres and a glial scar ends up filling the demyelinated plaque - this is known as astrocytic fibrillary gliosis (61,62). Therefore, in early active lesions, astrocyte hypertrophy is observed, as well as an increased expression of glial fibrillary acid protein (GFAP) (58).

In gliosis, it is possible to observe enlarged nuclei and the chromatin becomes less dense. In contrast, the nucleoli become more prominent and there is a higher production of GFAP, nestin and vimentin, resulting in more highly condensed glial processes and fibres. These replace injured CNS cells to form a gliotic scar. In addition, gliosis also results in the release of substances such as cytokines, growth factors and extracellular matrix proteins (63).

7. Pharmacological Therapies for Multiple Sclerosis

Therapies for MS include therapies for MS attacks, or relapses, and treatments to modify progression, the also known disease modifying therapies (DMTs). As for the treatment of relapses, the use of anti-inflammatory treatments such as corticosteroids (CS), adrenocorticotropic hormone (ACTH) and plasma exchange are approaches supported by the current evidence. The main goal for these treatments is to promote immunosuppression, accelerate recovery from the area of inflammatory demyelination and to mitigate the severity of the relapse (64,65).

7.1. Pharmacological strategies for MS relapses

Corticosteroids are the main treatment modality for MS relapses and can be administered orally or through IV. Both forms of administration are equally effective, although oral corticosteroids remain more convenient to the patient. They have anti-inflammatory and immunosuppressive properties and their effect on the immune system is thought to be dose and duration-dependent. When used to treat MS relapses, high-dose, short-term IV CS therapy provides relief of symptoms, as well as improves motor function and shortens the recovery phase of acute attacks (64,65). Currently, in order to induce this fast recovery from exacerbations, treatment with high-dose methylprednisolone is recommended (66). ACTH gel is an alternative for patients that don't respond or don't tolerate corticosteroids and is used less frequently that methylprednisolone as a treatment for MS relapses due to the unpredictable rise in serum concentrations of cortisol, since ACTH is an agonist in the melanocortin system and stimulates the production of cortisol in the adrenal cortex. It is relevant to MS relapse treatment because of its anti-inflammatory and immunomodulatory functions involving lymphocytes and macrophages, as well as reduction of proinflammatory cytokines (64,66). Plasma exchange is a second-line treatment option. One study showed that it led to a significant improvement of some patients who remained impaired after relapses treated with high-dose corticosteroids (64).

7.2. Pharmacological approaches for disease progression

Although DMTs have received a lot of attention when it comes to their role in the treatment of MS progression, the management of symptoms is also fundamental for the well-being of patients, since they play a role in their quality of life. The treatment of these symptoms is mainly pharmacological and are used to treat mobility related symptoms, bladder, bowel and sexual dysfunction, as well as fatigue, cognitive impairment and mood disturbance, for example (67). DMTs reduce but don't eliminate MS relapses and MRI activity. According to the Portuguese guidelines for the treatment of MS, published by the Direção-Geral de Saúde, updated in 2015, this therapeutic approach should be considered for patients with clinically isolated syndrome (CIS), RRMS, SPMS and PRMS (68,69). DMTs are summarised in the Table 3, according to the American, European and Portuguese guidelines for the treatment of MS.

Table 3 - DMTs for the treatment of disease progression in MS.

Disease stage	Pharmacological therapy	Administration	Important observations
RRMS	**First line**		
	Interferon-beta (1a/1b)	IM	For INF-beta, teriflunomide and dimethyl fumarate, liver function should be monitored (69).
	Glatiramer acetate	SC	
	Teriflunomide	Oral	
	Dimethyl fumarate	Oral	
	Second line		
	Natalizumab	IV	Patients under natalizumab when positive for John Cunningham virus (JCV) infection have higher risk of developing progressive multifocal leukoencephalopathy (PML) (68,69).
	Fingolimod	Oral	Patients under fingolimod and alemtuzumab should be monitored for Varicella-Zoster virus infection. In the first administration of fingolimod, patient should be monitored cardiotoxicity (69).
	Alemtuzumab	IV	
RRMS with rapid evolution	Alemtuzumab	IV	Azathioprine and cladribine may be recommended for people with relapsing forms of MS that don't have access to approved DMTs (68).
	Azathioprine	Oral	
	Cladribine	Oral	
PRMS	Ocrelizumab	IV	Only drug proven to alter disease progression in patients with PPMS (68).
	Mitoxantrone	IV	Mitoxantrone has shown high risk of cardiomyopathy, ovarian failure, male infertility, chromosomal aberrations and promyelocytic leukaemia. To be used only when the therapeutic benefits greatly outweigh the risks (68–71).
SPMS	Interferon-beta (1a/1b)	IM	Patients with SPMS who experience relapses and have MRI-active lesions benefit from DMTs (70,71).
	Mitoxantrone	IV	

8. Importance of the microbiome in Neurodegenerative Diseases

8.1. The role of the microbiome in neurodegenerative diseases

Environmental risk factors experienced during an individual's life impact the onset, severity and, subsequently, progression of neurodegenerative diseases. These diseases have, throughout the years, been linked to changes in diet and the gut microbiome. The microbiome is very related to the development and ability to recover in some diseases. Therefore, it has become a matter of much interest amongst the scientific community, not only because its alteration has proven to have influence on the progression of the disease, but also because this suggests that gut microbiome manipulation could potentially be an innovative therapeutic for these diseases (72).

As summarised in Figure 3, research on the human gut microbiome involves collection of faecal samples, metagenomic DNA extraction, massive DNA sequencing and bioinformatics data analysis. This assessment allows the identification of altered microbiomes that are associated with diseases and may play an important role in the personalised treatment (73).

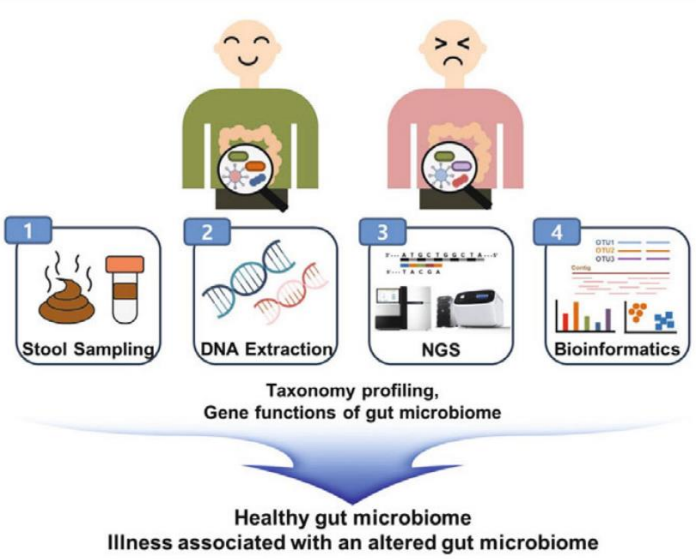

Figure 3 - Steps involving human gut microbiome assessment. From (73).

Microbiome evolution

The composition of the intestinal microbiome is settled in the first years of life, stabilised by the age of three and it can be shaped according to many factors, such as genetic background, diet (breastfeeding or not), stress, medication, especially antibiotics, gastrointestinal infections and maternal vertical transmission. The form of delivery has a serious impact on the microbiome of infants, since the birth is the first moment where large-scale colonisation of bacteria happens. As the infant passes through the vaginal canal, it is

exposed for the first time to the maternal vaginal microbiota. On the other hand, in case of a C-section, the newborn doesn't contact the vaginal canal and therefore doesn't contact the vaginal microbiota. This procedure is linked to a decreased colonisation rate of *Bifidobacterium*, *Bacteroides*, and *Lactobacillus*. Recent studies have shown that the mode of delivery can affect the microbiome of infants (74–76).

The gut microbiome is a diverse community of species constituted by more than 30 bacterial phyla, seven of which account for the vast majority of species. *Firmicutes* consist of the majority of microbiota, corresponding to approximately 51% of the total microbiome, including the *Clostridium coccoides* and *Clostridium leptum* groups and the *Lactobacillus* genera. *Bacteroidetes* are the second most abundant phyla, constituting about 48% of total microbiota, including the genera *Bacteroides* and *Prevotella*. In the other 1% there are other less populous phyla, such as *Actinobacteria*, including the genera *Bifidobacteria* genera, *Cyanobacteria*, *Fusobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Spirochaetes* and *Lentisphaerae*, as represented in Figure 4. There are at least 1000 species identified and more than 7000 strains of bacteria that compose the 10^{13} - 10^{14} microorganisms of the microbiome (76,77).

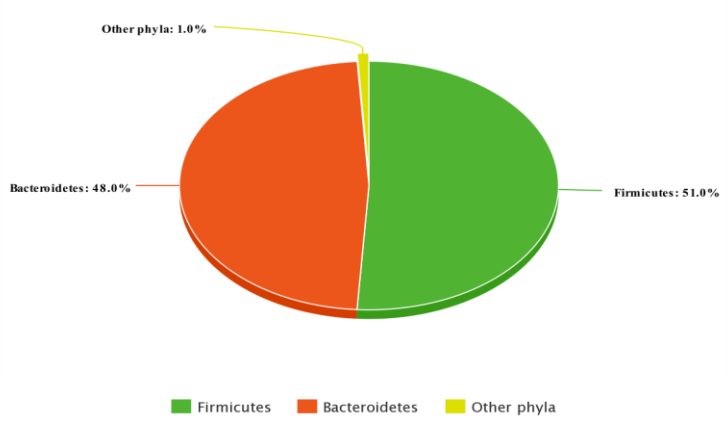

Figure 4 - Representation of the different phyla that compose the human gut microbiome. From (76,77).

The microbiome and ageing

There is a particular interest in the relation between ageing and alterations of the microbiome. Ageing is characterised by chronic inflammation, also known as "inflamm-ageing", increased intestinal permeability, disrupted nutrient absorption and impaired digestion. For example, a study showed that in old mice, impaired proliferation of B cells in the Peyer's patches was corrected through faecal microbiota transplantation from young mice, demonstrating that ageing is strongly associated with alterations in the gut microbiome. Besides this, the diversity and stability of intestinal microorganisms decreases progressively with age. Although some phylum remain dominant, such as *Firmicutes* and *Bacteroidetes*, the proportions change, as some increase and other decrease, creating an imbalance in the microbiome, as it will be discussed further (72,77,78).

Two hallmarks of immune ageing are immunosenescence and inflamm-ageing, as mentioned before. Immunosenescence refers to abnormal, impaired immune responses in the elderly and is also characterised by ageing of primary lymphoid organs, such as bone marrow and thymus, chronic antigenic overload, inflammation and gut dysbiosis. Dysbiosis is the term that refers to an imbalanced microbial community structure and the progression of ageing leads to a gradual weakening of the balance between pro- and anti-inflammatory responses. Subsequently, this impaired balance is called inflamm-ageing and it is a significant risk factor for mortality and morbidity. Inflamm-ageing increases the tendency for chronic diseases, such as neurodegenerative diseases (79–81).

Immunosenescence is promoted by chronic exposure to stressors and an important characteristic of inflamm-ageing is dysregulated immunity. This is reflected in the increased local and systemic inflammatory mediators, like IL-6, TNF-α, IL-1β and C-reactive protein. In the senescent gut, a decreased microbial diversity and increased pathobiont overgrowth is observed, as well as a decrease in tight junctions and increased gut permeability, which leads to a leakage of bacteria, bacterial products, such as LPS, toxins, DNA, mucus, 5-HT and histamine. Consequently, and in addition to the activated dendritic cells, macrophages and lymphoid cells, increased levels of pro-inflammatory cytokines are observed. Furthermore, this triggers a dysbiotic gut-brain communication and signalling. This communication system is called the "gut-brain axis" and it works through neural, immune and hormonal mediators, as it will be discussed below (79–81).

The gut-brain axis

The gut-brain axis is a two-way communication system that allows communication between the gut microbiome and the brain and between the brain and the intestine. This notion supports the idea that these microorganisms that exist in the human gut have the potential to influence the CNS by modulation of several functions. These include neuroimmune function, neuronal signalling and metabolic activity (72,74,75,77,78,82). The main players of the gut-brain axis consist of intestinal microbiota, enteric nervous system (ENS), parasympathetic and sympathetic nervous systems, CNS, neuroendocrine connections, humoral pathways, cytokines, neuropeptides and other signalling molecules (74,77).

When it comes to the gut-brain axis pathways, these can be divided into two main groups: neural pathways and humoral pathways. As summarised in Figure 5, the neural pathways include the ENS and CNS through the vagus nerve and/or spinal afferents, whereas humoral pathways include cytokines, hormones/neuropeptides and microbial active substances, such as bacterial metabolites (74). As for the neural pathway, neurologic modulation of afferent nerves directly produces molecules that can act as local neurotransmitters, such as serotonin, melatonin, histamine, GABA and acetylcholine. When it comes to the humoral pathway, it includes endocrine, metabolic and immune pathways. The endocrine pathway affects the gut-brain axis due to the fact that gut microbiome alters nutrient availability, influencing the release of biologically active peptides from enteroendocrine cells. For instance, galanin, a neuropeptide, is thought to be connected to a few neurobiological functions (e.g. sleep/wake cycle regulation, mood, blood pressure regulation) but also

stimulates the activity of the hypothalamic-pituitary-adrenal axis (HPA), enhancing the glucocorticoid secretion from the adrenal cortex (83).

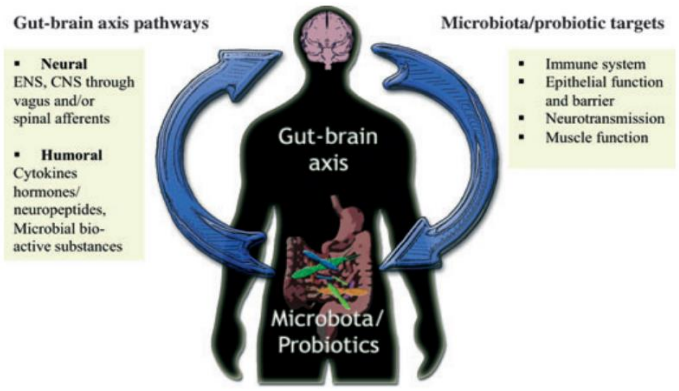

Figure 5 - The different pathways of the gut-brain axis. From (74).

On the other hand, the metabolic pathways include two fundamental bacterial metabolites, the short-chain fatty acids (SCFAs), such as acetate and butyrate, that are produced by bacterial fermentation of carbohydrates from the diet, and lipopolysaccharide (LPS), derived from the cell walls of Gram-negative enterobacteria. These metabolites are important humoral influencers, as they are known to have immunomodulatory and hormone-like activities and they also interact with nerve cells by stimulating the sympathetic branch of the autonomic nervous system (ANS), as summarised in Table 4 (83). SCAFs are the main metabolite produced by bacteria and are often reduced in a dysbiotic state which leads to an inflammatory environment (84).

Table 4 - Most important bacterial metabolites involved in the gut-brain axis and their functions in the metabolic pathways.

Metabolite	Function in metabolic pathway
SCFAs	Immunomodulatory activity; Hormone-like activity: interact with nerve cells, stimulating the sympathetic branch of the autonomic nervous system (ANS); Cross the BBB and regulate microglia homeostasis; Regulation of the release of gut peptides from enteroendocrine cells; Regulation of gut-derived serotonin from enterochromaffin cells; Regulation of the brain through G protein coupled receptors (78,83).
LPS	Enter the systemic circulation due to loss of epithelial tight junctions, showing high levels in patients with major depression (83).

The immune pathway is characterised by the release of cytokines, like IL-10 and IL-4 during times of dysbiosis in the intestine, and they can regulate the brain by activating the HPA axis and releasing cortisol (77,83). The gut-brain axis allows the communication between the gut microbiome and the CNS, which suggests that changes in the microbiome can deeply affect several functions. This is not only connected to diseases of the gastrointestinal tract, but also

neurodegenerative diseases, and others such as depression and anxiety. For example, about 95% of total serotonin present in plasma is provided by the gut (83).

It is known already that gut dysbiosis manifests in neurological disease as the microbiome plays a fundamental role for instance in microglial activity, BBB integrity and neurotransmitter production (77). Thus, the gut microbiome has several functions: it constitutes the intestinal barrier, produces mucus and sustains the mucosa, promotes the existence of more microbiota and stimulates epithelial cell regeneration, proving its effect on the brain not just through the nervous system but through other pathways, like endocrine, immune and metabolic pathways, as discussed previously (85).

8.2. Characterization of the Intestinal Microbiome in Multiple Sclerosis

The gut-associated lymphoid tissue (GALT) is the biggest immune reservoir and contains about 80% of the immune compartment. Mice raised in germ-free conditions have shown serious defects in their gut-associated and systemic lymphoid tissues, with hypoplasic Peyer's patches and reduced number of plasma cells producing IgA and CD4+ T cells. Microbiome MS studies have been mostly case-control examinations of patients versus healthy subjects. A systematic review by Mielcarz *et al.* analysed several human studies, which are summarised in Table 5 below (86).

Table 5 - Summary of studies regarding differences in the microbiome between MS patients and healthy individuals.

Study type	Subject	Conclusions
Case-control	7 RRMS patients	Different levels of *Firmicutes*, *Bacteroidetes* and *Proteobacteria*; Glatiramer acetate treatment alters *Firmicutes*; Vitamin D treatment increases *Enterobacteria* in both healthy subjects and patients, but patients show different levels of *Firmicutes*, *Actinobacteria* and *Proteobacteria* (86).
Case-control	53 patients (22 naïve, 13 glatiramer acetate and 18 INF-beta) versus 44 healthy subjects	Increase in inflammation-associated; *Methanobrevibacter smithii*; Decreased levels of *Firmicutes* and *Butyricimonas*, a butyrate producer that has anti-inflammatory (86).
Case-control	26 RRMS and 4 SPMS patients	Patients with an antibody against *Clostridium perfringens* epsilon toxin, in serum and CSF (86).

Other reviews of clinical trials have analysed the differences in MS patients' microbiome, as shown in Table 6. These studies have shown that MS patients have increased

levels of *Pedobacteria*, *Flavobacterium*, *Pseudomonas*, *Mycoplana*, *Dorea*, *Blautia*, *Streptococcus* and *Akkermansia*. On the other hand, MS patients appear to have lower levels of the following microbial populations: *Prevotella*, *Bacteroides*, *Parabacteroides*, *Haemophilus*, *Adlercreutzia*, *Coprobacillus*, *Lactobacillus* and *Clostridium*. The human gut microbiome is characterised by a lower relative abundance of regulating T cell-inducing bacteria, a higher activation of peripheral Th1 and Th17 cells, reduced production of SCFAs, such as butyrate, that maintain the integrity of the BBB, reduced levels of lipid 654 (87,88). Two systematic reviews of several clinical trials have been conducted by Schepici *et al*. and Mirza *et al.* and their conclusions are summarised in the table below.

In a study between RRMS patients and healthy subjects, the differences in microbiome composition were analysed. Among the *Actinobacteria* phylum, the genera *Adlercreutzia* and *Collinsella* were decreased in RRMS patients, when compared to healthy individuals. As for *Bacteroidetes*, such as *Pedobacter* and *Flavobacterium* genera, these had a higher abundance, while *Parabacteroides* had lower abundance in patients. *Blautia* and *Dorea* genera, belonging to the *Firmicutes* phylum, were increased, whereas *Lactobacillus* and *Coprobacillus* were decreased in RRMS patients. Regarding the *Proteobacteria* phylum, *Pseudomonas* and *Mycoplana* had higher abundance in patients, while *Haemophilus* was more abundant in controls. In general, healthy subjects presented higher levels of *Adlercreutzia*, *Collinsella*, *Lactobacillus* and *Parabacteroides*, while RRMS patients presented higher levels of *Pseudomonas*, *Pedobacter*, *Blautia*, *Dorea* and *Mycoplana* (89).

In another study investigating the relative abundances of the microbiome between MS patients (treated and untreated) and healthy individuals, the relative abundance of *Methanobrevibacter*, a genus of the *Euryarchaeota* phylum, and *Akkermansia*, a genus of the *Verrucomicrobia* phylum were increased in MS patients compared with healthy subjects. Additionally, *Butyricimonas*, of the *Bacteroidetes* phylum, had a decreased relative abundance. For untreated patients, the results were similar. *Collinsella* and *Slackia*, both from the *Actinobacteria* phylum, and *Prevotella*, from the *Bacteroidetes* phylum, were decreased in untreated patients. The separation of treated and untreated patients is important in this analysis because immunomodulatory therapy may alter the composition of the microbiome (90).

Table 6 - Microbiome alterations in MS patients observed in clinical trials.

Subject	Results	Conclusions
RRMS patients versus healthy subjects	↑*Firmicutes* and ↓*Bacteroidetes* in the relapse phase, vs. healthy subjects and patients in the remitting phase; ↓ *Prevotella* and ↑ *Streptococcus mitis* and *Streptococcus oralis*.	*Prevotella* produces propionate, an anti-inflammatory metabolite, being associated with the expansion of Th17 cells and disease activity, and so is *S. mitis*. This study concluded that the microbiome could regulate disease activity through the expansion of Th17 at the intestinal level (87,88).
RRMS patients versus healthy subjects	↓ genus *Clostridium*.	Decreased levels of *Clostridium* is directly linked to reduced production of SCFAs, and alteration of regulatory T cells and anti-inflammatory IL-10 (87,88).
MS patients versus healthy subjects	↑ *Firmicutes* (*Blautia* and *Dorea* genera) and *Bacteroidetes* (*Pedobacteria* and *Flavobacterium*); ↓ *Bacteroidetes* genera, like *Parabacteroides, Bacteroides* and *Prevotella*; ↓ *Adlercreutzia*.	*Bacteroidetes* commensals produce Lipid 654, a toll-like receptor ligand (TLR-2) involved in the regulation of immune responses, that is significantly reduced in serum of MS patients. Reduction of *Adlercreutzia* leads to an increased oxidative stress and inflammatory cytokines, contributing to progression of disease (87,88).
RRMS patients versus healthy subjects	↓*Parabacteroides distasonis*.	*Parabacteroides distasonis* may have a protective role in RRMS (87,88).
MS patients versus healthy subjects	↑*Methanobrevibacter* (*Euryarchaeota* phylum) and *Akkermansia* (*Verrucomicrobia* phylum) and ↓*Butyricimonas*	*Methanobrevibacter* is associated with inflammatory processes because of its ability to recruit dendritic and inflammatory cells; *Akkermansia* have pro-inflammatory activity, involved in the presentation of antigen; *Butyricimonas* produces butyrate, a SCFA that induces regulatory T cells, which can promote inflammation as in numerous autoimmune (87,88).

Lastly, the repair of microbial population levels in patients with RRMS reduces inflammatory events and the reactivation of the immune system (87). A summary of the phyla, genera and their differences amongst MS patients and healthy controls is described in Table 7 below.

Table 7 - Microbial population differences between MS patients and healthy controls, by phyla and genera.

Phylum	Genus	Microbial population levels of MS patients versus healthy subjects (86–90)
Firmicutes	*Dorea* *Blautia* *Streptococcus* *Coprobacillus* *Lactobacillus* *Clostridium* *Faecalibacterium*	↑ ↑ ↑ ↓ ↓ ↓ ↓
Bacteroidetes	*Pedobacteria* *Flavobacterium* *Prevotella* *Bacteroides* *Parabacteroides*	↑ ↑ ↓ ↓ ↓
Proteobacteria	*Pseudomonas* *Mycoplana* *Haemophilus*	↑ ↑ ↓
Actinobacteria	*Adlercreutzia* *Collinsella*	↓ ↓
Verrucomicrobia	*Akkermansia*	↑
Euryarchaeota	*Methanobrevibacter*	↑

The characterisation of the human microbiome is not only important to understand the development of MS, but also to understand the role of microbial populations in disease exacerbation. RRMS patients with active disease have shown differences in microbiome when compared to other patients in remission, which points towards the possibility that the human microbiome could play different roles when it comes to disease onset and disease exacerbation (89).

9. Modulation of the gut microbiome as a therapy for Multiple Sclerosis

9.1. Therapies for modulation of the gut microbiome

Modulation therapies for the gut microbiome include probiotics, prebiotics, synbiotics, antibiotics and faecal microbiota transplantation (FMT). Antibiotic treatment has been reported to change the course of disease of a few neurological disorders, whereas probiotics could potentially improve disease symptoms, although results are inconsistent. In fact, the most effective modulation therapy for gut microbiome is FMT, which will be described in more detail further (91,92).

Probiotics are living microorganisms that, when administered in adequate amounts, promote gut health and potentially modulate dysbiosis. Their beneficial effects depend on their metabolism and metabolic products that activate immune responses. They increase the integrity of the epithelial barrier, inhibit the adhesion of pathogenic agents to the intestinal mucosa, as well as eradicate pathogens by producing antimicrobial substances. For example, strains of *Lactobacillus* have shown to reduce IL-6 and IL-7 levels, which are pro-inflammatory cytokines, to control levels of *Enterobacteriaceae* pathobionts and restore the balance between regulating T cells and Th17 cells (93,94). A study of 54 MS patients receiving a probiotic capsule daily for 12 weeks containing *L. acidophilus, L.casei, B. bifidum* and *L. fermentum* revealed that subjects had favourable effects on EDSS, parameters of mental health, inflammatory factors and markers of insulin resistance (95).

Prebiotics are non-microbial and non-digestible dietary compounds that help probiotics grow, stimulating their activity. They include soluble/insoluble fibres, resistant starch, pectin and milk oligosaccharides. Dietary polyphenols, which are active substances found in colourful fruits, tea and cocoa, also have prebiotic properties and have shown to confer protection against animal models of autoimmune disease. For example, a study showed that polyphenols extracted from Jatoba, a South American herb, is favourable to EAE through the suppression of Th1 cells. This is suggestive that prebiotics could also have beneficial effects to patients with MS, since there is strong evidence that prebiotics can modulate gut microbiota, although in humans this is not studied enough yet (93,94).

FMT is another option in modulation of the gut microbiota, where a solution of faecal matter from a donor is administered into the intestinal tract of a recipient, with the objective of restoring the microbial community in the gut. It is a very efficacious treatment for recurrent *Clostridiodes difficile* infections, although it has been of great interest for the treatment of neurological disorders, including Parkinson's disease, Alzheimer's disease, autism, and others (91–94,96). As far as the preparation of FMT material goes, several studies have been done, although it is difficult to come to solid conclusions. However, preparations using water infusions have shown to achieve higher rates (98.5%) of *Clostridiodes difficile* infection

improvement compared to preparations using normal saline infusions (86%). Additionally, the faeces should be freshly produced, within 6 hours of treatment, in order to secure bacterial viability and should weigh at least 150 g. They are then directly covered in 500 mL of sterile saline 0.9% solution and filtered for a homogeneous solution. After preparation, the solution is administered to the patient, mostly through a duodenal tube, however, it can be administered through different routes. A bowel lavage with 1-2 L of macrogol must be performed first and prior antibiotic use in the recipient has not been proven to be clearly successful (97,98).

Respecting donors, a thorough selection must be considered, since the risk of transmission of unknown pathogens via FMT can't be excluded. Furthermore, a donor selection is fundamental to avoid a new disease in the recipient. A screening for transmittable diseases must be done, including in blood and faecal matter. In blood, the following pathogens must be researched: EBV, hepatitis A virus, hepatitis B virus, hepatitis C virus, HIV (human immunodeficiency virus), human T-lymphocytic virus, cytomegalovirus, *Strongyloides*, amoebiasis. On the other hand, in faecal matter, the pathogens to screen are: *Helicobacter pylori* antigen, *Yersinia*, *Campylobacter*, *Shigella*, *Salmonella*, rotavirus, adenovirus, enterovirus, parechovirus, sapovirus, norovirus, astrovirus, and parasites. More in depth criteria must be considered, like the recent use of medications, within 3 months, especially antibiotics, proton pump inhibitors, risk factors for transmittable diseases and symptoms of irritable bowel syndrome and abnormal defecation patterns (92,97,98).

The faecal microbiota can be transplanted through various routes, such as an upper tract endoscopy, a nasojejunal tube, a nasogastric tube, a colonoscopy, a enema or even orally, although enema or oral capsules are safer than endoscopic procedures, since there are additional risks associated with sedation and loop perforation (97,98). When it comes to the recipient and FMT, and since DMT can influence microbial communities, patients who are not under DMT are recommended (98).

9.2. Pre-Clinical Research

Pathophysiological and clinical characteristics of MS are most accurately simulated in the experimental autoimmune encephalomyelitis (EAE) mouse model, thus EAE mice are most frequently used in pre-clinical research regarding MS (91). Chronic inflammatory diseases have been deeply associated with altered profiles of intestinal microbiota and this causal relation was established by transplanting human-derived microbiota to rodents, which showed that recipients ended up developing equivalent conditions. The same strategy has been adopted to study chronic autoimmune and neurodegenerative diseases, such as MS (99). Regarding pre-clinical research, three studies will be mentioned and analysed, as summarised in Table 8.

Berer *et al*. transplanted faecal samples from MS-affected twins and healthy twins into germ-free mice, which lead to a rather curious observation: the human microbiome was able to trigger EAE in mice, but the MS-derived microbiome triggered EAE much more significantly

and at higher rates when compared to the healthy-derived microbiome. Berer *et al.* concluded that MS-derived microbiota precipitated an MS-like autoimmune disease in a transgenic mouse model, suggesting that gut microbiota is required to induce EAE, since the mice did not develop spontaneous EAE (99).

Li *et al*. conducted a study using mice raised in pathogen-free conditions and proceeded to EAE induction through injection of emulsified myelin oligodendrocyte glycoprotein, MOG35–55, and afterwards one group was transplanted with faecal microbiota. The mice were divided into two distinct groups: the FMT group was given fresh faecal supernantant through oral gavage daily for 42 days; the EAE group was transplanted with sterile saline and 42 days post immunisation, faecal samples from the mice were collected. The study showed that FMT modulates the microbiome in EAE, prevents BBB leakage in EAE, confers protection on myelin and axons in EAE, alleviates microglia and astrocyte activation in EAE, demonstrating its therapeutic effects on EAE. Li *et al*. also concluded that FMT appears to be the most direct way to reconstruct the microbiota (100).

In a study by Cekanaviciute *et al.*, the microbiome of MS patients was transplanted into germ-free mice immunised with MOG35-55 emulsion. The mice were divided into two groups: FMT-MS, where the transgenic mice were transplanted with the microbiota from MS patients and FMT-HC, where the transgenic mice were transplanted with the microbiota from healthy controls. The FMT-MS group showed a more severe clinical course of EAE when compared to the FMT-HC group, as well as a decrease in IL-10 levels (101).

Table 8 - Pre-clinical research studies performed on animal models.

Models	Follow-up	Pre-treatment	Route of administration	Effects of FMT	References
Transgenic, germ-free mice carrying a MOG-specific T cell receptor. Groups of mice: MS-FMT HT-FMT	12 weeks after FMT	No pre-treatment	Oral gavage	MS-FMT exhibited increased incidence of EAE onset; HT-FMT exhibited increased expression of IL-10	(99)
Germ-free, immunised mice with MOG35–55. Groups of mice: FMT group EAE group	42 days after immunisation	NA	Oral gavage	Alleviation of microglia and astrocyte activation in the FMT group	(100)
Germ-free, immunised mice with MOG35–55 Groups of mice: MS-FMT HC-FMT	70 days	Pre-treatment with antibiotics	Gavage	MS-FMT showed a more severe clinical course of EAE and decreased IL-10 levels when compared to HC-FMT	(101)

9.3. Clinical Research

As far as clinical research goes, there are only two case reports on the therapeutic effects of FMT (92,96). As of May of 2022, there are currently 4 clinicals trials ongoing (2 recruiting, 1 active but not recruiting and 1 not yet recruiting), according to the clinicaltrials.gov database. These studies are described in detail and then summarized in Table 9.

In a single-arm, non-randomized, time series, single-subject study, a 48 year-old caucasian male with active RRMS for two years, with symptoms of difficulty in walking and bloating, underwent evaluation for 12 months before and after FMT. Subject's stool and serum were collected before FMT and at weeks 3, 13, 26, 39 and 52, and during the year-long study, two FMTs interventions were performed. As a pre-treatment, a standard bowel prep was conducted and the morning before the first FMT no food or drink were consumed by the subject, in order to keep the colon empty. In this study, brain-derived-neurotrophic-factor (BDNF), as indicator of neuronal brain development and synaptic plasticity, and inflammatory biomarkers (IL-6, IL-8, IL-17 and TNF-α) were evaluated. The results of this study showed that the relative abundance of *Faecalibacterium prausnitzii*, butyrate-producing, increased significantly. *Collinsella aerofaciens* and *Eubacterium rectale* also increased after FMT. Besides this, three SCFAs were assessed (acetate, propionate and butyrate) and their concentrations, following two FMTs, significantly increased at weeks 13 and 39. These results suggest that FMT improved the subject's microbiome, leading to an increase of the relative abundance of anti-inflammatory butyrate-producing bacteria, particularly *Faecalibacterium prausnitzii*. It was also observed that increased BDNF levels could be explained by increased levels of butyrate-producers, since BDNF production is inhibited by an inflammatory state. Additionally, there was an improvement of walking matrices, which was the patient's main complaint and maintained normal GI symptoms. When it comes to the 12 months follow-up, the subject had no episode of RRMS symptoms or relapses (102,103).

In a randomised, controlled trial, 9 MS patients were provided monthly FMTs for up to 6 months. It is important to mention that, initially, there were 10 MS patients, but one of them progressed to SPMS. Two outcomes were evaluated: the primary outcome determined the concentration of inflammatory cytokines; the secondary outcome evaluated the composition of the microbiome, permeability of the intestine and safety, in parallel with EDSS and MRI. There were two donors selected and FMT was performed through an enema because of its safe delivery route for repeated administrations. The evaluation of cytokines included pro- and anti-inflammatory cytokines such as: IL-1β, IL-2, IL-4, IL-10, IL-13, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, TNF-α and TNF-β. As for the secondary outcome, the signs of disease activity or progression were measured using the EDSS, once a month, for 12 months and MRI. The patients underwent pre-treatment before the procedure and only one adverse effect was found to be related to FMT: a patient developed hives after the procedure with no need for treatment. As far as results go, there was no significant change in the concentrations of peripheral cytokines, but could be from the small

sample size. Although there were 9 patients, only 5 were included in the analysis of intestinal permeability. Two of them had abnormal small intestinal permeability that normalised after 6 FMTs. Regarding microbiome composition, the results showed that FMT was linked to alterations in the gut microbiota. MS patients had higher abundance of *Bacteroides, Blautia faecis* and *Bacteroides uniformis* and lower abundance of *Faecalibacterium*, whereas donors had higher abundance of *Prevotella* and *Paraprevotella*. Although this study shows good results in terms of FMT as a treatment for MS, it has a big limitation, which is the small number of patients. In order to further study the effect of FMT in MS patients, cohorts need to be more representative (104,105).

Only two case reports studying the therapeutic effects of FMT in MS patients have been conducted (92,96). Borody *et al.* conducted a case report with three patients with MS. First, a 30 year-old male with constipation, vertigo and a history of MS, with neurological symptoms, that had previous treatments with IFN-β, but with no results. The patient underwent 5 FMTs as a treatment for his constipation that was reverted in parallel with progressively improvements of MS-associated symptoms. The patient regained his ability to walk and remained well after 15 years, without relapses. The second patient was a 29 year-old wheelchair-bound male with chronic constipation and atypical MS diagnosis, reporting paresthesia and leg muscle weakness. He went through 10 FMT infusions that resolved his constipation, but he also noticed a significant progressive improvement of his neurological symptoms, including regaining his ability to walk. Three years later, he has normal motor, urinary and GI function. The third case was a 80 year-old female with chronic constipation, severe muscular weakness and difficulty in walking, diagnosed as atypical MS. 5 FMTs were performed on this patient and the constipation symptoms were rapidly solved, as well as neurological improvement. Eight months later, she is walking long distances with no assistance needed and, two years later, is asymptomatic. This study performed on three different MS patients, suggests that FMT has a therapeutic application for MS symptoms, as these patients had their neurological symptoms ameliorated and haven't experienced relapses (92,106).

This fourth study is a case report conducted by Makkawi *et al*. of a 61 year-old woman with MS, followed since 1988, at the age of 33. Between 1998 and 2001, she had 7 relapses and her MRI confirmed RRMS, with several lesions. In 2001, she started pharmacological therapy with glatiramer acetate and she has maintained relapse-free with no new lesions in her MRI since then. From 2001 to 2005, some of her symptoms, including balance, ambulation and lower limb power worsened and, subsequently, her EDSS increased from 2.0 to 3.0. This progression of symptoms suggested a diagnosis of SPMS. During the years of 2005 and 2006, she presented *C. difficile* enterocolitis and treatment with clindamycin for a gingival infection. After this, her EDSS score went up to 6.0. To treat her condition, she underwent a FMT in 2006, through rectal enema and the results show that her EDSS score stabilised immediately, with no need for other treatments. Ten years later, her Modified Multiple Sclerosis Functional Composite scores minimally improved. This case report suggests the long-term benefits of therapy using FMT on MS progression. Her recurrent *C. difficile* infections led to a dysbiosis, which led to an aggravation of MS symptoms, which progression was stopped by. FMT (92,107).

Table 9 - Summarisation of clinical research studies conducted.

Sample	Study type	Status of study	Conditions of study	Evaluated parameters and results	Reference
Caucasian male (48y) with active RRMS for two years	Single-arm, non-randomised, time series, single-subject	Completed	Patient underwent 2 FMT interventions; Pre-treatment with standard bowel prep was performed	Biomarkers: ↑ BDNF; ↑ IL-6, IL-8 and TNF-α first FMT; = IL-6 and TNF-α, ↓ IL-8 after second FMT. Microbiota assessment: ↑ *Faecalibacterium prausnitzii* and *Collinsella* and *Eubacterium* (producers of SCFAs) after FMT	(102)
9 RRMS patients	Randomised, controlled trial	Terminated	Patients underwent monthly FMTs for up to 6 months	Concentration of inflammatory cytokines in peripheral blood: No significant change Microbiome composition: ↓ *Blautia* and *Subdoligranulum*; ↑ *Phascolarctobacterium* which produces propionate.	(104)
MS male patient (30y) with constipation and vertigo Wheelchair-bound MS male patient (29y) with chronic constipation; MS female patient (80y) with chronic constipation	Case report	Completed	Previous treatments included IFN-β; and 5 FMTs. 10 FMT infusions for his constipation. 5 FMTs were performed.	Reversed constipation and MS improvement; regained his ability to walk and after a 15-year follow-up, remains without relapses. Reversed constipation and progressive improvement of neurological symptoms. Three-year follow-up with normal motor functions. Reversed constipation and neurological improvement. Two-year follow-up with no symptoms.	(106)
MS female patient (61y)	Case report	Completed	FMT to treat recurrent *C. difficile* infections.	EDSS score stabilised, no need for other treatments. Ten-year follow-up, her MMSFC scores minimally improved.	(107)

9.4. Differences in gut microbiome composition with disease-modifying treatments

Although it is known the importance of the microbiome in the immune pathways of MS, there is still limited data when it comes to the effect of DMTs on patients' microbial environment. Besides this, some studies have shown that MS patients undergoing DMTs, show alterations in the composition of the microbiome and theses alterations could contribute to the efficacy of treatments by stimulation of microbes with anti-inflammatory properties (108–110).

Sand *et al*. conducted a study with 168 RRMS patients, where 75 were treatment-naïve, 33 treated with DMF and 60 treated with GA, and patients undergoing DMTs had to be stable on GA or DMF for at least 3 months. The microbiome of all patients was composed predominately by *Firmicutes* and *Bacteroides*, and, in lower abundance, of *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia*, which is consistent with recent reports on the microbial profiles of MS patients. GA administration led to a decrease in the relative abundance of 7 genera and increase of 7 genera. On the other hand, all the 13 genera that were altered in DMF treated patients had decreased relative abundance and the most prominent decrease was in the order *Clostridiales*. The phylum *Bacteroidetes* showed an increase in abundance in the group of patients treated with DMF when compared to treatment-naïve patients. The genus *Bacteroides*, a group that has been shown to have a potential protective effect in MS, showed an increase. Other studies have shown that there is an increase in *Prevotella* associated with INF treatment. However, in the group of patients, no change in the abundance of *Prevotella* was noticed, when comparing treated patients with treatment-naïve patients, which suggests that this genus is less affected by GA or DMF than by IFN. Additionally, the abundance of *Lachnospiraceae* and *Veillonellaceae* was decreased in both DMF-treated and GA-treated patients. In conclusion, this study shows that both DMTs were linked to decreased abundance of the *Lachnospiraceae* and *Veillonellaceae* families. However, DMF was associated with decreased abundance of *Firmicutes* and the order *Clostridiales*, and increased *Bacteroidetes* (108,109).

Another study determined the effect of DMTs (e.g., rituximab, ocrelizumab, DMF, fingolimod and natalizumab) in the microbiome focusing on alterations in β-diversity. The results show that β-diversity of patients treated with DMT did not differ from treatment-naïve patients, but all MS treatment subgroups differed from healthy subjects, which suggests that the microbiome is more greatly influenced by disease status rather than DMTs. Furthermore, the study also concluded that anti-CD20 treatments, such as rituximab and ocrelizumab, increased *Faecalibacterium prausnitzii* and DMF increased *Roseburia intestinalis*. These two are butyrate producers that seem to be reduced in MS patients. Besides this, fingolimod and natalizumab seemed to increase *Ruminococcaceae* (111).

In a trial studying the effects of DMF, 36 RRMS patients underwent treatment with either DMF, GA or IFN (injectable group) for 12 weeks. Stool samples were analysed at baseline and at enpoint. There were 165 healthy controls included. At baseline, there were 16

genera that were altered in patients when compared with healthy controls. Two weeks after treatment with DMF, patients showed a decreased abundance of *Actinobacteria*, mainly *Bifidobacterium*. While in the beginning of the study, MS patients showed lower levels of *Faecalibacterium*, a producer of butyrate, after 12 weeks of treatment, *Faecalibacterium,* were increased and there was also an increase in the ratio between *Firmicutes* and *Bacteroidetes*. Besides this, in the injectable group no changes were noticed at phylum level at either two weeks or 12 weeks (110).

All of these studies suggest that a relationship between disease status in patients undergoing DMTs and the microbiome exists. Although there is some knowledge regarding the effect of GA, IFN and DMF on the microbiome of MS patients, the role of the microbiome in the failure of first-line treatment options still remains unclear and there is lack of data regarding the long-term effects on the microbiome of other therapeutic options, such as fingolimod and ocrelizumab, for instance (108,112).

10. Conclusion

MS is an autoimmune disease that affects a great number of people worldwide, especially in developed countries, where people have more access to health care systems and assistance. On the other hand, the lack of epidemiological data in developing countries could be from the lack of diagnosis, lower access to medical care and different diagnosis criteria, which makes it more difficult to provide information regarding disease incidence, prevalence and mortality in these countries. Besides this, MS development depends on genetic susceptibility and environmental factors, which could also explain these epidemiological differences.

As of today, most patients with MS are treated with pharmacological therapies, whether we are talking about relapses or disease progression. Amongst the pharmacological therapies currently available for relapses, CS, ACTH and plasma exchange are three options for the treatment of symptoms. For disease progression, first line therapies include IFN-beta (IM), GA (SC), teriflunomide (oral) and DMF (oral). Although pharmacological therapies act as immunomodulators or immunosuppressors, they can also modulate the microbiome of patients, as patients have shown to have alterations of their microbiome composition before and after initiating DMT. The ability of DMTs to modulate the microbiome proves that the microbiota plays an important part in MS.

The microbiome has been of great interest in autoimmune diseases, as immune responses are heavily modulated by these microorganisms that live in the human gut. The gut-brain axis, a two-way communication system, shows that there is a very strong connection between the brain and the gut. This communication, subsequently, can be influenced by substances produced by microbial organisms. The microbiome plays a fundamental role in the functions of the gastrointestinal tract, however, patients with neurodegenerative diseases have

shown to have distinct alterations in their microbiota. Because of this, the scientific community has shown a lot of interest in these organisms and in how they can favour the disease. The microbiome, by modulating the immune response, could potentially have a therapeutic role in these diseases.

MS patients have distinct microbial profiles, as certain phyla and genera are either increased or decreased in these patients when compared to healthy individuals. With that being said, the modulation of the gut microbiome in MS patients could be a therapeutic approach. Microbiome modulating therapies include probiotics, prebiotics, FMT and even diet alterations. FMT is a procedure that includes introducing a solution of faecal matter from a healthy donor into the intestine of the patient. It is commonly used to treat recurrent infections by *Clostridium difficile* but it could also have an interesting effect in MS. FMT has shown to have several beneficial effects in the treatment of symptoms as well as preventing disease progression, as patients have shown to regain some motor functions that were affected by the disease and to recover from relapses, many of them without having any since. This procedure could be revolutionary for patients with MS that present microbial alterations.

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