DOCTORAL COURSE IN
"Translational Neurosciences and Neurotechnologies"

CYCLE XXXIV

COORDINATOR Prof. Luciano Fadiga

**MULTIPLE SCLEROSIS AND THE "HYGIENE HYPOTHESIS":
FROM THE OUTDOOR EXPOSURE TO THE INDOOR EFFECTOR
EVIDENCE FROM EPIDEMIOLOGICAL SURVEYS AND AN EXPERIMENTAL STUDY
ON THE GUT MICROBIOTA**

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Candidate	**Supervisor**
Dott. Ferri Caterina	Prof. Pugliatti Maura

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Chapter 1. INTRODUCTION

1.1 Multiple sclerosis: epidemiology, course and clinical features

Multiple sclerosis (MS) is an inflammatory autoimmune and degenerative disease of the central nervous system (CNS) affecting mostly young adults. It represents the most frequent cause of neurological disability in young people after trauma. It is characterized by demyelinating process followed by axonal damage involving the white and also grey matter. The autoimmune response is mostly direct against myelin self-antigens. The MS onset occurs in most cases between 20 and 50 years of age, pediatric onset or later onset are uncommon [Giovannoni 2016].

About 2,8 million is the population affected by MS worldwide (35.9 per 100,000 population) [Walton 2020]. The prevalence of the disease is higher among women and depends on the latitude, increasing with the distance from the equator. Europe (especially countries of central and northern Europe), North America, Australia and New Zeeland feature the highest prevalence overcoming 100/100,000 inhabitants, while in regions with the lowest risk (Africa, East Asia) the prevalence is about 5/100,000 inhabitants [Browne 2014] (**Figure 1.1**). In Italy the estimated prevalence is 208/100,000 inhabitants while in Sardinia, where the risk is higher compared to the rest of the Country, it is 330 cases per 100,000 [Urru 2020]. The MS Italian incidence is 6 per 100,000 people per year while in Sardinia is about 12.
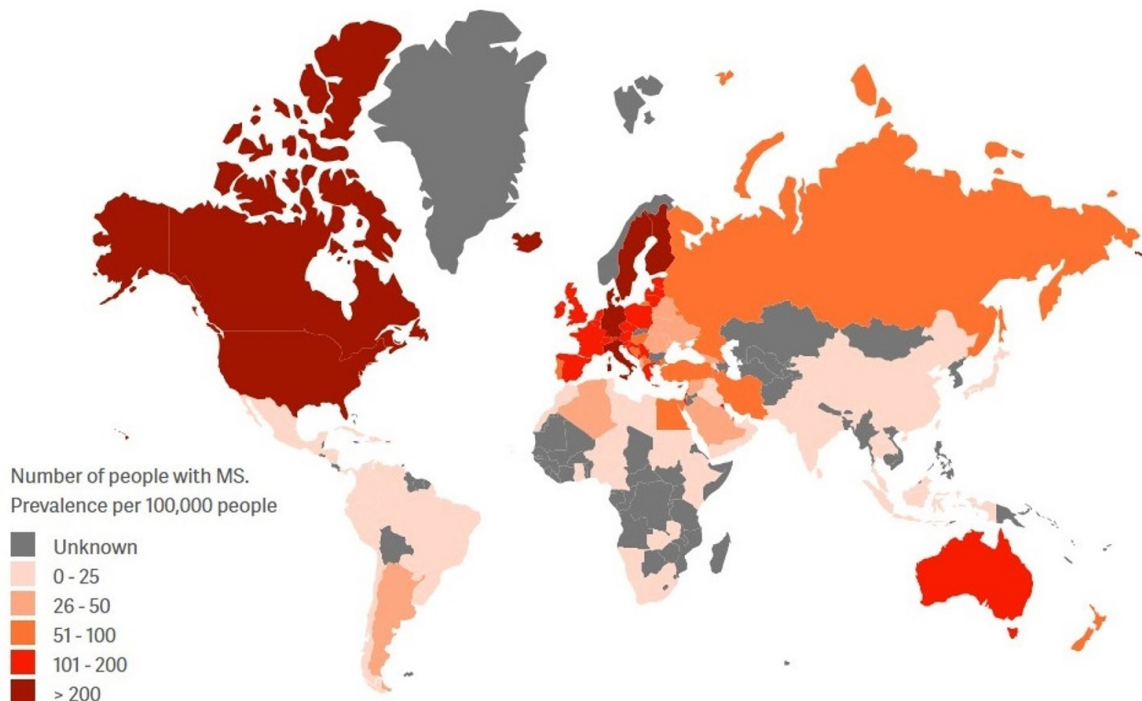

Figure 1.1 Worldwide prevalence of multiple sclerosis (MS) [Atlas of MS].

Basing on the course of the disease, we can differentiate the relapsing-remitting form of MS (RR), the primary progressive (PP) and the secondary progressive (SP) form. RRMS is the most frequent and is characterized by clinical relapses which resolve within weeks or months even if the recovery may not be complete, with clinical or subclinical residual deficiency. The first clinical relapse, when diagnostic criteria are not fulfilled, is named clinically isolated syndrome (CIS). The risk of conversion from CIS into MS is higher when brain MRI shows at least one typical lesion and when oligoclonal bands are identified in cerebrospinal fluid (CSF) [Gelfand 2014]. Most patients, after 15 years of disease duration, show a secondary progressive course characterized by disability accumulation without recovery in absence of clinical relapses (which sometimes may also be present). Rarely the disease course is progressive from the beginning (PPMS). The clinical onset usually occurs during the third decade of life for RRMS, in the fourth for PPMS.

The clinical presentation depends on the localization of the lesions which generally affect periventricular white matter (WM), corpus callosum, optic nerve, brainstem, cerebellum and spinal cord. WM is the principal target of the inflammatory process but also the grey matter (GM) is affected by the pathological process and the presence of cortical or juxta-cortical lesions, included in the MS diagnostic criteria, improve the diagnostic specificity. Clinical manifestations are very heterogeneous including vision loss, sensitivity or motor disturbances, cerebellar and bulbar symptoms, sphincter disorders, fatigue, mood deflection and cognitive decline.

During the early stages of the disease, when axonal damage is limited, it is still possible to assist to spontaneous remyelination, while in later phases the repair process tends to be incomplete and the damage become irreversible. Over time the disease causes brain atrophy and disability accumulation [Giovannoni 2016]. About 50% of patients require a wheelchair 25 years after diagnosis. The Expanded Disability Status Scale (EDSS) is a useful tool to quantify the disability and measure the disease clinical progression and it is widely used in clinical practice and clinical trials [Kurtzke 2015].

1.2 Immunopathogenesis

MS pathogenesis is not yet fully understood; the autoimmune process is supported by several histopathological, neurochemicals and neuroradiological evidences. Most of the knowledge on the inflammatory mechanisms of MS comes from studies on experimental autoimmune encephalomyelitis (EAE). The principal targets of the immune response are the myelin basic protein (MBP), the myelin oligodendrocyte glycoprotein (MOG) and the proteolipid protein (PLP). The traditional pathogenetic model is the "outside-in", according to it the loss of the tolerance of the immune system against myelin antigens represents the *primum movens* and it is followed by the activation of autoreactive cells which penetrate the blood-brain barrier (BBB). Molecular mimicry induced by Epstein Barr Virus (EBV) and Varicella Zoster Virus (VZV) infection or even vaccines may explain this loss of tolerance. On the other side, also a primary disfunction of the CNS could be

involved in inducing neurological damage: this is the "inside-out" model (**Figure 1.2**). It has been hypothesized that the primary stimulus may be an alteration of the myelin or oligodendrocyte with release of highly antigenic fragments [Stys 2013]. In EAE a mild myelin alteration is capable of triggering a severe inflammatory demyelinating response, therefore in humans subclinical modifications occurring at a prodromic phase could turn a susceptibility into a real inflammatory response direct against myelin [Caprariello 2018]. A combination of both models, "outside-in" and "inside-out", is probably the closest to reality: myelin degeneration induced by the immune response according to the "outside-in" model could be the manifestation of a subclinical myelin disease which, according to the "inside-out" model, predisposes to an accelerated inflammatory myelin disruption. Evidences of early myelin *in vivo* alterations are now increasing [Xia 2017, Giovannoni 2017].
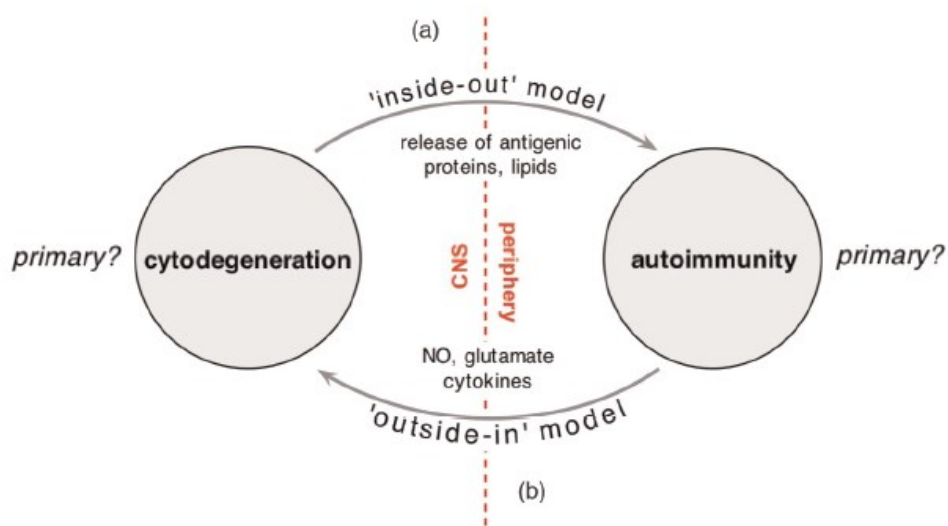
Figure 1.2: Pathogenetic models of multiple sclerosis [Stys 2013].

Regardless of the primary mechanism, the following phases of the inflammatory process are common to both models: autoreactive Th1 and Th17 CD4+ T lymphocytes cross the BBB and here they can be activated by the antigen presenting cells (APCs), like microglia, so inducing the inflammatory cascade through IFNγ and IL17 synthesis. After the increased permeability of the BBB, other immune cells (B lymphocytes, monocytes) are recruited and distribute in the perivenular region forming a perivascular sleeve and inducing demyelination of the surrounding areas. Microglia cells residing into the CNS are also activated causing further increase in inflammatory infiltrate into the damaged areas and thus perpetuating the inflammatory circle and demyelination through the release of proinflammatory cytokines, nitric oxide and matrix metalloproteinases [Dargahi 2017] (**Figure 1.3**). Besides CD4+ T lymphocytes, also CD8+ lymphocytes are involved in MS pathogenesis; they are distributed into demyelinating lesions and correlate with axonal damage. Among them, there are mucosal-associated invariant T (MAIT) cells

which synthetize IL17. In MS patients, MAIT cells concentration in peripheral blood tend to decrease, suggesting their migration into the CNS [Willing 2014].
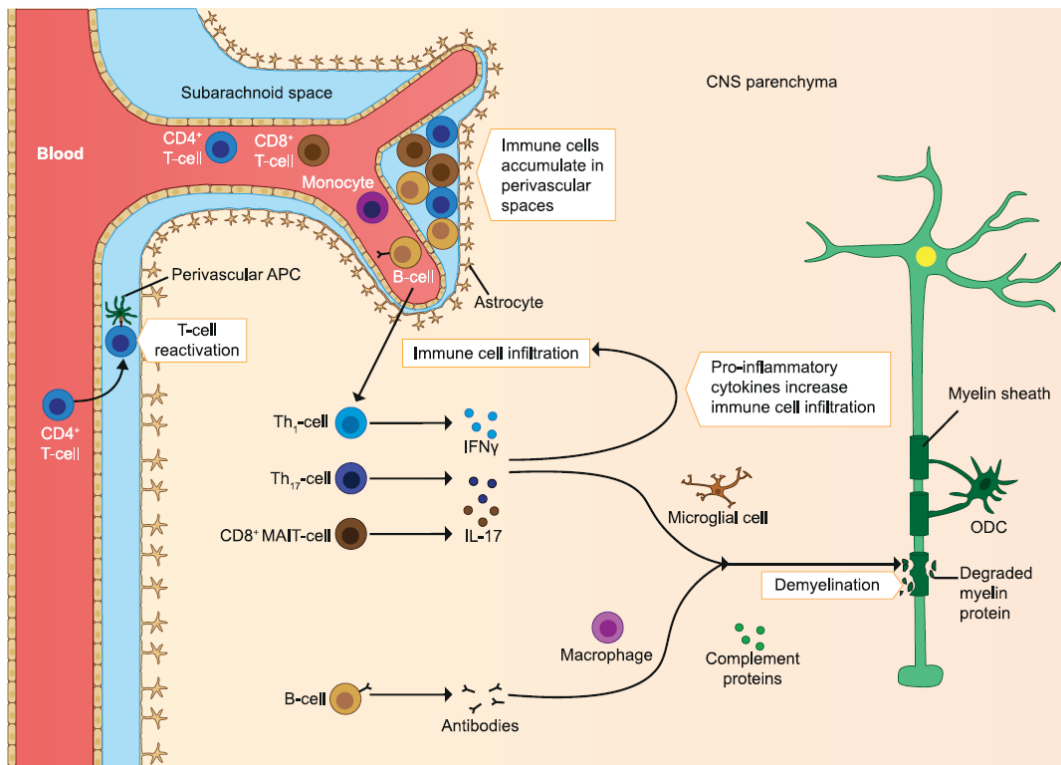
Figure 1.3: Pathogenetic mechanism of the early stage of MS [van den Hoogen 2017].
APC, antigen presenting cell; ODC, oligodendrocyte; MAIT, Mucosal-associated invariant T cell.

Figure 1.4: Pathogenetic mechanism of the advanced stage of MS [van den Hoogen 2017].
FDC, follicular dendritic cells; ODC, oligodendrocyte.

Another mechanism of myelin damage is mediated by autoreactive B lymphocytes secreting autoantibodies which activate complement and macrophages. Moreover, MS in characterized by Treg deficiency, these cells producing IL10 exert an anti-inflammatory function. In advanced stage of the disease the pathogen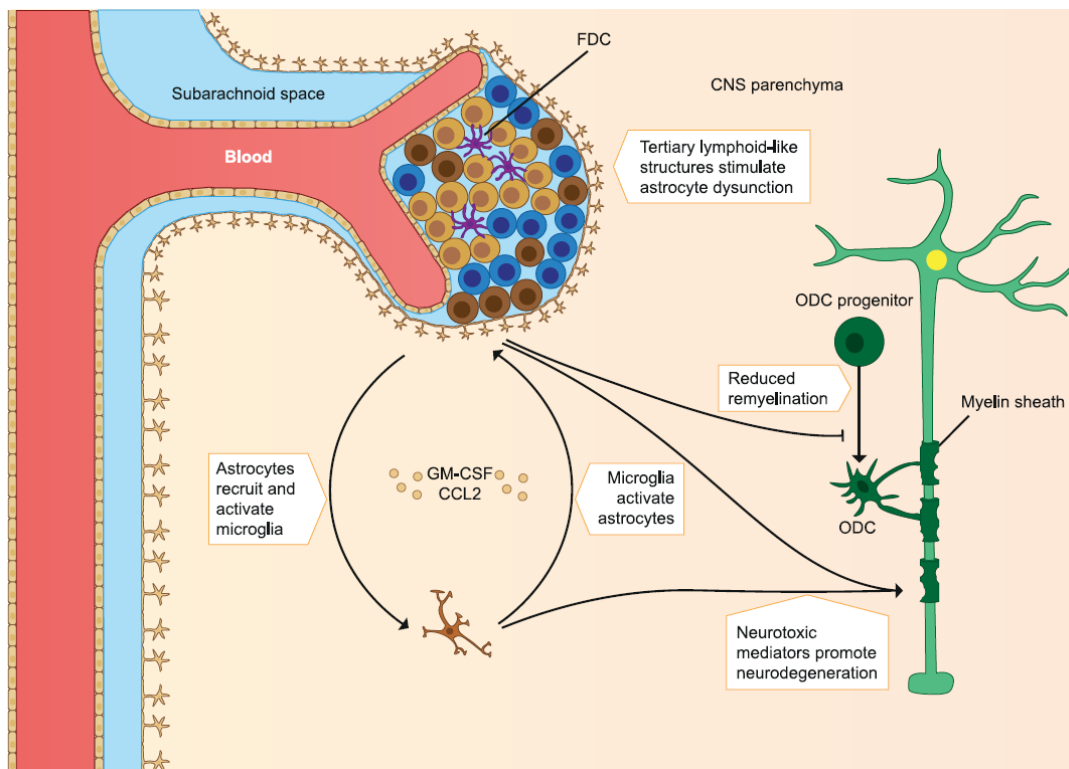etic mechanisms change: the inflammatory infiltrate is reduced while a chronic inflammation prevails with the creation of tertiary lymphoid-like structures in perivascular spaces, astrocyte and microglia disfunction. Microglia activation promotes the astrocytes production of CCL2 chemokine and granulocyte-macrophage colony-stimulating factor (GM-CSF) which cause further activation of microglial cells. In this phase, neurotoxic mediators derived from microglia and astrocytes, such as reactive oxygen species (ROS), promote neurodegeneration and the remyelination is limited by astrocytes which inhibits the maturation of oligodendrocytes' progenitors. [van den Hoogen 2017] (**Figure 1.4**).

1.3 Genetic and environmental risk factors

MS is a multifactorial disease, both environmental and genetic factors are implied in the etiopathogenetic mechanisms. The existing role of genetic impact on MS is well known: the individual probability of developing MS is highly influenced by ethnicity and familiar history [Hollenbach 2015], monozygotic twins share the diagnosis in 25% of cases. Over 200 loci have been associated to disease susceptibility, especially those of the class II of the Major Histocompatibility Complex (MHC), such as HLA-DR2 and HLA-DR4 alleles. MHC is a group of genes located on the short arm of the chromosome 6 coding for protein expressed on the surface of immune cells (dendritic cells, macrophages, B lymphocytes) presenting antigens to T lymphocytes. In Caucasians an association between MS and haplotipe DRB1*1501 of the allele DR2 has been demonstrated [Canto 2018]. The incomplete penetrance of the disease reflects the influence of others genes, post-transcriptional processes and the interaction of environmental factors.

The disease geographical latitudinal gradient and with higher prevalence in high-income countries support a major role of environmental exposures. Furthermore, the prevalence of MS has been steadily increasing over the past 5 decades, albeit without a clear latitudinal gradient, questioning the latitude effect and emphasizing the role of other environmental factors in determining the risk of developing MS [Puthenparampil 2021]. The phenomenon of migration has been traditionally used as a tool to understand the role of environmental factors in the risk of pathology. When individuals migrate from areas at low risk to areas at high risk for MS before the age of 15 years, they have been shown to acquire the high risk of the host population. On the basis of this evidence, crucial environmental factors are assumed to act during the early phases of life. Conversely, if migration occurs after puberty, the risk of MS remains similar to that of the country of origin [Munk Nielsen 2019]. More recently, the migration during adulthood has also been related to MS risk modification with a rising risk with the duration of exposure to a high-MS-risk environment, suggesting a dose-response effect [Rotstein 2019, Pugliatti 2020].

A lot of environmental exposures have been implied in the pathogenesis of MS: smoking habit, low serum vitamin D levels, low sun exposure, high body mass index (BMI) during adolescence, dietary habits, alcohol intake, socio-economics conditions, sexual hormones, viral infections and gut microbiota composition. The relationship between smoking and the risk of MS has been long investigated showing a relative risk for smokers of about 1.5 compared to never smokers [Degelman 2017]. Hypovitaminosis D, which is widespread in temperate countries, has been related to MS risk and so has low sun exposure. The month of birth and low vitamin D levels during pregnancy can affect the baby's future risk during the prenatal period: in the northern hemisphere people born at the end of autumn have a reduced MS risk cause of high sun exposure of the mother during pregnancy, on the contrary those born at the end of spring have an increased MS risk. In the southern hemisphere the risk period is reversed [Pierrot-Deseilligny 2017]. It has also been proposed that the higher level of vitamin D in male population could be responsible for the lower incidence of the disease in this gender. The overweight during adolescence and obesity have both been associated with higher risk of MS, especially in females; the causal mechanisms may be lower levels of vitamin D, which is sequestered in the adipose tissue, and the inflammatory chronic action exert by the adipose tissue itself as adipocyte are able to secrete inflammation mediators such as TNFα, interleukin-6 and leptin [Liu Z 2016, Langer-Gould 2013]. The diet and lifestyle role in MS risk is also proven by the geographical distribution of the disease which is prevalent in regions where the diet is rich in saturated fatty acids of animal origin. The education level and factors related to lower socioeconomic status are also associated with MS risk [Bjørnevik 2016]. Conversely, sexual hormones, both in men (testosterone) and in women (estrogens and progesterone) could exert a protective effect against MS [Bove 2014, Robinson 2012]. According to epidemiological evidence, Epstein Barr Virus (EBV) infection and infectious mononucleosis (IM) seem to be associated with a high risk of MS. Seropositive individuals for EBV, even without a history of IM, have a 15-fold greater risk of developing MS compared to seronegatives. In addition, the role of EBV is also supported by the frequent finding of antibodies and T lymphocytes against it in MS patients [Tarlinton 2019]. Finally, increasing evidence supports the role of gut microbiota in MS development [Castillo-Alvarez 2017].

1.4 The "hygiene hypothesis"

The human immune repertoire is constantly shaped by environmental exposures to infectious agents. This kind of stimuli are reduced in the context of the industrialized lifestyle which is characterized by decreased burden of infections in infancy and childhood due to higher standard of personal cleanliness, the improved health care (vaccinations, antibiotic use, caesarean section) and sanitation with limited exposure to microorganisms such as helminths and microbes, the declining family size and consequent reduced cross-infections in young families. An exaggerated protection against infective agents causes an insufficient development of the immune system which

predisposes to the onset of autoimmune and allergic diseases, this relationship has been named by Strachan "hygiene hypothesis" [Strachan 1989].

According to the "hygiene hypothesis", the increased rate autoimmune diseases and allergies is a consequence of the improved hygiene over the last decades (**Figure 1.5**). This hypothesis could also explain the gap in the incidence and prevalence of dysimmune conditions between high and low-income countries, where infectious diseases are much more frequent [Fleming 2007].

Traditionally, the immunological paradigm was centered on the dichotomy between CD4+ lymphocytes Th1 and Th2, both accompanied by a specific cytokine environment. The imbalance of the Th1/Th2 equilibrium and the lack of regulatory T-cell populations have been considered to led dysimmune diseases: the Th1 response promotes autoimmune disease while the Th2 predisposes to allergic disorders. Th1 cells are typically activated by antigen presenting cells (APCs), capable of producing IL12 and IL18 in response to intracellular pathogen infections. Activated Th1 lymphocytes secrete IL2, TNFα and INFγ, involved in both response against intracellular pathogen and autoimmune diseases pathogenesis. Th2 cells, on the other side, are stimulated by a IL18-poor and IL4 rich environment. IL4 with IL5, IL16 and IL13 promotes IgE production, which are fundamental in the pathogenesis of allergic diseases, and suppress INFγ production.
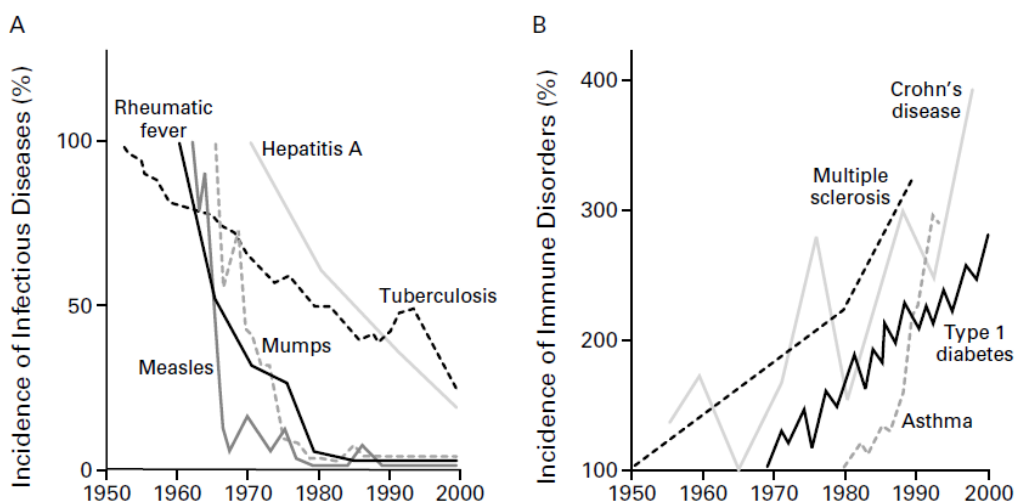
Figure 1.5 Inverse relation between the incidence of prototypical infectious diseases (Panel A) and the incidence of immune disorders (Panel B) from 1950 to 2000 [Bach 2002].

It has been hypothesized that the Th1-mediated processes (including autoimmune diseases) are able to inhibit the Th2 response and protect against the development of allergic diseases and vice versa atopic subjects could be protected against Th1-mediated autoimmune diseases. Apparently, one process seems to exclude the other and according to the immunological paradigm, the "hygiene hypothesis" would not explain the parallel increase in allergy and autoimmunity. Nevertheless, over time new immunological acquisitions have shown that the simple Th1 / Th2 dichotomy is an oversimplification: Th2 cells are actually involved in the Th1-mediated autoimmune diseases, as well as Th1 cells are involved in Th2 mediated allergic diseases. Strachan's theory does not

include lifestyle factors (e.g., diet, microbiota, sun exposure, smoking habits) and more specifically identified microorganism. The immunologist Graham Rook tried to make "hygiene hypothesis", microbiota and diet converge so as to explain the increase in autoimmune diseases and developed the "old friends hypothesis": lifestyle changes in high-income countries have led to decrease the exposure to environmental organisms ("old friends") which always featured the mammal's evolution over the last millennia and are responsible for the immunoregulatory mechanism [Rook 2012]. Rook's idea was supported by the results from the ISAAC study suggesting that an increased prevalence in asthma, rhinitis and neurodermitis is highly influenced by lifestyle and environmental changes [Asher 2006]. Among "old friends" we may recognize both pathogens and symbionts. Chronic infections with helminths and mycobacteria are hypothesized to induce regulatory cells which downregulate inflammation and the expansion of the autoreactive T-cells protecting against Th1 mediated autoimmune disease. Helicobacter Pylori, whose infection is acquired early in life, also appears to be inversely associated with the risk of MS [Jaruvongvanich 2016]. A protective role has also been attributed to the parasite Toxoplasma gondii. Conversely common childhood infections and vaccinations do not seem to affect the risk of MS [Wendel-Haga 2017]. It has also been speculated that the increased incidence of MS in the Sardinian population, compared to that of Africa, may be due to the lack of influence of the malaria parasite on the immune system [Sotgiu 2008]. The protective effect of the interaction between pathogen antigens and the innate immune system is also supported by the finding that the administration of the BCG (Bacillus Calmette-Guerin) in MS patients has an immunoregulatory and therapeutical effect, which also support the role of mycobacteria as an "old friend" [Ristori 2018]. Beside the exposure to infective agents and parasites, symbiotic bacteria are now considered as modulators of immune system development and function. Moreover, a higher hygienic level in industrialized countries may impact on the gut microbiota, which has an important role in keeping the health state of the organism and the tolerance against non-self-antigens [Freedman 2018].

Assuming that immunoregulatory mechanisms settling occurs early in life, even prenatally, Kramer revised Strachan's theory and proposed the "early immune challenge hypothesis" suggesting that the early exposure to a wide spectrum of "old friends" is essential for the maturation of the immune system, especially parasites can modulate the inflammatory and allergic reactions by downregulating the immune response [Kramer 2013]. Those hypotheses have a great potential for population-based prevention strategies through educational programs and for new therapeutical strategies based on developing immune modulating compounds from crucial organisms.

Allergy and the risk for MS

The allergy is a form of immunological reactivity in which reactive antibodies are quickly produced in response to exposure to common allergens, typically harmless environmental substances. Allergic diseases include skin allergies (urticaria, angioedema, hay fever, allergic asthma, food allergies and systemic involvement with potential fatal consequences like anaphylaxis. As for MS, also allergic diseases share genetic predisposition: the concordance in homozygous twins is about 70% while it drops to 15% in heterozygous twins, moreover the risk is increased in subjects with affected parents. The genetic influence is exerted by genes for the cytokines IL3, IL4, IL5, IL9, IL13 and GM-CSF, residing on the chromosome 5q31 and probably by the locus 6q, near the HLA complex. Environmental risk factors for allergy include instead dietary habit, air pollution, air recycling and humidity, in addition to the proposed limited exposure to microbial agents during early stage of life as suggested by the "hygiene hypothesis". The hypersensitivity of the immune system is mediated by type 1 or immediate hypersensitivity reaction which involves immunoglobulin E antibodies (IgE). Their binding to an allergen and then to a receptor on mast cells or basophils triggers the release of inflammatory chemicals such as histamine. This abnormal immunological response is underpinned by the Th2 lymphocytes which are higher in atopic subjects compared to general population, as well as IgE levels. The prevalence of allergic disorders is over 40% of the American and European population and is increasing over the years giving rise to an epidemic phenomenon [Kramer 2013]. Similarly to autoimmune diseases, the causal role of early life exposures has also been shown for allergic disorders: children grown up in the countryside, and exposed to many more allergens than those grown up in the city, are less predisposed to allergic diseases, both during childhood and in adulthood. Moreover, the protective effect increased with the amount and the duration of the exposure and is higher for exposures during the first year of life [Riedler 2001].

According to the Th1/Th2 paradigm, autoimmune and allergic diseases should be mutually protective. Nevertheless, their inverse association is still debated and some authors even supported a positive association (**Table 1.1**). Several studies have been published across years, mostly case-control studies. Often researchers focused strictly on respiratory tract allergies, at the same time, different kind of allergies have frequently been considered together without distinguishing between respiratory, alimentary allergies and atopic manifestations. Thus, overall allergies were variably found to have an equal [Solaro 2001, Alonso 2006, Alonso 2008, Hughes 2013, Karimi 2013, Bourne 2017], higher [Mansouri 2014] or lower prevalence in MS population than in controls [Pedotti 2009, Sahraian 2013, Ren 2017]. Similarly, asthma was found to be more prevalent in MS people [Edwards 2004, Ponsonsby 2006], in controls [Tremlett 2002, Conradi 2011] or equally prevalent among the two groups [Manouchehrinia 2015, Bourne 2017].

Table 1.1 Studies on the association between MS and allergies (published from 2000 onwards).

Risk factor	Reference	Study design	Population size	Geographical locations	Results
Bronchodilators and inhaled corticosteroids prescription	Evans 2000	Case-control study	216 MS patients, 216 controls	Wales, UK	Significantly fewer bronchodilators and inhaled corticosteroids were prescribed in MS population.
Allergies	Solaro 2001	Case-control study	312 MS patients, 312 controls	Genoa, Italy	There was no association between MS and allergies.
Asthma, eczema	Tremlett 2002	Case-control study	320 MS patients, 320 matched controls	Wales, UK	There was an inverse association between MS and asthma. No association between MS and eczema.
Asthma, eczema, hay fever, atopies	Edwards 2004	Cohort study	658 MS patients, 252,538 controls	UK	The MS population had significantly increased rates of asthma.
Multiple allergies	Alonso 2006	Case-control study	163 incident MS patients, 1,523 matched controls	UK	History of any allergic condition in the 3 years before the index date was not associated with MS risk. Specific allergic conditions were not clearly associated with the risk of MS.
Asthma, hay fever, other allergies	Ponsonby 2006	Case-control study	136 MS patients, 272 matched controls	Tasmania, Australia	MS cases were more likely than controls to have asthma which began before age of onset of MS symptoms. The absence of younger sibling exposure by age 6 years potentiated the association between asthma and MS.
Multiple allergies	Alonso 2008	Case-control study	298 women with MS, 1,248 matched controls, 248 women with history of breast cancer	UK	History of allergy was not associated with MS risk.
Multiple allergies	Pedotti 2009	Case-control study	423 MS patients, 643 controls	Northern Italy	A history of atopic allergies seems to confer protection against MS. No association was found between MS and nonatopic allergies.
Atopic diseases (allergic respiratory diseases, ARDs)	Bergamaschi 2009	Case-control study	200 MS patients, 200 controls	Pavia, Italy	MS patients had less probability to suffer from ARDs and allergic rhinitis.
Cow's milk allergy (CMA) in infancy	Ramagopalan 2010	Cohort study	6,638 MS index cases, 2,509 spousal controls	Canada	There was no association between CMA during childhood and MS risk.
Asthma	Conradi 2011	Case-control study	245 MS patients, 296 controls	Berlin, German	MS patients had a reduced risk of asthma compared to controls.
Cow's milk allergy (CMA) – specific IgE antibody	Ashtari 2013a	Case-control study	48 MS patients, 48 HC	Iran	There was no difference in the detection of IgE specific for CMA between MS patients and controls.
Fish and egg allergies	Ashtari 2013b	Case-control study	48 MS patients, 48 HC	Iran	Neither cases nor controls had allergies for fish and egg as documented by specific-IgE.
Multiple allergies	Sahraian 2013	Case-control study	195 MS patients, 195 HC	Iran	A positive history of respiratory allergies or food /drug allergies was inversely associated with the risk of MS. A history of both increased the inverse association.
Asthma, eczema, hay fever	Hughes 2013	Case-control study	279 patients with first clinical diagnosis of CNS demyelination (FCD), 539 controls	Australia	Allergies were not overrepresented among people presenting with FCD.
Multiple allergies	Karimi 2013	Case-control study	40 new diagnosed MS patients, 40 HC	Iran	There was no association between MS and allergies.
Allergies	Mansouri 2014	Case-control study	1,403 MS patients, 883 controls	Iran	There was a positive association between MS and history of any allergic condition.
Asthma	Manouchehrinia 2015	Case-control study	680 MS patients (of whom 88 had comorbid asthma, controls from health survey England 2010	UK	No difference in the prevalence of asthma between our MS cohort and the England general population.
Food and environmental allergies, asthma, animal contact	Bourne 2017	Case-control study	271 MS patients with pediatric onset, 418 controls	Boston, Massachusetts, USA	In unadjusted analyses, pediatric MS patients reported fewer environmental and food allergies in the first 5 years of life. After adjustment there was no difference in prevalence of allergies or asthma between cases and controls.
Respiratory tract allergies and other allergies	Ren 2017	Case-control study	829 MS patients and 2,441 matched controls	USA	Both respiratory tract allergies and other allergies were associated with a reduction of the risk of MS.

One study measured the prevalence of asthma through the prescription of bronchodilators and inhaled corticosteroids which were reduced in MS patients [Evans 2000]. Another Italian study found an inverse association between MS and respiratory tract allergies [Bergamaschi 2009]. As for

food allergies, no association has been detected between MS and fish and egg allergy [Ashtari 2013b] and cow's milk allergy [Ramagopalan 2010, Ashtari 2013a], conversely, an inverse association was found by an Iranian study [Sahraian 2013] and an American study in unadjusted analysis [Bourne 2017].To summarize, these results are highly conflicting and with special concern to respiratory allergies, asthma and overall allergies. At individual level, food allergies were found to be a protective factor for MS or to not influence the disease risk. Interestingly, some evidence has been published regarding the potential impact of allergy on the course of MS (**Table 1.2**). History of food allergy has been associated with lower relapse rate [Bourne 2017] but also with more relapses and higher chance of gadolinium-enhancing lesions [Fakih 2019] while it was not related to MSSS [Albatineh 2020]. We must consider that these studies used different types of comparators ranging from patients without food allergies to those without known allergies. Allergic respiratory diseases, on the other hand, showed a tendential association with lower EDSS and MS severity score (MSSS), while asthma alone was not associated with disability [Bergamaschi 2009, Manouchehrinia 2015]. As seen from these conflicting results, the role of allergy in MS prognosis is far from being understood.

Table 1.2 Studies on the prognostic role of allergies in MS.

Prognostic factor	Reference	Study design	Population size	Geographical locations	Outcome	Results
Atopic diseases (allergic respiratory diseases, ARDs)	Bergamaschi 2009	Case-control study	200 MS patients, 200 controls	Pavia, Italy	EDSS, MSSS (MS severity score)	MS tends to be less severe (lower EDSS and MSSS) when associated to ARDs, but not significantly.
Asthma	Manouchehrinia 2015	Case-control study	680 MS patients (of whom 88 had comorbid asthma)	UK	EDSS, Multiple Sclerosis Impact Scale (MSIS-29) scores	There was no association between having asthma and the risk of reaching EDSS scores 4.0 and 6.0. MS patients with asthma reported higher level of psychological impairments.
Food and environmental allergies, asthma, animal contact	Bourne 2017	Case-control study	193 MS patients with pediatric onset, 418 controls	USA	Annualized relapse rate (ARR)	Patients with food allergies in the first 5 years of life had fewer relapses compared to patients without food allergies. There was no association between relapse rate and other types of allergies.
Food, environmental and drug allergies	Fakih 2019	Cohort study	1,349 MS patients	Boston, Massachussets, USA	Clinical (number of attacks, expanded disability status scale (EDSS), MS severity score (MSSS)) and radiological variables (presence of gadolinium-enhancing lesions and lesion count)	MS patients with food allergy had more relapses and a higher likelihood of gadolinium-enhancing lesions compared with patients with no known allergy.
Food allergies	Albatineh 2020	Cross-sectional study	128 MS patients	Kuwait	MSSS	Food allergies were not associated with MSSS.

The sibling effect

The "sibling effect" or "sibship size effect" or "birth order effect" has been proposed as an indirect indicator of the overall amount of microbial exposition during childhood and it was used to prove the "hygiene hypothesis". It was first described by Golding and Peters in 1986: in

"The British Birth Survey" they found a significant reduction of the risk of eczema and hay fever in subjects with higher number of siblings [Golding 1986]. Following the "hygiene hypothesis", this concept has been applied to the autoimmune field: the exposure to infant siblings may act as a protective factor for the risk of autoimmune and allergic diseases. Nevertheless, literature evidences regarding sibship and autoimmune inflammatory diseases such as MS are quite inconsistent mostly because the definition of "sibling effect" is not unique and different surrogate for the exposure to infant siblings have been used: birth order position, total number of siblings (sibship size) or the number of older and younger siblings [Karmaus 2002]. Most studies on birth order regards type 1 diabetes mellitus and chronic inflammatory bowel disease (IBD). The risk of childhood onset type 1 diabetes was decreased with increasing birth order, which could reflect the higher infections' exposure in second- or later born children [Cardwell 2011]. The risk of IBD has also been related positively to low birth rank [Hampe 2003]. As for MS, evidence is weak and the "sibling effect" is still debated (**Table 1.3**). One of the first study on this subject found that the risk of the disease was inversely related to the birth order position and first- and second-born had a greater risk of MS [Isager 1980]. In agreement with the previous study, a German case-control study supported the protective effect of having older siblings on MS risk [Conradi 2011]; moreover, an Israeli work pointed out a lower birth order in MS patients, even if the patients born abroad showed an opposite trend, thus suggesting the role of different causal factors in different populations [Zilber 1988]. A greater birth order in MS patients has also been shown by a French study [Alperovitch 1981]. Other researchers' groups found no significant difference in birth order between MS patients and controls [Visscher 1982, Gaudet 1995, Sadovnik 2005, Ahlgren 2005]. Similarly, a Danish population-based cohort study, which investigated beside the birth order also the sibship size, the number of older and younger siblings and the duration of the exposure to younger siblings under 2 years of age, did not find an association with MS [Bager 2006]. A Swedish case-control study described instead an inverse association between MS risk and having 3 or more siblings regardless of whether they were younger or older [Montgomery 2004]. The hypothesis of a protective role of younger siblings has mainly been supported by two Australian studies [Ponsonby 2005a, Hughes 2013]. Interestingly, the work by Ponsonby and colleagues focused on the duration of the exposure to younger siblings aged less than 2 years showing that higher infant sibling exposure in the first 6 years of life was associated with a reduced risk of MS. Only younger siblings were the responsible for this inverse association between number of siblings and MS while older siblings did not affect the disease risk. Nevertheless, when the difference between siblings was more than 6 years the protective effect was lost. In addition, higher infant contact in early childhood delayed MS onset. These results

Table 1.3 Studies on the association between MS and sibship.

Outcome	Reference	Study design	Population size	Geographical locations	Results
Birth order	Isager 1980	Case-control study	46 MS patients born between 1930 and 1950, 138 matched controls	Copenaghen, Denmark	There was an inverse association between risk of MS and birth order position: birth order 1 and 2 were more frequent in MS, birth order greater than 2 occurred more commonly in controls.
Birth order	Alperovitch 1981	Comparison of birth order with expected distribution	107 MS patients	France	The observed birth order was significantly greater in MS patients than expected.
Birth order	Visscher 1982	1) Case-control study 2) Comparison of birth order with expected distribution	1) 65 MS patients who had at least one sibling (born between 1905 and 1956), 65 matched controls with at least one sibling 2) 2,119 MS patients born between 1887 and 1954	USA	1) The proportion of birth-order positions 1 and 2 in MS cases (75.38) was higher than that in controls (61.54) but without statistical significance. 2) The distribution of birth-order positions was not significantly different from that expected.
Birth order	Zilber 1988	1) Case-control study 2) Comparison of birth order with expected distribution	93 MS patients, 93 matched controls, 369 MS patients who were born abroad	Israel	1) The mean birth order among Israel-born MS patients was significantly smaller ($p < 0.007$). 2) The mean expected birth order for all patients was 2.38 while the mean observed birth order was 2.11 ($p < 0.065$) due to an excess of first-born among patients. In MS patients born abroad but who developed the disease in Israel, MS was connected with an excess of last-born and high birth order.
Birth order	Gaudet 1995	Case-control study	88 sibships having 2 or more cases of MS (187 siblings with MS -88 'first' cases; 99 'additional' cases- and 205 'unaffected' siblings born after the 'first' case of MS)	Ontario, Canada	MS patients were randomly ordered by birth. Affected siblings were no more likely to be closer in birth order position than expected by chance.
Sibship size, n° of older and younger siblings	Montgomery 2004	Case-control study	4,443 MS patients, 24,194 controls	Sweden	There was an inverse association between MS risk and number of siblings: having 3 or more younger or older siblings, compared with none, is associated with reduced MS risk. The risk of MS was reduced among different-sex twins.
Birth order	Ahlgren 2005	1) Comparison of birth order with expected distribution 2) Population-controlled Study	258 MS patients with at least one sibling (of whom 211 with definite or probable MS).	Sweden	1) There was no association between MS (n = 211) and birth order ($p = 0.1411$). The observed number of first-born patients did not differ significantly from the expected number (p = 0.0871). When definite, probable and possible MS (n = 258) were included, there was a significant low number of first-borns ($p = 0.0475$) and a significant high birth order compared with the expected values ($p = 0.0381$). 2) No significant association was found between birth order and MS; possible tendency for a higher birth order the MS patients ($p = 0.0742$).
Birth order	Sadovnik 2005	Longitudinal cohort study	10,995 MS patients and 26,336 healthy siblings Simplex group: 9,381 index cases, 24,014 healthy siblings	Canada	There was no association between birth order and MS. In simplex sibships of at least 7 siblings, slightly more siblings who were born late in the birth order had MS; the same was found for the first-born sibling with MS in a multiplex sibship. Siblings with MS were slightly younger than those without MS.
Duration of contact with younger siblings aged less than 2 years in the first 6 years of life	Ponsonby 2005a	Case-control study	136 MS patients, 272 controls	Tasmania, Australia	Increasing duration of contact with a younger sibling aged less than 2 years in the first 6 years of life was associated with reduced MS risk: increasing number of younger siblings was strongly associated with a reduced risk of MS with a dose-response relation, no younger sibling effect was evident if the nearest younger sibling was born more than 6 years after the subject. Among cases, higher infant contact in early childhood was associated with delayed MS onset.
Sibship size, n° of older and younger siblings, age distance from the nearest younger sibling, years exposed to younger siblings < 2 years of age	Bager 2006	Population-based cohort study	1,036 MS patients out of 1,903,625 people	Denmark	There was no association between MS and n° of older or younger siblings, total siblings, being a member of a multiple birth and years exposed to younger siblings <2.
Birth order	Conradi 2011	Case-control study	245 MS patients, 296 controls	Berlin, German	Having at least two older siblings was associated with a lower MS risk: late birth order protects against MS.
Birth order, sibship size	Hughes 2013	Case-control study	279 patients with first clinical diagnosis of CNS demyelination (FCD), 539 controls	Australia	Having younger siblings was associated with reduced FCD risk, but there were no associations between sibship size, having older siblings and FCD.

support the hypothesis that contacts with infants in early childhood can modulate the infection patterns and the related immune response, reducing the risk of MS [Ponsonby 2005a]. The conflicting evidence between studies could be explained by different methodologies used including the outcome measure. The birth order, which has been widely used as outcome especially by older works, is subject to error as a proxy measure for exposure to younger infant siblings and probably should not be used alone as a surrogate for childhood infections [Ponsonby 2005b]. Moreover, it must be considered that the protective effect of infections in early life is possibly modulated by many factors: the kind of infection, the age at the infection, the stage of immunological development, history of infectious exposure, and the sequence of viral infections, which could all be influenced by the sibship. Regardless of the effect that sibship may have on MS risk, it appears to influence the development of the immune response against different microorganisms.

The exposure to infant siblings has indeed been associated with a reduced IgG response to EBV and reduced risk of infectious mononucleosis (IM) which is a risk factor for MS [Ponsonby 2005a]. Moreover, having siblings has been related to the gut microbiota composition during early phases of life as infants with siblings differed from only children in the microbiota profile [Martin 2016, Hasegawa 2017].

1.5 The gut microbiota

Microbiota immunomodulant effect

The term microbiota refers to the set of microorganisms collected within an anatomical niche; these are symbiotic microorganisms that coexist with the human organism without causing damage. The entire genetic heritage of the microbiota is known as microbiome. The gastro-intestinal (GI) tract contains more than 100 trillion microorganisms, mostly located in the colon and the microbiome is composed of at least 100 times more genes than the human genome [Glenn 2016]. The microbiotic community is made up of bacteria, archaea, eukaryotes, fungi and viruses. Over 400 different species of bacteria belonging to 10 phyla constitute the gut microbiota; the main phyla are Firmicutes, Bacteroides, Proteobacteria and Actinobacteria, the first two are the most represented ones in healthy adults (**Table 1.4**). Some bacteria have been indeed associated to a healthy condition: Bacteroides, Bifidobacterium, Clostridium cluster XIVa and IVa (which are butyrate producers), Eubacterium, Faecalibacterium, Lactobacillus and Roseburia [Hollister 2014]. An unbalanced ratio between Bacteroidetes and Firmicutes, on the contrary, has been involved in dysbiosis development, which is related to various pathological conditions [Costea 2018]. The colonization of the developing GI tract begins through the swallowing of the amniotic fluid by the fetus. After childbirth, microbiota diversity

gradually increases to culminate during adolescence, then it remains almost stable over the years. The first colonization is crucial because it promotes the development of the intestinal epithelium and the immune system [Glenn 2016]. During childhood, microbiota is mostly constituted of Bifidobacterium, with growth its composition changes towards that of adulthood. Many factors may influence the microbiota composition across the lifespan, including early-life factors (mode of delivery, gestational age, breastfeeding, sibship), geographic location, lifestyle (especially diet), stress, diseases and treatments (e.g.., antibiotic therapy, proton pump inhibitors) [Cresci 2015]. The early-life factors most predictive of a "beneficial" gut microbiota (highest abundance of Bifidobacteria and lowest abundance of Clostridium difficile and Escherichia Coli) were term birth, natural birth, home birth and exclusive breastfeeding [Penders 2006].

Table 1.4 Bacterial phyla and most represented genera of the gut microbiota.

Phylum	Gram stain	Main genera
Actinobacteria	Gram +	Bifidobacterium, Collinsella, Eggerthella, Slackia, Propionibacterium
Bacteroidetes	Gram -	Bacteroides, Prevotella, Corynebacterium, Butyricimonas, Parabacteroides, Alistipes
Cyanobacteria	Gram -	
Firmicutes	Gram +	Mycoplasma, Bacillus, Clostridium, Dorea, Faecalibacterium, Ruminococcus, Eubacterium, Staphylococcus, Streptococcus, Lactobacillus, Gemella, Lactococcus, Enterococcus, Sporobacter, Roseburia, Lachnospira, Blautia, Anaerostipes
Fusobacteria	Gram -	Sneathia
Lentisphaerae	Gram -	
Proteobacteria	Gram -	Escherichia, Klebsiella, Shigella, Salmonella, Citrobacter, Helicobacter, Serratia, Sutterella
Spirochaeates	Gram -	
Tenericutes	Gram -	
Verrucomicrobia	Gram -	Akkermansia

Naturally born infants show a preponderance of Bacteroidetes over Firmicutes compared to those born by cesarean section [Jakobsson 2014]. Moreover, having older siblings has been associated with a greater proportion of Bifidobacteria compared to having no siblings [Penders 2006]. These results were confirmed by a subsequent study finding that infants with siblings tended to have a Bifidobacterium-dominant profile of the gut microbiota while children without siblings had an Escherichia-dominant profile [Hasegawa 2017]. The maturation steps of the gut microbiota occur from birth during early infancy until 3-5 years of age when the microbiome reaches an adult-like composition [Dogra 2021].Many conditions may affect the microbiota causing dysbiosis; infectious and antibiotic use are responsible for acute dysbiosis while unbalanced diets (high-protein diets or diets with too many carbohydrates), unhealthy lifestyles protracted over time (limited physical activity, smoking, alcohol abuse) and pharmacological treatments (oral contraceptives, proton pump inhibitors, cortisone) may induce chronic dysbiosis. As dysbiosis may cause endotoxemia and a consequent intestinal or systemic inflammatory condition, it could also trigger neuroinflammation. The gut microbiota exerts several actions in order to maintain the human well-being:

- it guarantees the integrity of the intestinal barrier;
- it acts as a barrier against the colonization of pathogens;
- it acts as a "metabolic organ" allowing the use of energy resources, the vitamins biosynthesis, the transformation of bile salts and the metabolism of xenobiotics;
- it is involved in the development and maturation of the CNS;
- it influences the host's immune response [Castillo-Alvarez 2017].

The intestinal barrier consists of the intestinal epithelium and the adherent mucus layer; epithelial cells are connected to each other via intercellular junctional complexes, known as tight junctions. Those junctions guarantee to the barrier a certain selectivity, they preserve the integrity and regulate the paracellular permeability. An increase in the barrier permeability can cause greater exposure of the organism to toxic substances, allergens and pathogens. The gut microbiota and its metabolites, such as short chain fatty acids (SCFAs), are capable to influence the intestinal barrier permeability. A high-fiber diet promotes the proliferation of commensal bacteria capable of degrading plant polysaccharides into SCFAs and limits the pathogens access to the gut epithelium. The members of the Bacteroidetes phylum (Bacteroides, Prevotella) mainly produce acetate and propionate, while Firmicutes produces butyrate (Coprococcus, Clostridium, Dorea, Eubacterium, Lachnospira, Roseburia, Ruminococcus, Faecalibacterium). The SCFAs have both metabolic and immune functions and may modulate the brain function through different pathways (**Figure 1.6**), furthermore, butyrate constitutes the main nutrient of the cholic epithelium. Through the activation of free fatty acid receptors (FFARs) of immune cells, SCFAs locally influence intestinal mucosal immunity and barrier [Dalile 2019]. SCFAs

immunomodulating actions consists in: i) induction of mucus production by epithelial cells, ii) induction of IgA secretion by B lymphocytes, iii) stimulation of tissue repair, iv) development of Treg cell facilitating immunological tolerance by inducing FoxP3 transcriptional factor, v) inhibition of the proinflammatory transcriptional factor NF-kB and vi) enhancement of epithelial integrity by upregulating the expression of tight junction proteins and augmenting transepithelial electrical resistance (TEER) [Thorburn 2014]. SCFAs may also influence systemic immunity through interleukins secretion regulation and brain activity and human behavior (e.g., memory, learning, food intake, mood) through the gut hormones glucagon-like peptide 1 (GLP1) and peptide YY (PYY) produced by enteroendocrine cells. SCFAs can cross the blood-brain-barrier (BBB) and influence its integrity inhibiting inflammatory pathways; moreover, their property of modulating neurotrophic factors makes them capable of regulate growth, survival and differentiations of neurons [Dalile 2019]. At last, SCFAs are able to cross the placenta reaching the developing fetus or be delivered via breast milk influencing gene expression and the development of the immune system. Beside SCFAs, other products of the gut microbiota with symbiotic action are polysaccharide A, which stimulates the host's immunological development, and the microbial anti-inflammatory molecule (MAM), an inhibitor of NF-kB, produced by Faecalibacterium prausnitzii [Quévrain 2016]. Lactobacilli are instead capable of synthetizing K and B vitamins and are involved in the metabolism of cholesterol and other substances (e.g., xenobiotics, drugs, antibiotics) [Kang 2013]. Commensal flora may synthetize neuroactive substances such as serotonin, melatonin, GABA, histamine, acetylcholine, dopamine and noradrenaline [Evans 2013]. The concept of "gut-brain axis" refers to this bidirectional connection between the CNS and the gastro-enteric system mediated by nutrients, neuroendocrine and immunological signals. The nervous system itself can affect the microbiota by the control over food intake through satiety signals; the hypothalamic-pituitary-adrenal axis and the autonomic nervous system may instead influence the intestinal motility, secretions and permeability [Fung 2017, Wang 2014]. The gut microbiota has been therefore proposed to be involved in several neurological diseases through the activation of the NRLP3 inflammasome by the enteric bacteria with a secondary inflammatory response generating CNS neuroinflammation and neurodegeneration [Pellegrini 2020]. In particular, studies on animal models demonstrated a direct role of the gut microbiota in determining the immune response against nervous system influencing the development and the course of the experimental autoimmune encephalomyelitis (EAE) in mice. The oral administration of broad-spectrum antibiotics has a protective effect against EAE, specifically the mice treated with oral antibiotics develop a milder disease than untreated ones; this effect is lost when antibiotic therapy is administered intraperitoneally suggesting a direct role of the gut microbiota [Ochoa-Repáraz 2010a].
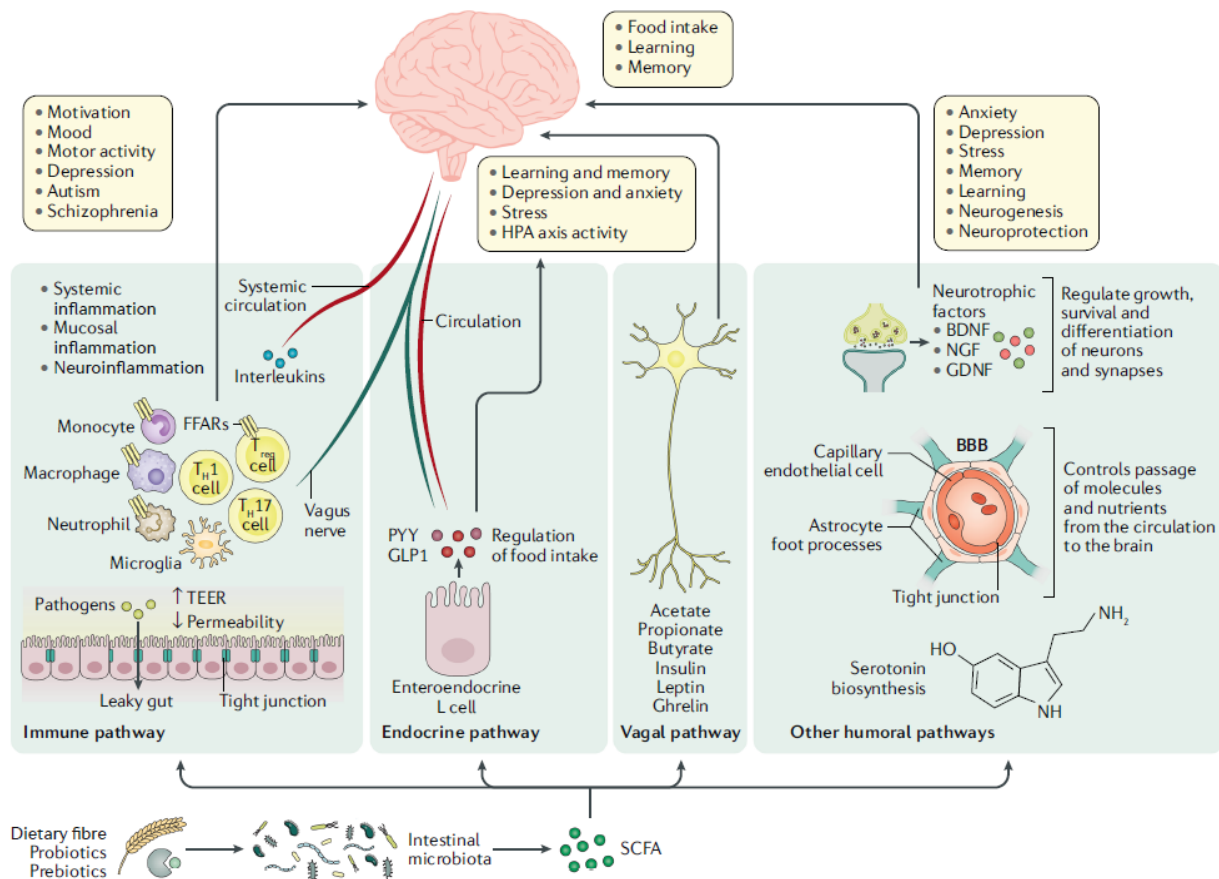
Figure 1.6 Actions of SCFAs on brain function through immune, endocrine, vagal and other humoral pathways [Dalile 2019].
FFARs, free fatty acid receptors; TEER, transepithelial electrical resistance; BDNF, brain- derived neurotrophic factor; GDNF, glial cell line- derived neurotrophic factor; HPA, hypothalamus–pituitary–adrenal; NGF, nerve growth factor; TH1, T helper 1; TH17, T helper 17; Treg cell, regulatory T cell.

Animals raised in germ-free conditions are resistant to the induction of EAE and show an attenuated immunological response, this effect is lost following repopulation of the intestine by the commensal bacterial flora [Berer 2011, Lee 2011]. Moreover, the transfer of the gut microbiota from MS subjects to transgenic mice expressing a myelin-specific T-cell receptor induced the development of an autoimmune reaction directed against the CNS while no immune response was induced after a fecal transplant from healthy subjects [Berer 2017, Cekanaviciute 2017]. Capsular polysaccharide A, derived from Bacteroides fragilis, has been demonstrated to have immunoregulatory properties and suppresses the EAE neuroinflammation through the expansion of CD4 CD39 + Treg cells [Ochoa-Reparáz 2010b].

Considering these findings, it is easy to see why interest in the role of the gut microbiota in the etiopathogenesis of MS is rapidly increasing.

Microbiota analysis methods

In the past, through the use of conventional techniques based on fecal culture examination, it was possible to isolate only 10-25% of the microbiota bacteria since most of the gastrointestinal microorganisms are anaerobes. After the improvement of the cultivation techniques of anaerobic germs, it has become possible to identify some abundant genera such as Bacteroides, Clostridium and Bifidobacterium. More recently, thanks to high-throughput genetic sequencing techniques, great strides have been made in defining the composition of the microbiota. Currently, two different approaches are used for the microbiota analysis: the complete sequencing of the microbiome (metagenomic approach) and the sequencing of some genes considered phylogenetically significant, defined as markers. The first approach consists in the sequencing of the genome of all intestinal microorganisms, it is rarely performed as it has high costs. In most cases, the analysis aimed at characterizing bacterial communities in terms of species, is carried out by sequencing the 16S ribosomal RNA (rRNA) gene. The 16S ribosomal gene is present in all living organisms, it is small in size (1.5 Kb) and has both conserved and hypervariable regions. The conserved regions are common to all organisms and allow to distinguish the 16S gene from the entire genome while the variable regions let to identify the taxonomic identity of organisms down to family or genus level. The mainly used variable regions are V3, V4, V6 and V8. The 16S rRNA is sequenced through Next Generation Sequencing (NGS) platforms. Leading NGS technologies include Roche-454, Illumina and AB Solid [Liu 2012]. NGS sequencing is capable of providing a large amount of data with good accuracy but it is not free from errors, which most likely stem from library preparation methods and primer choice [Shirmer 2015]. Once the sequencing is complete, bioinformatics analysis follows: similar sequences are grouped into Operational Taxonomic Units (OTUs), a process known as "clustering". The OTUs abundance is estimated on the basis of the number of corresponding sequences [Jandhyala 2015]. OTUs with a diversity up to 3% are generally considered representative of the same species and up to 5% of the same genus. The number of obtained OTUs is an index of the abundance and taxonomic diversity of the microbial community. The term biodiversity means the variety of living organisms that populate a certain environment; we can distinguish three subtypes of biodiversity: α, β, and γ. The α diversity represents the diversity within a habitat or community on a local scale, it describes the number of species and the degree of distribution of abundances among the individual species of a community. The β diversity is the ratio between regional and local species diversity and it is a measure of the variations in the composition and abundance of species between two distinct habitats of an ecosystem. Finally, the γ diversity represents the total specific diversity of a large area, it is calculated by the product between the diversity α and β. In general, the number of species observed through a sampling is not always the best way to estimate the real specific

richness of a certain area; one of the most used methods to manage an unequal sampling intensity is the rarefaction of data. Plotting the total number of sampled species, we obtain an accumulation curve: at first the curve will have an exponentially rising trend due to the collection of the most common species, subsequently, when all the present species are sampled, it will reach an asymptote which identifies the optimal number of samples (reads) to be obtained. The rarefaction is a resampling procedure that randomly selects *n* sampling units, until all sampling units of the reference sample have been accumulated. The rarefaction curve can be considered as a statistical estimate of the respective accumulation curve, it shows the expected number of species in a small group of samples, taken randomly from the total number *n* of the samples detected (**Figure 1.7**). The raw data of specific diversity can only be compared when the curve reaches the asymptotic phase.
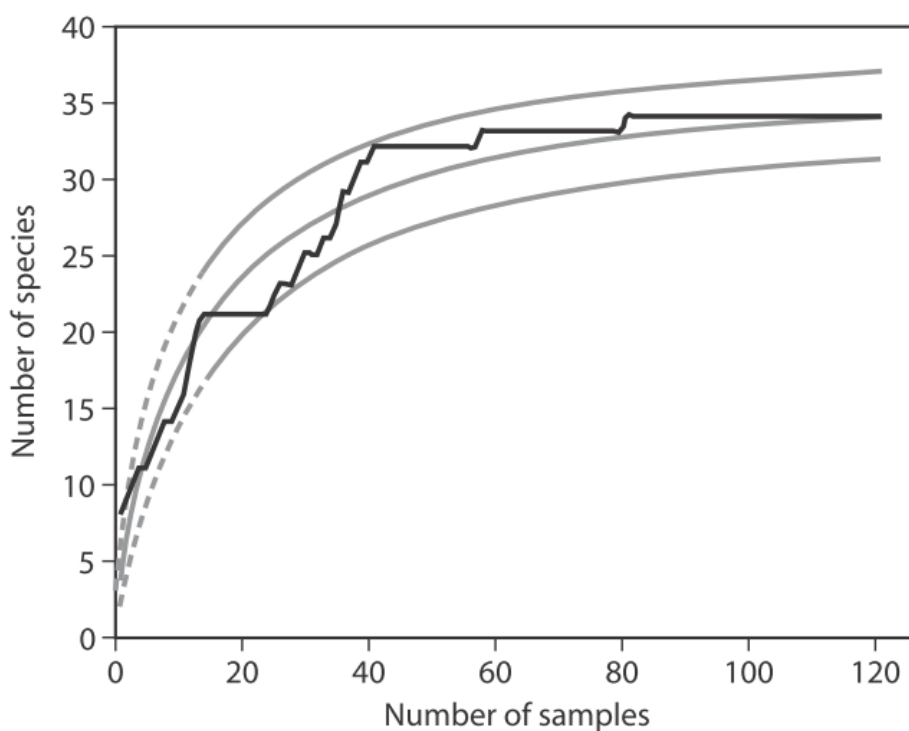
Figure 1.7 Example of rarefaction curves (gray) and of the respective accumulation curve (black). The abscissa axis represents the number of samplings or reads, the ordinates axis the number of observed species.

Different indices have been developed to measure the biodiversity including the Shannon, Simpson, Evenness and Chao1 indices. These non-parametric indicators take into account, not only the absolute abundance, but above all the relative abundance of OTUs, therefore rare OTUs are also important for biodiversity: the number of rare species found in a sample is used to calculate the likelihood that there are other undiscovered species [Colwell 1994]. Chao1 calculates the real estimated diversity of the species of a sample through the equation:

$$S_1 = S_{obs} + \frac{F_1^2}{2F_2}$$

S_{obs} is the number of observed species within the sample; F1 and F2 represent the number of species with a single occurrence (singletons) and two occurrences (doubletons) respectively in the examined sample. As part of the sampling, the occurrence of singletons, which are rare species, raises the suspicion that there are still other species to be discovered; as soon as all the species have been sampled twice, it is highly probable that there are no more other species to be identified within the sample.

Microbiota characteristics in MS patients

Microbiota richness and biodiversity are recognized as health indicators; however, a clear definition of a healthy microbiota is lacking due to the high interindividual variability on the microbiota composition, even within healthy people; this uniqueness of each individual's microbial community may even constitute a healthy property [Human Microbiome Project Consortium 2012, Le Chatelier 2013]. Some findings suggest that in neurodegenerative diseases the microbiota is different from that of healthy subjects and especially oriented toward a proinflammatory profile [Pellegrini 2018]. As for MS, most of evidence derives from preclinical investigations or from studies with limited sample size of patients [Castillo-Alvarez 2017]; nevertheless, it has been suggested that MS patients, both adults and children, have unbalanced gut microbiota which is different from that of healthy controls (HC) (**Table 1.5**) [Chen 2016, Miyake 2015, Jangi 2016, Cantarel 2015, Tremlett 2016a, Cekanaviciute 2017]. Even if hypothesis have been made on the unbalanced ratio between the phyla Bacteroidetes and Firmicutes in MS patients, this has not been demonstrated by clinical researches. A few evidence support a greater abundance of the phylum Actinobacteria in MS while most studies did not find significant differences in phyla representation. Most of differences between MS and controls regards families or genera relative abundance. Overall, in MS population have been described:

-higher level of:

> genus Bifidobacterium (phylum: Actinobacteria);
>
> genera Bacteroides, Parabacteroides (phylum: Bacteroidetes)
>
> genera Dorea, Blautia, Ruminococcus, Christensenellaceae, Streptococcus (phylum: Firmicutes);
>
> genera Psuedomonas, Mycoplana, Haemophilus, Bilophilia, Desulfovibrio, Acinetobacter (phylum: Proteobacteria);
>
> genus Akkermansia (phylum: Verrucomicrobia);
>
> Methanobrevibacter (Archaea)

-lower level of:

> genera Collinsella, Adlercreutzia, Slackia (phylum: Actinobacteria);

> Bacteroidaceae family, genera Bacteroides, Parabacteroides, Butyricimonas (phylum: Bacteroidetes)

> Lachnospiraceae, Ruminococcaceae family, Clostridium cluster XIVa, Clostridium cluster IV, genera Faecalibacterium, Prevotella, Anaerostipes, Faecalibacterium, (phylum: Firmicutes, order: Clostridiales).

Table 1.5 Studies on the gut microbiota characteristics in MS population.

Reference	Study design	Population size	Geographical location	Results
Chen 2016	Case-control study	31 RRMS patients (14 treated with IFNβ, 5 with NTZ, one with GA, 11 patients not treated) of whom 12 patients with active disease and 19 in remission; 36 HC	USA	MS patients had higher level of Psuedomonas, Mycoplana, Haemophilus, Blautia and Dorea. HC were more enriched with Parabacteroides, Adlercreutzia and Prevotella.
Miyake 2015	Case-control study	20 RRMS patients, 40 HC. 18 HC for longitudinally sampling (158 samples).	Japan	Greater interindividual variability in MS patients compared to HC. Non statistically significant higher abundance of the phylum Actinobacteria and lower abundance of Bacteroidetes and Firmicutes. At genus level Bacteroides, Faecalibacterium, Prevotella and Anaerostipes were reduced in MS patients while Bifidobacterium and Streptococcus were increased compared to HC. 21 species were statistically different between MS and HC, 15 species belonged to Firmicutes of which 14 to Clostridium cluster XIVa and Clostridium cluster IV, all of them were reduced in MS.
Jangi 2016	Case-control study	60 MS patients (28 non treated, 18 treated with IFNβ and 14 with GA), 43 HC	USA	Increased abundance of Methanobrevibacteriaceae (Archaea) and Akkermansia, decreased Butyricimonas in MS compared to HC. Collinsella, Slackia and Prevotella were decreased in untreated MS patients vs HC.
Cantarel 2015	Case-control cross-sectional study and longitudinal prospective study (samples collected at baseline and 90 days after starting vitamin D supplmentation)	7 RRMS patients with vitamin D deficiency (5 treated with GA, 2 untreated), 8 HC with vitamin D deficiency Samples of 4 RRMS patients (2 treated with GA) were available for longitudinal evaluation	USA	Different microbiota composition between MS patients and HC: in the group of MS patients, before the start of vitamin D supplementation, there were a lower number of OTUs classified as Bacteroidaceae and Faecalibacterium and a higher number of Ruminococcus OTUs.
Tremlett 2016a	Case-control study	18 children ≤18 years old within two years of MS onset (5 treated with GA, 3 with IFNβ, 1 with NTZ), 17 HC	USA, Canada	MS patients had greater abundance of the phylum Actinobacteria, the genera Akkermansia, Parabacteroides, Bacteroides, family Desulfovibrionaceae (Bilophilia, Desulfovibrio), Christensenellaceae and Methanobrevibacter (Archaea). Conversely, the orders Bacteroidales and Clostridiales (Lachnospiraceae and Ruminococcaceae) were lower compared to HC.
Cekanaviciute 2017	Case-control study	71 untreated MS patients, 71 HC	USA	Akkermansia muciniphila and Acinetobacter calcoaceticus were increased and Parabacteroides distasonis was reduced in MS patients

IFN, interferon; GA, Glatiramer acetate; NTZ, Natalizumab; HC, healthy controls.

Clostridiales order have received particular attention in the field of demyelinating diseases. Clostridium perfringens is an anaerobic bacillus which can be classified into 5 subclasses based on the type of exotoxin produced. Type A usually colonize the human intestine, its prevalence

is about 63% of healthy individuals while type B and D which produce epsilon toxin (ETX) colonize mostly the intestines of ruminant animals (e.g., sheep, goats, cattle). ETX is a strong neurotoxin secreted as an inactive precursor, after the cleavage by trypsin, chymotrypsin or lambda toxin enzymes, it crosses the intestinal barrier, enters the bloodstream and binds to receptors on the surface of brain endothelial cells. Here it oligomerizes to form a heptameric pore, responsible for an increase in the permeability of the BBB [Popoff 2011]. Clostridium perfringens may also act directly on oligodendrocytes, binding and damaging the myelin [Wioland 2015]. Rumah and colleagues, for the first time, described a case of human carrier of Clostridium perfringens type B in a MS patient with disease onset within the previous 3 months. They then decided to study the prevalence of Clostridium perfringens type A in 30 MS patients and 31 HC founding a reduced prevalence of type A in MS group compared to HC (23% vs 52%). They also evaluated the immunoreactivity against ETX in serum and CSF obtained from banked samples: an increased immunoreactivity to ETX was detected in MS population (10% vs 1%) [Rumah 2013]. Other data support these results suggesting a potential role of ETX in MS etiology [Wagley 2018].

Effects of the DMTs on the gut microbiota

Giving the increasing relevance of the immunomodulant role of the gut microbiota in MS pathogenesis, and the suggested different microbiota composition in MS patients compared to HC, it has been hypothesized that DMTs could normalize the commensal flora restoring a healthy balance. Indeed, some researchers' groups found microbiota modifications induced by immunomodulators, supporting their normalizing effect on the microbiota composition (**Table 1.6**) [Jangi 2016, Cantarel 2015, Tremlett 2016a, Katz Sand 2018, Storm-Larsen 2019]. Nevertheless, strong and conclusive results are lacking: most studies included a limited sample of patients and considered together treated and untreated MS patients or patients taking different DMTs regardless of their mechanism of action. Moreover, confounding lifestyle factors such as diet and smoking were never considered. Dimethyl fumarate (DMF) is one of the oral first-line DMTs used to treat RRMS. It is the methyl ester of fumaric acid, it is converted in the intestine to its active metabolite, monomethyl fumarate. Its action consists in the activation of the transcription pathway of nuclear factor erythroid 2 (Nrf2) which induces the expression of antioxidant gene, e.g., NAD (P) H dehydrogenase (quinone 1). DMF exert therefore neuroprotective action mediated by the increased glutathione levels and by the suppression of proinflammatory cytokines [Albrecht 2012]. The short-term modification of the transcriptome of mononuclear cells in peripheral blood, which reflects activation of Nrf2 and inhibition of the transcription factor NFkB, characterizes MS patients who will show a good response to treatment [Gafson 2018]. DMF has been also shown to possess an additional

immunomodulant action which is independent of Nrf2 [Schulze-Topphoff 2016]. There is some evidence on the effect of DMF on the gut microbiota from animal experiments: on mouse models DMF is capable of ameliorating the intestinal barrier function and integrity and has a favorable impact on biodiversity and on the abundance of bacteria producing SCFAs (e.g., Gemella, Roseburia, Bacillus and Bacteroides) [Ma 2017].

Table 1.6 Studies on the impact of DMTs for MS on the gut microbiota.

Reference	Study design	Population size	Geographical location	Results
Jangi 2016	Case-control study	60 MS patients (28 non treated, 18 treated with IFNβ and 14 with GA), 43 HC	USA	Treated MS patients had increased Prevotella and Sutterella, which were either significantly reduced or showed a trend of reduced populations in untreated patients compared with HC. The genus Sarcina was instead reduced in treated patients vs untreated.
Cantarel 2015	Case-control cross-sectional study and longitudinal prospective study (samples collected at baseline and 90 days after starting vitamin D supplmentation)	7 RRMS patients with vitamin D deficiency (5 treated with GA, 2 untreated), 8 HC with vitamin D deficiency Samples of 4 RRMS patients (2 treated with GA) were available for longitudinal evaluation	USA	Difference between treated with GA and untreated MS patients in the abundance of the family Bacteroidaceae and the genera Faecalibacterium, Ruminococcus, Lactobacillaceae and Clostridium. GA could affect vitamin D changes in the microbiota: treated MS subjects had increases in Janthinobacterium and decreases in Eubacterium and Ruminococcus after high-dose vitamin D supplementation. Compared to HC and GA-treated MS subjects, untreated MS patients had an increase in the Akkermansia, Faecalibacterium, and Coprococcus genera after vitamin D supplementation.
Tremlett 2016a	Case-control study	18 children ≤18 years old within two years of MS onset (5 treated with GA, 3 with IFNβ, 1 with NTZ), 17 HC	USA, Canada	Treated MS patients showed a greater biodiversity even if without statistical significance.
Katz Sand 2018	Cross-sectional study	168 RRMS patients (75 treatment-naive, 33 treated with DMF and 60 with GA)	USA	Both therapies were associated with decreased relative abundance of the Lachnospiraceae and Veillonellaceae families. DMF was associated with decreased relative abundance of the phyla Firmicutes and Fusobacteria and the order Clostridiales and an increase in the phylum Bacteroidetes.
Storm-Larsen 2019	Longitudinal prospective pilot study	36 RRMS (27 treated with DMF, 9 with injectable DMTs)	Norway	Trend towards normalization of the low abundance of butyrate-producing Faecalibacterium after 12 weeks treatment. In the DMF patients there was also a trend of reduced Actinobacteria at two weeks, mainly driven by Bifidobacterium.

Only two studies have focused on the effect of DMF on the gut microbiota in human MS population, one of these with longitudinal stool sampling during treatment up to 12 weeks of follow-up [Katz Sand 2018, Storm-Larsen 2019]. The findings by Katz Sand and collaborators were a reduced abundance of the phyla Firmicutes and Fusobacteria, the Lachnospiraceae and Veillonellaceae families and specifically the order Clostridiales while the phylum Bacteroidetes was increased in patients taking DMF [Katz Sand 2018]. Storm-Larsen described after the initiation of DMF a longitudinal increase in the abundance of butyrate-producing Faecalibacterium after 12 weeks and decrease of the phylum Actinobacteria after 2 weeks, mainly driven by Bifidobacterium [Storm-Larsen 2019].

Teriflunomide (TFN), another oral first-line DMTs, is an active metabolite of leflunomide which selectively and reversibly inhibits the dihydroorotate dehydrogenase, necessary for the synthesis of pyrimidines, with cytostatic effect on T and B cells [Dargahi 2017]. The therapeutic effect of TFN could also depends on other mechanisms: it reduces the expression of

APCs in the gut-associated lymphoid tissue (GALT) in the mouse. The GALT is a well-known reservoir of inflammatory cells which are involved in the induction of EAE. TFN-treated mice also showed a relative increase in CD39 + Foxp3 + T reg lymphocytes in the GALT compared to controls and those lymphocytes, when transplanted to other mice, could reduce EAE severity [Ochoa Repáraz 2016]. TFN is also capable of affecting the microglia density and oligodendrocyte differentiation by reducing inflammation and axonal damage [Pol 2019]. There is currently no evidence of an effect of TFN on the microbiota other than indirect evidence due to the absence of studies. Noteworthy is that oral DMTs, including DMF, TFN and Fingolimod, have been shown to suppress the growth of Clostridium perfringens *in vitro* [Rumah 2017].

As far as known, different treatments can act differently on the gut microbiota but further investigation are needed to clarify their effect and how it can contribute to therapeutic efficacy.

1.6 Environmental prognostic factors for MS and their interaction with the gut microbiota

Most of the environmental risk factors cited for the development of MS have also been shown to act as prognostic factors influencing disease progression, the risk of relapse or brain MRI activity.

Smoking

Smoking has been associated with several outcomes for MS: higher EDSS, accelerated conversion to SPMS, decreased brain volume and increased number and volume of contrast-enhancing lesions on MRI [Heydarpur 2018, Ramanujam 2015, Healy 2009, Manouchehrinia 2013, Kappus 2016, Pichler 2019, Horakova 2013, Ivashynka 2019]. Smoking habit is also capable of influencing the microbiota composition apparently decreasing the biodiversity and variably increasing the abundance of Proteobacteria and Bacteroidetes phyla, the genera Clostridium, Bacteroides, Prevotella and Ruminococcus and reducing Actinobacteria and Firmicutes phyla, the genera Bifidobacterium, Faecalibacterium prausnitzii, Lactococcus and Akkermansia muciniphila [Capurso 2017, Savin 2018, Yan 2021]. The effect of smoking may be mediated by oxidative stress, increased permeability of the intestinal barrier, alterations of mucin composition and changes in acid-base balance. Thus acting, it has been proposed that smoking causes inflammation through the increase of proinflammatory bacteria and decrease of microorganisms capable of producing SCFAs [Yan 2021].

Air pollution

In addition to smoking, although less studied, air pollutants have also been involved in MS prognosis, especially in the risk of clinical or neuroradiological relapses [Ashtari 2018, Bergamaschi 2017, Cortese 2020, Angelici 2016, Roux 2017, Jeanjean 2018, Mehrpour 2013, Oikonen 2003, Vojinović 2015, Carmona 2018]. Among other functions, the gut microbiota acts as a scavenger for toxic compounds including environmental pollutants. Those substances may, on the other hand, alter the microbiota composition: the GI tract is exposed to inhaled particulate matter (PM) as a consequence of the mucociliary clearance or ingestion of contaminated food and water. In animal models the exposure to gasoline vehicle exhaust caused reduced ileum biodiversity and increased Firmicutes compared to those exposed to wood-smoke or filtered air (control group), on the contrary the Firmicutes/Bacteroidetes ratio was decreased in the wood-smoke exposed group and about 1 in the control group. Moreover, the exposed mice showed increase of various Clostridia, Turcibacter, and Romboutsi genera, and decreased levels of unclassified Lachnospiraceae genera; those alterations were associated with increased markers of inflammatory response [Fitch 2020]. Other studies performed in animal models found an association between PM10 exposure and reduced microbiota biodiversity, increase of Verrucomicrobia and Akkermansia, Firmicutes, and decrease of Bacteroidetes and Bifidobacterium [Feng 2020]. It has been suggested that pollutant-induced alterations od the gut bacteria may contribute to their toxicity [Claus 2016].

Sun exposure and vitamin D

Sun exposure and vitamin D levels, which have been widely involved in MS pathogenesis, have also been proposed as protective factors for MS worsening and progression as they have been related to EDSS improvement and reduced clinical and MRI activity [Hempel 2017, Ascherio 2014, Mowry 2012, Simpson 2010, Mandia 2014, Horakova 2013, Vojinović 2015]. The vitamin D is crucial for both the immune system and the gut homeostasis. Vitamin D receptor (VDR) is expressed by several immune cells: CD4+ and CD8+ T cells, B cells, neutrophils and APCs. Vitamin D and VDR are strongly connected to the gut microbiota: they cooperate in the maintenance of intestinal barrier integrity reinforcing intracellular junctions, ensure clearance of pathogen bacteria and suppress the Th1/Th17 cells favoring Treg cells [Fakhoury 2020]. VDR knockout mice are indeed more vulnerable to lipopolysaccharides (LPS) and express higher levels of inflammatory cytokines [Malaguarnera 2020]. Moreover, the disruption of the VDR signaling promotes the development of chronic inflammatory condition of the intestine such as inflammatory bowel disease (IBD). The role of vitamin D in the maintenance of immune homeostasis seems to occur at least partially by interacting with the gut microbiota. Low vitamin D intake has been indeed related to increased LPS, while high vitamin D intake

was associated with an increase of the genus Prevotella and decrease of the genera Haemophilus and Veillonella. The abundances of Coprococcus and Bifdobacterium were on the contrary inversely correlated with vitamin D [Luthold 2017]. The microbiota may also influence vitamin D effect as microbial metabolites like SCFAs (e.g., butyrate) can upregulate the VDR signaling [Battistini 2020].

Diet and BMI

Both dietary habit and BMI can influence MS prognosis: calorie restriction has hormonal, metabolic and cytochemical consequences which modulate the immune system. Fat intake, nutritional state, overweight have been related to EDSS progression, T1-lesions volume and to therapeutical response to IFN-β [Swank 1990, Da Costa Silva 2018, Kvistad 2015, Kappus 2016]. Diet is indeed known to modulate the inflammatory state: food may contain various substances capable of activating or inhibiting nuclear receptors, transcriptional factors or enzymes which in turn orientate towards anabolism or catabolism condition. Catabolism, resulting from caloric restriction, is generally associated with health, conversely anabolism may predispose to inflammatory conditions. Therefore, a proinflammatory diet is characterized by high-calorie intake, it is rich in animal fats, red meat, simple sugars and salt and low in fiber; a low-calorie and basically vegetarian diet can be considered on the contrary anti-inflammatory [Riccio 2015, Riccio 2018, García-Montero 2021]. A low-calorie diet seems to reduce disease activity while a diet rich in salt increases the production of Th17 lymphocytes in mice and worsens the course of the disease in mice and humans [Kleinewietfeld 2013, Farez 2015]. In addition to eating habits, individual nutritional factors have been studied in the prognosis of MS, such as PUFAs, retinoic acid, polyphenols, curcumin, resveratrol, milk proteins and gluten [von Geldern 2012] (**Figure 1.8**). Saturated fatty acids (SFAs) have a molecular structure similar to that of LPS, they act by binding to toll-like receptor (TLR) 2 and TLR-4 and activating the transcription factor NFkB. *Trans* fatty acids, which are unsaturated fatty acids containing one or more double bonds with *trans* isomerism between two carbon atoms, are mostly present in hydrogenated vegetal oils and may interfere with the metabolism of natural unsaturated fatty acids, which have a cis configuration; their assumption causes inflammation and Th17 lymphocytes activation. In the mouse model, fatty acids modulate the development of EAE through their effect on the T cells of the small intestine [Haghikia 2015]. Moreover, in mice a salt-rich diet increases the production of Th17 lymphocytes and worsen the Red meat processed and preserved with nitrites, the formation of nitrous compounds may be high and N-glycolylneuraminic acid (Neu5Gc) and arachidonic acid may be present. The first is a salic acid not synthesized by the human body which could activate an inflammatory response, the arachidonic acid is a precursor of proinflammatory eicosanoids (e.g., prostaglandins, thromboxane, leukotrienes) which activates the Th17 pathway.
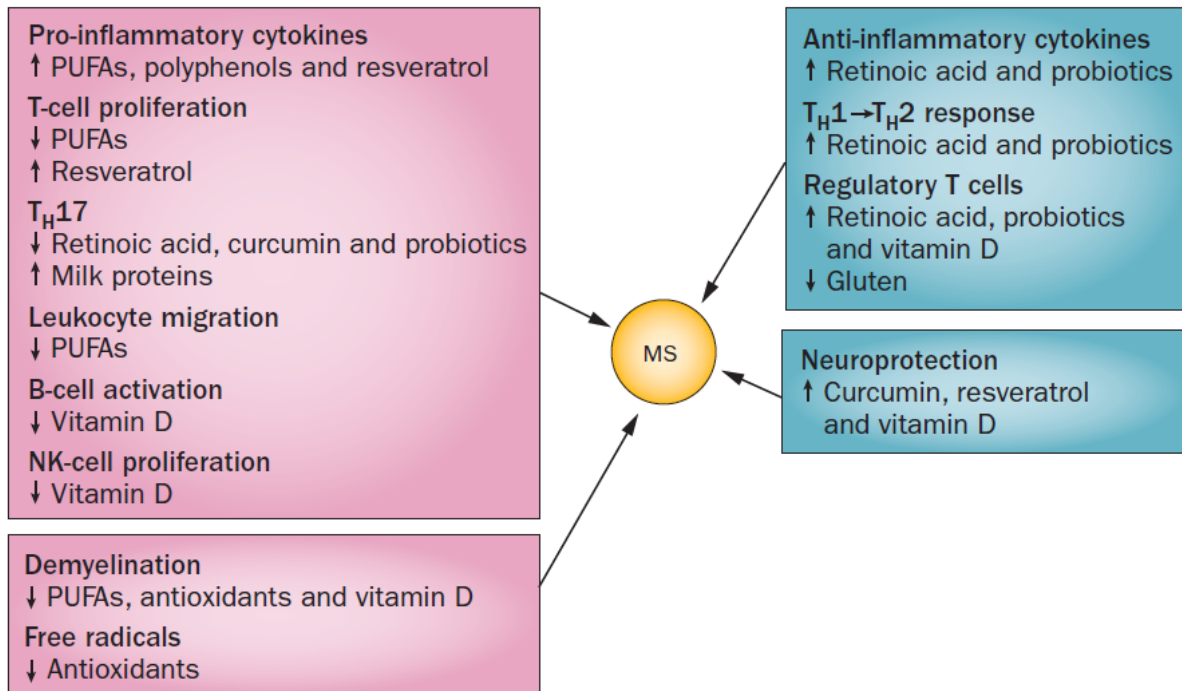
Figure 1.8 Nutritional factor and their potential influence on MS [von Geldern 2012].
On the left are shown the mechanisms promoting inflammation and demyelination, on the right those inhibiting the inflammatory activity and promoting neuroprotection.

The abundant intake of sugar and refined grains, along with a low-fiber diet, stimulates the production of insulin which, in turn, induces the biosynthesis of arachidonic acid. The proteins of the milk fat globule membrane (MFGM), like butyrophilin, are important for the development of the digestive, nervous and immune systems in infants. Butyrophilin is worthy of particular interest due to its similarity to MOG; both proteins may induce EAE and they share cross-reactive antibodies in MS which is the reason why adult people with MS should drink skimmed milk [Riccio 2018]. An anti-inflammatory effect is exerted by polyunsaturated fatty acids (PUFAs), Omega-3 fatty acids, vitamins (D, A, B12, C, E, PP), carotenoids, trace elements, thiol compounds (lipoic acid, glutathione, N-acetyl-cysteine) and polyphenols. Omega-3 fatty acids have an opposite action to Omega-6 and their ratio should be about 1; in the "Western diet", however, the Omega-6 / Omega-3 ratio tends to be increased. Among the carotenoids, lycopene is a powerful antioxidant and can form β-carotene and retinoic acid which, in turn, activates the anti-inflammatory retinoid X receptor (RXR). The trace elements selenium, zinc and magnesium contribute to the anti-inflammatory, immunomodulating and antioxidant mechanisms; zinc is directly involved in myelin formation. Polyphenols, contained in fruit, vegetables, cereals, legumes, herbs, wine, coffee, tea and chocolate, can be metabolized but not synthesized or stored by the host. They possess anti-inflammatory, antiangiogenic, antiviral properties and promote catabolism. Dietary tryptophan, an essential amino acid, is metabolized by the microbiota into agonists of the aryl hydrocarbon receptor (AHR); these

metabolites reduce inflammation in the CNS through the activation of the AHR signaling pathway in astrocytes. A reduction in circulating levels of AHR agonists has been demonstrated in MS patient [Rothammer 2016]. Probiotic and prebiotics can also influence the gut-brain-axis. Probiotics (e.g., Lactococcus lactis, Bifidobacterium lactis and Clostridium butyricum) may reduce the severity of EAE clinical symptoms [van den Hoogen 2017] and promote an anti-inflammatory response. Prebiotics like inulin and fructo-oligosaccharides (FOS) are non-digestible organic substances that act as nutrients for the bacterial flora by selectively stimulating the growth or activity of certain symbiotic species. It is known that diet deeply affect the microbiota composition and function. Fiber intake enhance the SCFAs production promoting the proliferation of the "good" commensal bacteria. Most Bacteroidetes, which are Gram negative, uses resistant starch and oligosaccharides from undigested fiber as an energy substrate; such bacteria produce SCFAs. The Gram positive Firmicutes are mostly unable to break down complex carbohydrates and prefer macronutrients of animal origin. This phylum is indeed most represented in people with a pro-inflammatory "Western diet" [Riccio 2018, Tilg 2009, De Filippo 2010]. A healthy diet should contain a high amount of fiber (above 10-15 g per day) and should be as varied as possible to encourage the growth of a greater number of different intestinal microbial species. On the contrary a high meat intake is associated with a reduction in intestinal microbial species. A high fats intake leads to a greater production of bile acids (e.g., deoxycholic and lithocholic acid); this environment leads to a selection of species resistant to bile acids while many of the bacteria that prefer a vegetarian diet do not survive [David 2014]. SFAs, similarly to LPS, can cause dysbiosis and endotoxemia. On the contrary, people with high vegetables intake, a greater abundance of Lachnospiraceae, a species of bacteria capable of promoting the differentiation of Treg lymphocytes and the production of IL10 and TGFβ, was found. This finding was also associated with lower relapse rate over 12 months of observation compared to patients on a "Western diet" [Saresella 2017]. Probiotics may act beneficially reducing the abundance of some bacterial genera linked to dysbiosis (Akkermansia, Blautia) and increase some taxa whose abundance is generally reduced in MS, such as Lactobacillus [Tankou 2018]. To summarize, the "Western diet" has been associated with higher bile acid production and reduced biodiversity while the vegetarian diet, rich in fiber, with greater biodiversity and increase in bacteria with saccharolytic properties (**Figure 1.9**). Dietary changes have therefore been proposed to influence MS prognosis via the gut microbiota [Vieira 2015].

Regardless of diet, obesity and BMI have been independently related to the gut microbiota composition: obesity is associated with reduced biodiversity and increased relative abundance of the phylum Bacteroidetes. Low biodiversity, on the other side, has been related to insulin resistance, high levels of cholesterol and marker of inflammation, weight gain and reduced

compound of butyrate-producing bacteria resulting in reduced production of Treg lymphocytes [Le Chatelier 2013, Mirzaei 2021]. These findings help explaining the association between obesity and overweight during childhood and MS pathogenesis.

VEGETARIAN/VEGAN DIET (V-DIET)
anti-inflammatory

Complex carbohydrates (fibers), vegetables, fruit, fish, legumes, + [probiotics, vitamins D & A, lipoic acid, caloric restriction, physical exercise].

WESTERN DIET (W-DIET)
pro-inflammatory

Animal fat, trans fatty acids, red meat, sweetened drinks and sugar, high salt.

GUT MICROBIOTA   **GUT MICROBIOTA**

GUT EUBIOSIS – HEALTH, WELLNESS

Increase of:
V-diet bacteria , Microbial diversity, SCFA, Butyrate, Polysaccharide A (PSA), Microbial anti-inflammatory molecule (MAM); Histone deacetylase inhibitor; AHR receptor agonists, Treg/Th17 ratio.

GUT DYSBIOSIS - ENDOTOXEMIA

Increase of:
W-diet bacteria, Energy harvest, Bile acids, LPS, TNF, IL-6, IL-17, Gut barrier permeability, BBB permeability.

Decrease of:
Microbial diversity, VDR availability.

Figure 1.9 Effect of diet on the gut microbiota [Riccio 2018].

As for alcohol intake, it has been suggested to have a protective effect against the progression of the disability in RRMS and brain atrophy even if some authors found also a faster increase in T2-lesion volume in subjects consuming from one to three glasses of red wine per week compared to nondrinkers [D'Hooghe 2012, Foster 2012, Diaz-Cruz 2017]. It is well known that alcohol is deeply related to several gastro-intestinal diseases; in the small intestine and in the colon, it can cause bacteria overgrowth, disruption of the intestinal barrier with LPS and endotoxins translocation, changes of mucus composition and also microbiota modifications with depletion of bacteria with anti-inflammatory properties. In human and animal studies, alcohol consumption has been mostly related to increase of Proteobacteria and Actinobacteria phyla and decrease of Firmicutes, Bacteroidetes and Akkermansia muciniphila. Some bacteria are able to produce alcohol themselves such as those belonging to the phylum Proteobacteria, which are increased in some pathological conditions [Capurso 2017].

Physical activity

Physical exercise has a positive impact on long-term progression of MS and has been associated to lower MS severity score (MSSS) [Stuifbergen 2006, Albatineh 2020]. It has been suggested that this positive effect of physical activity on various pathological conditions could be due in part to its anti-inflammatory effect mediated by the microbiota; it has even been suggested that the microbiota could mediate the cognitive impairment due to physical activity in elderly people [Sanborn 2020]. In animal models, moderate exercise can increase the biodiversity and modify the abundance of some genera [Carbajo-Pescador 2019, Yang 2021], moreover, the microbiota could improve the physical performances of mice: germ-free mice had worse exercise performance while mice colonized by multiple harmless bacteria showed higher endurance capacity [Codella 2017]. Human studies on this field included mostly professional athletes, little evidence exists on MS patients considering the influence of physical activity alone [Aya 2021], even so, physical exercise seems to increase the biodiversity and the production of SCFAs. It has even been proposed the existence of a bidirectional cross-talk between the skeletal muscle and the gut (gut-muscle axis) (**Figure 1.10**) [Ortiz-Alvarez 2020].
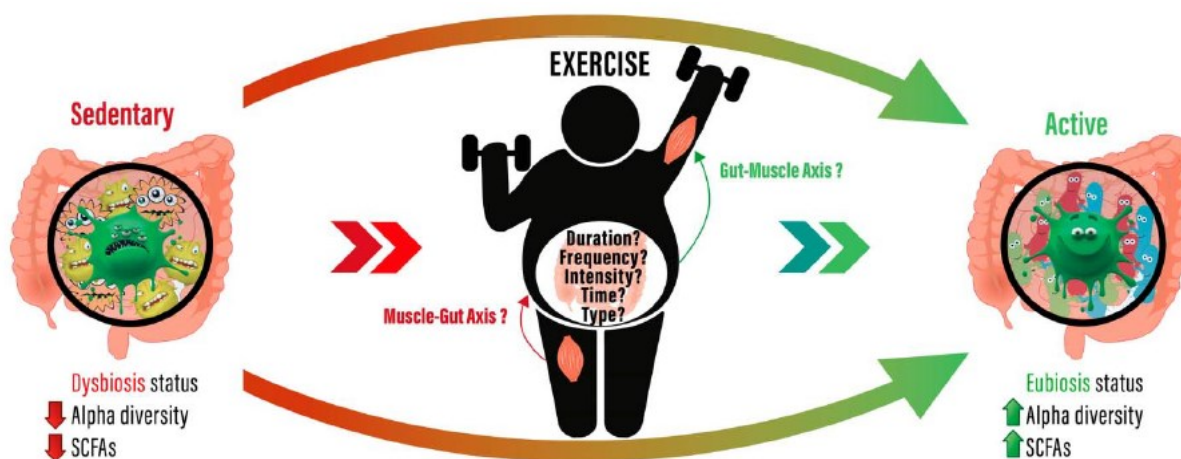
Figure 1.10 Effect of physical exercise on the gut microbiota [Ortiz-Alvarez 2020].

The prognostic role of the gut microbiota

This *excursus* on environmental risk or protective factors for MS points out to how the external environment and the gut microbiota do interact. Indeed, the gut microbiota has been proved to be susceptible to the action of almost all environmental factors involved in MS development and prognosis. The microbiota in turn is capable of modulating the immune system function, thus affecting the neuroinflammation. Some studies have demonstrated that microbiota composition tends to be different according to the MS phase (activity or remission) which could underlie a direct action of the microbiota on MS. A study including pediatric MS patients found

that Fusobacteria depletion was associated with relapse risk [Tremlett 2016b]; another work by Chen and colleagues detected a lower biodiversity in patients with recent clinical relapse compared to that in remission phase [Chen 2016]. Finally, a recently published study showed that both individual and network of gut microbes may influence MS activity measured with clinical relapse or new gadolinium enhancing lesions and new or enlarging T2-hyperintense lesions on MRI [Horton 2021].

Assuming that environmental factors can act through the microbiota on the MS prognosis, it follows that acting on the gut microbiota might affect the course of the disease. This is what some researchers have tried to do with dietary or lifestyle intervention [Barone 2021]. A different and drastic proposed approach consists in acting directly on the gut microbiota through fecal microbiota transplantation (FMT) from healthy donors to affected individuals. Consistent evidence on the efficacy of FMT in treating MS is still lacking even though promising results came from case reports and one longitudinal single-subject study (**Table 1.7**) [Borody 2011, Makkawi 2018, Engen 2020].

Table 1.7 Studies on the prognostic role of the gut microbiota in MS.

Prognostic factor	Reference	Study design	Population size	Geographical locations	Outcomes	Results
Microbiota	Tremlett 2016b	Cohort study	17 RR pediatric MS (9 relapses)	Canada	Clinical relapse	A shorter time to relapse was associated with Fusobacteria depletion, expansion of the Firmicutes and presence of the Archaea Euryarchaeota. After covariate adjustments for age and immunomodulatory drug exposure, only absence (vs presence) of Fusobacteria was associated with relapse risk.
Microbiota	Chen 2016	Case-control study	31 RRMS patients (14 treated with IFNβ, 5 with NTZ, one with GA, 11 patients not treated); of whom 12 patients with active disease and 19 in remission; 36 HC	USA	Clinical relapse (within one month prior to collection of the stool sample)	Patients with active disease tended to show a lower richness of microbial species as opposed to those who were in remission, who presented a biodiversity comparable to that of the control subjects.
Microbiota	Horton 2021	Cohort study	55 pediatric-onset MS patients	USA	Clinical relapse, MRI measures: new gadolinium enhancing lesions, new or enlarging T2-hyperintense lesions	Microbiota (individual and network of gut microbes) may influence MS activity: 5 microbes were associated with all three outcomes; 2 networks of cooccurring microbes were associated with a higher hazard of both MRI outcomes.
Microbiota - Fecal Microbiota Transplantation (FMT)	Borody 2011	Case report	3 MS patients	Australia	MS symptoms	Indirect evidence: dysbiosis may influence MS symptoms. Three patients with MS achieved durable symptom reversal with FMT for constipation.
Microbiota - Fecal Microbiota Transplantation (FMT)	Makkawi 2018	Case report	1 SPMS patient	Calgary, Canada	MS functional system score, EDSS	Indirect evidence: dysbiosis may influence MS course and disability. After FMT the EDSS score stabilized and over the following 10 years Functional System scores minimally improved.
Microbiota - Fecal Microbiota Transplantation (FMT)	Engen 2020	Single-subject longitudinal study	1 RRMS patient	Chicago, USA	Serum brain-derived-neurotrophic factor (BDNF), gait metric	FMT was associated with increased abundances of putative beneficial stool bacteria and SCFA metabolites, which were associated with increased BDNF levels and gait/walking metrics.

Chapter 2. Evidence from the EnvIMS study on the "hygiene hypothesis" in multiple sclerosis

2.1 Rationale and objectives

The concomitant increase in prevalence of both MS and allergic diseases in the last decades may find an explanation in the "hygiene hypothesis". The reduced contact with microorganisms and improved hygienic standard in high-income countries, especially during the early phases of life, contribute to deregulate the immune system which, receiving little stimuli from the outside, tends to improperly react against self or harmless antigens. This is the basis of atopic reactions and autoimmune diseases. MS and allergic diseases are mediated by different immune pathways: MS is mainly supported by a Th1-response while allergy by a Th2-pathway. These paths can inhibit each other, which raised the hypothesis that allergies could be protective against MS. To clarify the pathogenetic mechanisms underlying MS, many studies tried to assess whether this inverse association could indeed be observed. Most of the studied allergic disorders in association with MS were those involving the respiratory tract and asthma. The overall findings do not support a clear inverse association being quite conflictual and ranging from the absence of association to the negative or positive association. As for food allergies, definitely less studied, evidence supports a null or negative association with MS.

Beside the association between MS and allergies, another way to investigate the "hygiene hypothesis" and its influence on MS risk is to measure the impact of indicators of exposure to microorganisms during childhood. This goal has been achieved over the years through two approaches: i) starting from infection biomarkers (e.g., markers of previous or present infection by viruses, bacteria or parasites) which are however difficult to measure on a large scale, or ii) detecting early life factors suggestive of exposure to the well-known "old friends", more easily adapted to large-samples studies. Based on the evidence a reduced frequency of allergic conditions (eczema and hay fever) in subjects with higher sibship size, the sibship has been proposed as a risk factor for autoimmune diseases including MS. The sibship in MS patients has been therefore investigated over the past century. In particular, birth order has been the most frequently used outcome, but whether there is an association with MS onset in the general population is still under debate. While having older siblings has been reported in association to a reduced risk of MS onset in the general population, other findings suggest instead a lower such risk among individuals with younger siblings. Based on these premises, we aimed to investigate those factors related to the "hygiene hypothesis" to shed more light on the complex mechanisms underlying MS risk.

A case-control study is presented which has two main focuses, and namely the Study A on allergies and Study B on birth order and sibship. These investigations have been conducted over two populations of cases and controls, ie, the Italian and the Norwegian populations, potentially

differing from one another for distribution of environmental factors and MS incidence. Starting from the "hygiene hypothesis", the main objective of the Study A is to assess if a negative association exists between allergies during early phases of life or adolescence and MS incidence. Likewise, the main purpose of the Study B is to identify whether and to what extent sibship affect the risk for MS by reducing it.

2.2 Material and methods

The data have been collected through the EnvIMS study (Environmental risk factors in Multiple Sclerosis) that is a wide population-based multinational case-control study aimed to investigate past environmental exposures potentially influencing MS risk in the general population. Two thousand eight hundred patients with MS and 5,012 population-based controls were enrolled in 5 countries: Canada, Italy, Norway, Serbia and Sweden. For the current studies we analyzed the Italian and Norwegian populations. In these countries cases were identified from national or regional population-based MS registries. Inclusion criteria for cases were the diagnosis of MS according with McDonald or Poser criteria, age of 18 years or older, disease onset 10 years or less before the inclusion in the study. Population-based sources were also used to identify European controls, those of Canada were instead selected through random digit dialing using local telephone area code. Four controls matched on sex, age and geographical area were randomly selected for each case [Magalhaes 2015]. A 6-page self-administered questionnaire (EnvIMS-Q) was mailed to MS patients and control subjects to collect information regarding past environmental exposures and life-style characteristics, including sibship, education, smoking habit, diet, sun exposure, history of infectious mononucleosis, allergies, medical history and hormonal factors for women. The questionnaire was the same for cases and controls while differed between men and women for the presence in the female version of questions about pregnancy, hormone therapy and hirsutism. The EnvIMS-Q was shown to be cross-culturally acceptable, feasible and reliable [Pugliatti 2012].

The current study focused on allergies and sibship. The question on comorbid allergies allowed to collect the age of onset and the type of allergen involved (domestic dust, pollen, animal hair, food or other unspecified allergens). The age of onset was collected through a closed question consisting of six age ranges which were 0-5, 6-10, 11-15, 16-20, 21-25 and 26-30 years, respectively, for the Italian version (**Figure 2.1**). In the Norwegian versions of EnvIMS-Q age ranges referred to school periods, ie., 0-6, 7-12, 13-15, 16-18, 19-24 and 25-30 years, respectively.

The item on sibship was given by six spaces to enter the years of birth of siblings for participants who were not only children. This information, together with the year of birth of the study participant, whether a case or a control, allowed us know the participants' birth order and sibship size up to 6 siblings. (**Figure 2.2**).

6. Soffre o ha mai sofferto di allergia a qualcuna delle seguenti sostanze (per esempio ha mai avuto congiuntiviti, riniti, eczema, asma)?
Se si, indichi per cortesia a quale età ha avuto i primi sintomi di allergia.

	No	Non so	Si	0-5 anni	6-10 anni	11-15 anni	16-20 anni	21-25 anni	26-30 anni
Pollini	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Polvere domestica	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Forfora (pelo) di animale	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Cibi particolari	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Altro	☐	☐	☐ →	☐	☐	☐	☐	☐	☐

6. Har du hatt allergiske reaksjoner (øyekatarr, eksem, høysnue, astma) mot noen av det som er nevnt under? I så fall, angi omtrent hvilken alder du først merket disse symptomene

	Nei	Vet ikke	Ja	0-6 år	7-12 år	13-15 år	16-18 år	19-24 år	25-30 år
Pollen	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Husstøv	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Allergi mot kjæledyr og husdyr	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Mat	☐	☐	☐ →	☐	☐	☐	☐	☐	☐
Annen allergi	☐	☐	☐ →	☐	☐	☐	☐	☐	☐

Figure 2.1 EnvIMS-Q-Italian and Norwegian versions: questionnaire item on history of allergies.

5. Indichi l'anno di nascita dei Suoi fratelli, delle Sue sorelle e/o eventuali altri bambini che vivevano con Lei durante la Sua infanzia? Sono figlio unico ☐

	1	2	3	4	5	6
Anno di nascita:						

4. Fyll ut kjønn og fødselsår for hvert søsken (inkludert halvsøsken og adoptivsøsken): Jeg er enebarn ☐

	1	2	3	4	5	6
Fødselsår:						

Figure 2.2 EnvIMS-Q-Italian and Norwegian versions: questionnaire item on birth order and sibship size.

Statistical method

Descriptive statistics were used to present the demographic characteristics of the subjects: frequencies and percentages for categorical variables and average or median and standard deviation for continuous variables. Controls were randomly assigned an "index age" based on the distribution of age of MS onset in order to match them with cases for similar opportunity of exposure given that the MS cases would not be included in the analysis after clinical onset. Each population, ie., Italian and Norwegian, was analyzed separately.

The chi-square test was used to compare categorical variables and the Student's T test for continuous variables between cases and controls. The crude risk between MS and previous exposure to allergies or to siblings was estimated as odds ratios (ORs) with 95% confidence intervals (95%CI). The exposure to allergies was also analyzed by allergen: pollen, dust, animal hair and food. We also analyzed the association between allergies and MS by age of allergy onset (Study A). In the Study B, we considered the following variables: only child vs having siblings, having only younger vs having only older siblings, sibship size and interbirth interval between index children (study participants) and younger siblings (0-2 years, 3-6 years and 6+ years) compared with subjects

without younger siblings. Siblings with the same year of birth as the index child (interbirth interval=0 years) were included in the analysis along with younger siblings.

When applicable for deemed confounding, analyses were adjusted for age at study time, index age, sex, smoking habit, educational level (no/primary school, junior high school, high school and university degrees), exposure to mononucleosis (history of the disease vs no history of the disease), breastfeeding (no/less than 4 months vs. 4+ months), low sun exposure (at age 13 to 18 years in the Norwegian population and at age 0-5 years in the Italian population (Bjørnevik 2014)). The adjusted risk (OR_{adj}) for these potentially confounders or effect modifiers was calculated using logistic regression.

Statistical analysis was performed with SPSS Statistics for Windows, version 27 (IBM Corporation, Armonk, New York, USA).

Ethical approval

Ethics approval for the study of human subjects was obtained at each of the participating sites. Return of the questionnaire was considered to be implied consent.

2.3 Study A. Multiple sclerosis and history of allergy

Results

The studies were conducted on the Italian and Norwegian EnvIMS population of 2040 (707 cases and 1322 controls) and 2674 subjects (957 cases and 1717 controls), respectively. Of all the participants, we then excluded those who returned questionnaires with no information on allergy history. Therefore, the analyses were performed on 611 (86.4%) Italian cases vs 1161 (87.8%) controls, and 886 (92.6%) Norwegian cases vs 1616 (94.1%) controls. Demographic and clinical characteristics of the studied population are shown in the **Table 2.1**.

Table 2.1: Demographic and clinical characteristics of the study population of Italy and Norway.

	Italy			Norway		
	Cases N=707	**Controls N=1333**	*p*	**Cases N=957**	**Controls N=1717**	*p*
Age, years, mean (SD)	38.8 (10.1)	39.3 (10.7)	*0.310*	44.8 (10.5)	46.0 (10.8)	*0.006*
Female sex, N (%)	460 (65.1)	910 (68.3)	*0.143*	671 (70.1)	1256 (73.2)	*0.096*
Age at MS onset, years, mean (SD)	33.2 (10.1)	NA	-	37.6 (10.2)	N/A	-
Disease duration, years, mean (SD)	5.6 (2.7)	NA	-	7.2 (2.7)	N/A	-
Level of education, N (%)						
None/primary school	27 (3.9)	45 (3.5)	*0.057*	153 (16.3)	201 (11.9)	*0.000003*
Junior High School	236 (34.2)	403 (31.2)		385 (41.0)	601 (47.4)	
Senior High School/ University	428 (61.9)	842 (65.3)		402 (42.8)	890 (52.6)	
Smoking habit, N (%)						
Ever smoker	368 (54.3)	540 (42.5)	0.000001	637 (68.8)	885 (52.6)	*<0.000001*
Smoking habit at age 15 years N (%)						
Ever smoker	91 (13.4)	152 (12.0)	*0.346*	258 (27.9)	229 (13.6)	*<0.000001*
History of infectious mononucleosis, N (%)	63 (10.6)	70 (6.2)	*0.002*	134 (17.8)	155 (9.5)	*<0.000001*
Sun exposure during summer*, N (%)						
Seldom/Never	75 (11.3)	118 (9.3)	*0.023*	74 (11.4)	116 (9.2)	
Sometimes	163 (24.5)	263 (20.8)		159 (24.5)	262 (20.9)	
Quite often	262 (39.5)	499 (39.4)		253 (39.0)	496 (39.5)	
Very often	164 (24.7)	385 (30.4)		162 (25.0)	382 (30.4)	
Sun exposure during summer*, N (%)						
Low exposure	238 (35.8)	381 (30.1)	*0.010*	146 (17.8)	216 (14.7)	*0.051*
Breastfeeding, N (%)						
0-3 months	305 (55.8)	506 (48.7)	*0-008*	280 (29.3)	531 (30.9)	*0.380*
≥4 months	242 (44.2)	533 (51.3)		677 (70.7)	1186 (69.1)	

* Between 0 and 5 years of age in the summer in Italy; and between 13 and 18 years of age in Norway.

No significant differences were observed between cases and controls in the history of allergy for both populations. Food and respiratory allergies (allergy to pollen, dust and animal hair) were then considered separately. In the Italian EnvIMS population MS was inversely significantly associated with past exposure to food allergy after adjusting for sex, index age, cigarette smoking habit, history of mononucleosis, exposure to no or breastfeeding less than 4 months and low sun exposure between age 0 and 5 years (OR=0.64, p=0.036). Both crude and adjusted analyses, on both sexes and by sex, showed a tendency for an inverse association between MS and past exposure to food allergy. As for exposure to respiratory allergy no association was observed (**Table 2.2a**).

In the Norwegian EnvIMS population MS cases declared less frequent exposure to food allergy than controls (OR=0.77, p=0.033). In particular, such exposure in male MS patients was nearly half that in male controls even after adjusting for index age, cigarette smoking habit, history of mononucleosis, exposure to no or breastfeeding less than 4 months, low sun exposure at teen-age (OR_{adj}=0.51, p=0.040). Although all crude ORs were smaller than 1, no significant association was observed between MS and past history of respiratory allergy in both crude and adjusted analyses (**Table 2.2b**).

Exploring the Italian population for potential confounders or effect modifiers to the association between allergies and the risk for developing MS, an association was observed with some demographic and lifestyle factors. In particular, for respiratory allergies age at the study time, at age MS onset and disease duration were significantly higher in participants with no history of allergies (p=0.000006, p=0.0001, p=0.006, respectively). A history of allergy was also associated to female sex (OR=1.28, p=0.028), to history of mononucleosis (OR=1.80, p=0.003) and no/reduced breastfeeding (OR=1.33, p=0.014). Respiratory allergy was inversely associated to smoking habit (OR=0.70, p=0.001).

For food allergies, age at the study time and at age MS onset were significantly higher in participants with no history of allergies (p=0.000003, p=0.000008, respectively). A history of food allergy was also strongly associated to female sex (OR=2.15, p=0.000008), to history of mononucleosis (OR=3.16, p<0.000001) and also no/reduced breastfeeding (OR=1.52, p=0.013). Food allergy was not associated to smoking habit (p=0.768). Neither a history of respiratory nor of food allergies was found associated to low sun exposure nor level of education nor smoking habit in adolescence.

Interestingly, when low sun exposure at age 0-5 years was included in the regression analysis, a more significant association was observed between MS and past exposure to food allergy.

The analysis for interaction showed significant interactions between low sun exposure at age 0-5 years and i) having had mononucleosis (OR=2.93, p=0.014), ii) ever smoker condition (OR=1.85, p=0.002), and iii) no or less than 4 month-breastfeeding (OR=1.45, p=0.043).

Such interactions appeared to modify the effect of the association between MS and past exposure to food allergy. No such observations could be applied to past history of respiratory allergy.

Table 2.2a Total and sex-specific Crude (OR) and adjusted odds ratio (OR_{adj}) with 95% confidence interval (95%CIs) for the association between MS and past exposure to allergies to respiratory and food allergens in the Italian EnvIMS population.

	Cases	Controls	OR (95% CIs)	p	OR_{adj} (95% CIs)	p
ITALY						
Both sexes						
No history of allergy	388 (63.5)	733 (63.1)	1.0	0.917	1.0	0.119
History of allergy (any)	223 (36.5)	428 (36.9)	0.98 (0.80, 1.21)		0.82 (0.63, 1.05)	
No history of allergy	388 (67.1)	733 (66.5)	1.0	0.828[a]	1.0	0.242
History of respiratory allergy[b]	190 (32.9)	369 (33.5)	0.97 (0.79, 1.21)		0.85 (0.66, 1.11)	
No history of allergy	388 (83.8)	733 (83.3)	1.0	0.877[a]	1.0	**0.036**
History of food allergy	75 (16.2)	147 (16.7)	0.96 (0.71, 1.31)		0.64 (0.42, 0.97)	
Men						
No history of allergy	146 (67.9)	250 (68.5)	1.0	0.926[a]	1.0	0.901
History of allergy (any)	69 (32.1)	115 (31.5)	1.03 (0.72, 1.48)		0.97 (0.61, 1.55)	
No history of allergy	146 (69.5)	250 (70.8)	1.0	0.744	1.0	0.800
History of respiratory allergy[b]	64 (30.5)	103 (29.2)	1.06 (0.73, 1.55)		1.06 (0.67, 1.72)	
No history of allergy	146 (91.2)	250 (89.0)	1.0	0.515[a]	1.0	0.317
History of food allergy	14 (8.8)	31 (11.0)	0.77 (0.40, 1.50)		0.61 (0.24, 1.60)	
Women						
No history of allergy	242 (61.1)	483 (60.7)	1.0	0.900[a]	1.0	0.106
History of allergy (any)	154 (38.9)	313 (39.3)	0.98 (0.77, 1.26)		0.77 (0.57, 1.06)	
No history of allergy	242 (65.8)	483 (64.5)	1.0	0.689[a]	1.0	0.175
History of respiratory allergy[b]	126 (34.2)	266 (35.5)	0.95 (0.73, 1.23)		0.80 (0.58, 1.11)	
No history of allergy	242 (79.9)	483 (80.6)	1.0	0.784	1.0	0.086
History of food allergy	61 (20.1)	116 (19.4)	1.05 (0.74, 1.48)		0.67 (0.42, 1.06)	

OR=odds ratio;

ORadj (both sexes): adjusted for index age (see text for explanation), sex, smoking habit (ever smoker vs. never smoker), history of mononucleosis (yes vs. no), exposure to breastfeeding (no/less than 4 months vs. 4+months), sun exposure at age 0-5 years in the summer (never/seldom vs. quite often/always), level of education (see text).

ORadj (men, women): adjusted for index age (see text for explanation), smoking habit (ever smoker vs. never smoker), history of mononucleosis (yes vs. no), exposure to breastfeeding (no/less than 4 months vs. 4+months), sun exposure at age 0-5 years in the summer (never/seldom vs. quite often/always), level of education (see text).

[a] Fisher test

[b] Allergy to pollen, dust and animal hair

Table 2.2b Total and sex-specific Crude (OR) and adjusted odds ratio (OR$_{adj}$) with 95% confidence interval (95%CIs) for the association between MS and past exposure to allergies to respiratory and food allergens in the Norwegian EnvIMS population.

	Cases	Controls	OR (95% CIs)	p	OR$_{adj}$ (95% CIs)	p
NORWAY						
Both sexes						
No history of allergy	563 (63.5)	965 (59.7)	1.0	0.065[a]	1.0	0.165
History of allergy (any)	323 (36.5)	651 (40.3)	0.85 (0.72, 1.01)		0.87 (0.72, 1.06)	
No history of allergy	563 (66.9)	965 (63.8)	1.0	0.149[a]	1.0	0.420
History of respiratory allergy[b]	279 (33.1)	547 (36.2)	0.87 (0.73, 1.04)		1.09 (0.89, 1.33)	
No history of allergy	563 (81.1)	965 (76.9)	1.0	**0.033[a]**	1.0	0.068
History of food allergy	131 (18.9)	290 (23.1)	0.77 (0.62, 0.98)		0.78 (0.59, 1.02)	
Men						
No history of allergy	185 (71.2)	289 (66.0)	1.0	0.180[a]	1.0	0.088
History of allergy (any)	75 (28.8)	149 (34.0)	0.79 (0.56, 1.10)		0.71 (0.49, 1.05)	
No history of allergy	185 (73.1)	289 (69.0)	1.0	0.258[a]	1.0	0.166
History of respiratory allergy[b]	68 (26.9)	130 (31.0)	0.82 (0.58, 1.16)		0.75 (0.50, 1.13)	
No history of allergy	185 (89.8)	289 (84.0)	1.0	0.073[a]	1.0	**0.040**
History of food allergy	21 (10.2)	55 (16.0)	0.60 (0.35, 1.02)		0.51 (0.27, 0.97)	
Women						
No history of allergy	378 (60.4)	676 (57.4)	1.0	0.229[a]	1.0	0.384
History of allergy (any)	248 (39.6)	502 (42.6)	0.88 (0.73, 1.08)		0.91 (0.72, 1.13)	
No history of allergy	378 (64.2)	676 (61.8)	1.0	0.369[a]	1.0	0.678
History of respiratory allergy[b]	211 (35.8)	417 (38.2)	0.91 (0.74, 1.11)		0.95 (0.75, 1.20)	
No history of allergy	378 (77.5)	676 (74.2)	1.0	0.193[a]	1.0	0.218
History of food allergy	110 (22.5)	235 (25.8)	0.84 (0.65, 1.09)		0.83 (0.62, 1.12)	

OR=odds ratio;

ORadj (both sexes): adjusted for index age (see text for explanation), sex, smoking habit (ever smoker vs. never smoker), history of mononucleosis (yes vs. no), exposure to breastfeeding (no/less than 4 months vs. 4+months), sun exposure at age 13-18 years in the summer (never/seldom vs. quite often/always), level of education (see text).

ORadj (men, women): adjusted for index age (see text for explanation), smoking habit (ever smoker vs. never smoker), history of mononucleosis (yes vs. no), exposure to breastfeeding (no/less than 4 months vs. 4+months), sun exposure at age 13-18 years in the summer (never/seldom vs. quite often/always), level of education (see text).

[a] Fisher test

[b] Allergy to pollen, dust and animal hair

The age of onset was known for 79.4% of cases vs 84.2% of the controls for the Italian population. The distribution of allergies by age of onset showed a tendency for a higher frequency in age classes 0 to 10 years among female cases for dust (*p* for trend =0.042) and food (*p* for trend =0.035), respectively. Both the trends reversed when the age of onset of the allergy was above 10 years. Distribution did not differ significantly between cases and controls (**Table 2.3a**).

For the Norwegian population the age of onset of allergy was known for 82.7-87.0% of the population. The distribution of allergies by age of onset showed among female cases a tendency for a higher frequency in age ranges 11 to 20 years for pollen and for a higher frequency in age ranges 16 to 25 years for food allergy. Both the trends reversed when the age of onset of the allergy was above 20 and 25 years respectively. Conversely among male cases a higher frequency in age ranges 6 to 15 years was found for animal hair allergy (**Table 2.3b**).

Table 2.3a Distribution of past exposure to different allergies by age of onset among cases and controls in the Italian EnvIMS population.

	Both sexes			Men			Women		
	Cases	Controls	p^a	Cases	Controls	p^a	Cases	Controls	p^a
ITALY									
History of pollen allergy, N=383									
Age at onset, N=304 (79.4%)									
0-5 years	12 (11.5)	21 (10.5)	0.373	4 (10.5)	10 (15.4)	0.724	8 (12.1)	11 (8.1)	0.184
6-10 years	9 (8.7)	16 (8.0)		5 (13.2)	9 (13.8)		4 (6.1)	7 (5.2)	
11-15 years	16 (15.4)	17 (8.5)		5 (13.2)	7 (10.8)		11 (16.7)	10 (7.4)	
16-20 years	16 (15.4)	38 (19.0)		3 (7.9)	11 (16.9)		13 (19.7)	27 (20.0)	
21-25 years	17 (16.3)	47 (23.5)		8 (21.1)	12 (18.5)		9 (13.6)	35 (25.9)	
26-30 years	34 (32.7)	61 (30.5)		13 (34.2)	16 (24.6)		21 (31.8)	45 (33.3)	
History of dust allergy, N=337									
Age at onset, N=275 (81.6%)									
0-5 years	15 (17.0)	20 (10.7)	0.298	5 (16.7)	10 (22.7)	0.656	10 (17.2)	10 (7.0)	0.111
6-10 years	11 (12.5)	14 (7.5)		5 (16.7)	8 (18.2)		6 (10.3)	6 (4.2)	
11-15 years	7 (8.0)	25 (13.4)		2 (6.7)	5 (11.4)		5 (8.6)	20 (14.0)	
16-20 years	15 (17.0)	43 (23.0)		4 (13.3)	9 (20.5)		11 (19.0)	34 (23.8)	
21-25 years	18 (20.5)	37 (19.8)		5 (16.7)	5 (11.4)		13 (22.4)	32 (22.4)	
26-30 years	22 (25.0)	48 (25.7)		9 (30.0)	7 (15.9)		13 (22.4)	41 (28.7)	
							p for trend = 0.042		
History of animal hair allergy, N= 194									
Age at onset, N=159 (82.0%)									
0-5 years	9 (15.5)	12 (11.9)	0.974	2 (10.0)	5 (19.2)	0.803	7 (18.4)	7 (9.3)	0.807
6-10 years	9 (15.5)	15 (14.9)		6 (30.0)	6 (23.1)		3 (7.9)	9 (12.0)	
11-15 years	6 (10.3)	13 (12.9)		1 (5.0)	3 (11.5)		5 (13.2)	10 (13.3)	
16-20 years	9 (15.5)	18 (17.8)		2 (10.0)	4 (15.4)		7 (18.4)	14 (18.7)	
21-25 years	11 (19.0)	21 (20.8)		4 (20.0)	4 (15.4)		7 (18.4)	17 (22.7)	
26-30 years	14 (24.1)	22 (21.8)		5 (25.0)	4 (15.4)		9 (23.7)	18 (24.0)	

History of food allergy, N=222
Age at onset, N=187 (84.2%)

0-5 years	5 (7.9)	10 (8.1)	*0.491*	0 (-)	7 (25.0)	*0.461*	5 (10.0)	3 (3.1)	*0.118*
6-10 years	7 (11.1)	12 (9.7)		2 (15.4)	4 (14.3)		5 (10.0)	8 (8.3)	
11-15 years	4 (6.3)	10 (8.1)		1 (7.7)	2 (7.1)		3 (6.0)	8 (8.3)	
16-20 years	14 (22.2)	21 (16.9)		2 (15.4)	5 (17.9)		12 (24.0)	16 (16.7)	
21-25 years	18 (28.6)	25 (20.2)		4 (30.8)	5 (17.9)		14 (28.0)	20 (20.8)	
26-30 years	15 (23.8)	46 (37.1)		4 (30.8)	5 (17.9)		11 (22.0)	41 (42.7)	
							p for trend=0.035		

[a]chi-square test

Table 2.3b Distribution of past exposure to different allergies by age of onset among cases and controls in the Norwegian EnvIMS population.

	Both sexes			Men			Women		
	Cases	Controls	p^a	Cases	Controls	p^a	Cases	Controls	p^a
NORWAY									
History of pollen allergy, N=651									
Age at onset, N=544 (83.6%)									
0-5 years	19 (10.3)	31 (8.6)	*0.005*	7 (15.9)	10 (11.4)	*0.372*	12 (8.6)	21 (7.7)	*0.003*
6-10 years	29 (15.8)	69 (19.2)		14 (31.8)	23 (26.1)		15 (10.7)	46 (16.9)	
11-15 years	29 (15.8)	37 (10.3)		9 (20.5)	12 (13.6)		20 (14.3)	25 (9.2)	
16-20 years	20 (10.9)	15 (4.2)		3 (6.8)	4 (4.5)		17 (12.1)	11 (4.0)	
21-25 years	33 (17.9)	63 (17.5)		3 (6.8)	16 (18.2)		30 (21.4)	47 (17.3)	
26-30 years	54 (29.3)	145 (40.3)		8 (18.2)	23 (26.1)		46 (32.9)	122 (44.9)	
History of dust allergy, N=301									
Age at onset, N= 249 (82.7%)									
0-5 years	10 (13.0)	17 (9.9)	*0.647*	1 (5.3)	4 (10.5)	*0.667*	9 (15.5)	13 (9.7)	*0.737*
6-10 years	13 (16.8)	31 (18.0)		6 (31.6)	10 (26.3)		7 (12.1)	21 (15.7)	
11-15 years	10 (13.0)	12 (7.0)		4 (21.1)	3 (7.9)		6 (10.3)	9 (6.7)	
16-20 years	6 (7.8)	13 (7.6)		0 (-)	1 (2.6)		6 (10,3)	12 (9.0)	
21-25 years	16 (20.8)	41 (23.8)		3 (15.8)	9 (23.7)		13 (22.4)	32 (23.9)	
26-30 years	22 (28.6)	58 (33.7)		5 (26.3)	11 (28.9)		17 (29.3)	47 (35.1)	
History of animal hair allergy, N= 407									
Age at onset, N= 351 (87.0%)									
0-5 years	17 (13.4)	41 (18.3)	*0.213*	5 (16.1)	13 (25.5)	*0.041*	12 (12.5)	28 (16.2)	*0.663*
6-10 years	30 (23.6)	45 (20.1)		9 (29.0)	11 (21.6)		21 (21.9)	34 (19.7)	
11-15 years	23 (18.1)	23 (10.3)		7 (22.6)	4 (7.8)		16 (16.7)	19 (11.0)	
16-20 years	9 (7.1)	13 (5.8)		1 (3.2)	1 (2.0)		8 (8.3)	12 (6.9)	
21-25 years	17 (13.4)	41 (18.3)		0 (-)	11 (21.6)		17 (17.7)	30 (17.3)	
26-30 years	31 (24.4)	61 (27.2)		9 (29.0)	11 (21.6)		22 (22.9)	50 (28.9)	

History of food allergy, N=421
Age at onset, N=354 (84.1%)

0-5 years	23 (20.5)	41 (16.9)	*0.001*	6 (30.0)	13 (26.0)	*0.202*	17 (18.5)	28 (14.6)	*0.009*
6-10 years	18 (16.1)	38 (15.7)		5 (25.0)	9 (18.0)		13 (14.1)	29 (15.1)	
11-15 years	9 (8.0)	19 (7.9)		1 (5.0)	2 (4.0)		8 (8.7)	17 (8.9)	
16-20 years	9 (8.0)	13 (5.4)		1 (5.0)	1 (2.0)		8 (8.7)	12 (6.2)	
21-25 years	33 (29.5)	35 (14.5)		5 (25.0)	5 (10.0)		28 (30.4)	30 (15.6)	
26-30 years	20 (17.9)	96 (39.7)		2 (10.0)	20 (40.0)		18 (19.6)	76 (39.6)	

[a]chi-square test

The study of the association between MS and different allergies based on the age of onset (e.g., 0-10 years vs 21-30 years), did not yield significant adjusted risks. In the Italian sample a tendency for a direct association with an earlier exposure to food allergy among cases was detected (OR=3.04, p=0.076) (**Table 2.4a**).

In the Norwegian population instead, MS was significantly associated with allergy to pollen with onset between 11-20 years old, especially females. On the other side, an inverse association was evident for allergy onset in the age range 21-30 years for both genders. For allergy to dust, after adjustment for confounders, we reported a non-significant trend (p=0.050) towards the positive association with MS for men with allergy onset between 11-20 years old. Allergy to animal hair was instead positively associated with MS, especially for men with allergy onset 11-20 years old (ORadj=12.70, p=0.009). Finally, food allergy was non statistically associated with MS even if all the crude and adjusted ORs were over 1 (**Table 2.4b**).

Table 2.4a. Association between MS onset and history of allergy by age at onset and sex expressed with crude and adjusted odds ratio and 95% confidence interval (95%CI) in the Italian EnvIMS population.

	Cases	Controls	OR (95% CIs)	p	OR_{adj}^{1} (95% CIs)	p	OR_{adj}^{2} (95% CIs)	p
Respiratory allergy								
POLLEN								
Both sexes								
Age of onset:								
- 21-30 years	51 (49.0)	108 (53.7)	1.0	0.739	1.0	0.978	1.0	0.880
- 0-10 years	21 (20.2)	37 (18.4)	1.20 (0.64, 2.26)	0.568	0.99 (0.45, 2.17)	0.976	0.79 (0.31, 2.01)	0.625
- 11-20 years	32 (30.8)	56 (27.9)	1.21 (0.70, 2.09)	0.495	1.07 (0.53, 2.15)	0.856	0.98 (0.40, 2.41)	0.959
Men								
Age of onset:								
- 21-30 years	21 (55.3)	28 (43.1)	1.0	0.489	1.0	0.098	1.0	0.506
- 0-10 years	9 (23.7)	19 (29.2)	0.63 (0.24, 1.67)	0.355	0.39 (0.12, 1.32)	0.130	0.94 (0.22, 3.93)	0.932
- 11-20 years	8 (21.1)	18 (27.7)	0.59 (0.22, 1.62)	0.593	0.24 (0.05, 1.02)	0.053	1.86 (0.57, 6.12)	0.306
Women								
Age of onset:								
- 21-30 years	30 (45.5)	80 (58.8)	1.0	0.200	1.0	0.287	1.0	0.663
- 0-10 years	12 (18.2)	18 (13.2)	1.78 (0.77, 4.13)	0.181	1.66 (0.54, 5.12)	0.375	0.54 (0.11, 2.62)	0.445
- 11-20 years	24 (36.4)	38 (27.9)	1.68 (0.87, 3.26)	0.122	1.98 (0.84, 4.67)	0.118	0.54 (0.10, 3.00)	0.480
DUST								
Both sexes								
Age of onset:								
- 21-30 years	40 (45.5)	85 (45.2)	1.0	**0.050**	1.0	**0.038**	1.0	0.121
- 0-10 years	26 (29.5)	34 (18.1)	1.63 (0.86, 3.06)	0.133	1.43 (0.66, 3.10)	0.371	1.19 (0.46, 3.12)	0.719
- 11-20 years	22 (25.0)	69 (36.7)	0.68 (0.87, 1.25)	0.211	0.51 (0.25, 1.06)	0.070	0.44 (0.17, 1.17)	0.100
Men								

Age of onset:								
- 21-30 years	14 (46.7)	12 (27.3)	1.0	*0.219*	1.0	*0.243*	1.0	*0.312*
- 0-10 years	10 (33.3)	18 (40.9)	0.48 (0.16, 1.42)	*0.183*	0.71 (0.20, 2.50)	*0.591*	1.13 (0.22, 5.91)	*0.886*
- 11-20 years	6 (20.0)	14 (31.8)	0.37 (0.11, 1.26)	*0.110*	0.27 (0.06, 1.25)	*0.094*	0.25 (0.03, 1.84)	*0.174*

Women

Age of onset:								
- 21-30 years	26 (44.8)	73 (50.7)	1.0	***0.017***	1.0	*0.090*	1.0	*0.292*
- 0-10 years	16 (27.6)	16 (11.1)	2.81 (1.23, 6.41)	***0.014***	2.09 (0.74, 5.90)	*0.163*	1.04 (0.27, 4.08)	*0.953*
- 11-20 years	16 (27.6)	55 (38.2)	0.82 (0.40, 1.67)	*0.579*	0.66 (0.28, 1.55)	*0.340*	0.43 (0.12, 1.49)	*0.182*

ANIMAL HAIR
Both sexes

Age of onset:								
- 21-30 years	25 (43.1)	43 (42.6)	1.0	*0.763*	1.0	*0.951*	1.0	*0.758*
- 0-10 years	18 (31.0)	27 (26.7)	1.15 (0.53, 2.49)	*0.729*	0.90 (0.36, 2.22)	*0.811*	1.30 (0.43, 3.96)	*0.643*
- 11-20 years	15 (25.9)	31 (30.7)	0.83 (0.38, 1.83)	*0.648*	0.87 (0.36, 2.15)	*0.769*	0.84 (0.28, 2.54)	*0.759*

Men

Age of onset:								
- 21-30 years	9 (45.0)	8 (30.8)	1.0	*0.503*	1.0	*0.649*	1.0	*0.606*
- 0-10 years	8 (40.0)	11 (42.3)	0.65 (0.17, 2.42)	*0.646*	0.54 (0.12, 2.36)	*0.414*	1.66 (0.20, 13.92)	*0.643*
- 11-20 years	3 (15.0)	7 (26.9)	0.38 (0.07, 1.99)	*0.381*	0.44 (0.50, 3.85)	*0.456*	0.41 (‘.02, 7.72)	*0.548*

Women

Age of onset:								
- 21-30 years	16 (42.1)	35 (46.7)	1.0	*0.823*	1.0	*0.966*	1.0	*0.742*
- 0-10 years	10 (26.3)	16 (21.3)	1.37 (0.51, 3.67)	*0.535*	1.03 (0.30, 3.55)	*0.965*	0.56 (0.11, 2.94)	*0.495*
- 11-20 years	12 (31.6)	24 (32.0)	1.09 (0.44, 2.72)	*0.847*	1.14 (0.41, 3.17)	*0.801*	1.02 (0.27, 3.88)	*0.983*

FOOD
Both sexes
Age of onset:

- 21-30 years	33 (52.4)	71 (57.3)	1.0	*0.810*	1.0	*0.737*	1.0	*0.733*
- 0-10 years	12 (19.0)	22 (17.7)	1.17 (0.52, 2.65)	*0.701*	0.55 (0.04, 8.18)	*0.664*	7.08 (0.09, 535,72)	*0.375*
- 11-20 years	18 (28.6)	31 (25.0)	1.25 (0.61, 2.55)	*0.540*	0.45 (0.06, 3.47)	*0.439*	0.74 (0.02, 22.95)	*0.861*

Men
Age of onset:

- 21-30 years	8 (61.5)	10 (35.7)	1.0	*0.251*	1.0	*0.639*	-	-
- 0-10 years	2 (15.4)	11 (39.3)	0.23 (0.04, 1.34)	*0.101*	0.10 (0.00, 15.22)	*0.372*	-	-
- 11-20 years	3 (23.1)	7 (25.0)	0.54 (0.10, 2.78)	*0.456*	0.50 (0.01, 34.74)	*0.747*	-	-

Women
Age of onset:

- 21-30 years	25 (50.0)	61 (63.5)	1.0	*0.229*	1.0	*0.591*	-	-
- 0-10 years	10 (20.0)	11 (11.5)	2.22 (0.84, 5.88)	*0.109*	1.44 (0.03, 69.60)	*0.855*	-	-
- 11-20 years	15 (30.0)	24 (25.0)	1.53 (0.69, 3.38)	*0.298*	0.28 (0.02, 4.86)	*0.382*	-	-

OR =odds ratio;
[1]Model 1 = OR adjusted for allergy to food (for analysis on respiratory allergies) or respiratory allergens (for analysis on food allergy), and index age
[2]Model 2 = OR adjusted for allergy to food (for analysis on respiratory allergies) or respiratory allergens (for analysis on food allergy), index age, cigarette smoking habit (ever vs. never smokers), breastfeeding (no/less than 4 months vs. 4+ months), history of mononucleosis, low sun exposure at age 0-5 years

Table 2.4b Total and sex-specific adjusted odds ratio (OR_{adj}) with 95% confidence interval (95%CIs) for the association between MS and past exposure to allergies to respiratory and food allergens in the Norwegian EnvIMS population, stratified by age of onset.

NORWAY	Cases	Controls	OR (95% CIs)	p	OR_{adj}^{1} (95% CIs)	p	OR_{adj}^{2} (95% CIs)	p
Respiratory allergy[a] **POLLEN** **Both sexes** Age of onset:								
- 21-30 years	87 (47.3)	208 (57.8)	1.0	*0.002*	1.0	*0.015*	1.0	*0.017*
- 0-10 years	48 (26.1)	100 (27.8)	1.15 (0.75, 1.76)	*0.526*	1.39 (0.86, 2.25)	*0.185*	1.33 (0.77, 2.31)	*0.313*
- 11-20 years	49 (26.6)	52 (14.4)	2.25 (1.42, 3.58)	*0.001*	2.16 (1.28, 3.64)	*0.004*	2.38 (1.32, 4.32)	*0.004*
p for trend = 0.001								
Men Age of onset:								
- 21-30 years	11 (25.0)	39 (44.3)	1.0	*0.098*	1.0	*0.189*	1.0	*0.045*
- 0-10 years	21 (47.7)	33 (37.5)	2.26 (0.95, 5.36)	*0.065*	2.38 (0.93, 6.12)	*0.072*	5.16 (1.40, 18.99)	*0.014*
- 11-20 years	12 (27.3)	16 (18.2)	2.66 (0.97, 7.26)	*0.056*	1.92 (0.63, 5.83)	*0.252*	3.13 (0.74, 13.22)	*0.120*
p for trend = 0.041								
Women Age of onset:								
- 21-30 years	76 (54.3)	169 (62.1)	1.0	*0.004*	1.0	*0.020*	1.0	*0.024*
- 0-10 years	27 (19.3)	67 (24.6)	0.90 (0.53, 1.51)	*0.681*	1.14 (0.63, 2.08)	*0.670*	0.99 (0.50, 1.95)	*0.974*
- 11-20 years	37 (26.4)	36 (13.2)	2.29 (1.34, 3.89)	*0.002*	2.35 (1.28, 4.33)	*0.006*	2.48 (1.25, 4.94)	*0.010*
p for trend = 0.009								
DUST **Both sexes** Age of onset:								
- 21-30 years	38 (49.4)	99 (57.6)	1.0	*0.374*	1.0	*0.143*	1.0	*0.275*
- 0-10 years	23 (29.9)	48 (27.9)	1.25 (0.67, 2.33)	*0.485*	1.76 (0.84, 3.67)	*0.134*	1.58 (0.62, 3.99)	*0.341*

- 11-20 years	16 (20.8)	25 (14.5)	1.67 (0.80, 3.46)	*0.170*	2.19 (0.91, 4.87)	***0.083***	2.09 (0.82, 5.30)	*0.122*
Men								
Age of onset:								
- 21-30 years	8 (42.1)	29 (52.6)	1.0	*0.537*	1.0	*0.058*	1.0	*0.143*
- 0-10 years	7 (36.8)	14 (36.8)	1.25 (0.37, 4.25)	*0.721*	3.33 (0.69, 16.19)	*0.136*	2.36 (0.20, 28.44)	*0.498*
- 11-20 years	4 (21.1)	4 (10.5)	2.5 (0.5, 12.51)	*0.265*	15.61 (1.54, 158.17)	***0.020***	12.51 (1.00, 157.09)	***0.050***
Women								
Age of onset:								
- 21-30 years	30 (51.7)	79 (59.0)	1.0	*0.595*	1.0	*0.594*	1.0	*0.672*
- 0-10 years	16 (27.6)	34 (25.4)	1.24 (0.60, 2.57)	*0.564*	1.48 (0.62, 3.52)	*0.372*	1.61 (0.55, 4.69)	*0.384*
- 11-20 years	12 (20.7)	21 (15.7)	1.50 (0.66, 3.43)	*0.331*	1.44 (0.56, 3.69)	*0.450*	1.30 (0.44, 3.86)	*0.640*
ANIMAL HAIR								
Both sexes								
Age of onset:								
- 21-30 years	48 (37.8)	102 (45.5)	1.0	*0.100*	1.0	*0.066*	1.0	***0.046***
- 0-10 years	47 (37.0)	86 (38.4)	1.16 (0.71, 1.90)	*0.553*	1.45 (0.81, 2.58)	*0.210*	1.34 (0.68, 2.63)	*0.403*
- 11-20 years	32 (25.2)	36 (16.1)	1.89 (1.05, 3.40)	***0.034***	2.16 (1.13, 4.13)	***0.020***	2.53 (1.21, 5.31)	***0.014***
	p **for trend=0.044**							
Men								
Age of onset:								
- 21-30 years	9 (29.0)	22 (43.1)	1.0	*0.142*	1.0	***0.027***	1.0	***0.033***
- 0-10 years	14 (45.2)	24 (47.1)	1.43 (0.52, 3.94)	*0.494*	2.05 (0.58, 7.28)	*0.265*	2.36 (0.45, 12.48)	*0.312*
- 11-20 years	8 (25.8)	5 (9.8)	3.91 (1.00, 15.24)	***0.049***	9.80 (1.85, 51.83)	***0.007***	12.70 (1.86, 86.58)	***0.009***
Women								
Age of onset:								
- 21-30 years	39 (40.6)	80 (46.2)	1.0	*0.372*	1.0	*0.457*	1.0	*0.455*
- 0-10 years	33 (34.4)	62 (35.8)	0.09 (0.62, 1.93)	*0.763*	1.34 (0.69, 2.59)	*0.390*	1.17 (0.54, 2.53)	*0.684*

			Model 1[1]		Model 2[2]			
- 11-20 years	24 (25.0)	31 (17.9)	1.59 (0.82, 3.06)	*0.167*	1.56 (0.76, 3.20)	*0.231*	1.71 (0.73, 3.97)	*0.214*

Food allergy
Both sexes
Age of onset:

- 21-30 years	53 (47.3)	131 (54.1)	1.0	*0.479*	1.0	*0.174*	1.0	*0.315*
- 0-10 years	41 (36.6)	79 (32.6)	1.28 (0.78, 2.10)	*0.323*	3.21 (0.94, 10.94)	*0.063*	3.31 (0.70, 15.67)	*0.132*
- 11-20 years	18 (16.1)	32 (13.2)	1 39 (0.72, 2.69)	*0.328*	2.06 (0.25, 16.69)	*0.500*	1.96 (0.20, 18.82)	*0.560*

Men
Age of onset:

- 21-30 years	7 (35.0)	25 (50.0)	1.0	*0.505*	1.0	*0.999*	1.0	*0.999*
- 0-10 years	11 (55.0)	22 (44.0)	1.79 (0.59, 5.40)	*0.305*	-	-	-	-
- 11-20 years	2 (10.0)	3 (6.0)	2.38 (0.33, 17.17)	*0.389*	-	-	-	-

Women
Age of onset:

- 21-30 years	46 (50.0)	106 (55.2)	1.0	*0.707*	1.0	*0.338*	1.0	*0.471*
- 0-10 years	30 (32.6)	57 (29.7)	1.21 (0.69, 2.13)	*0.501*	2.85 (0.71, 11.54)	*0.141*	2.96 (0.50, 17.62)	*0.233*
- 11-20 years	16 (17.4)	29 (15.1)	1.27 (0.63, 2.56)	*0.502*	1.66 (0.19, 14.48)	*0.649*	2.19 (0.19, 24.83)	*0.526*

OR=odds ratio;
[1]Model 1 = OR adjusted for allergy to food (for analysis on respiratory allergies) or respiratory allergens (for analysis on food allergy), and index age
[2]Model 2 = OR adjusted for allergy to food (for analysis on respiratory allergies) or respiratory allergens (for analysis on food allergy), index age, cigarette smoking habit (ever vs. never smokers), breastfeeding (no/less than 4 months vs. 4+ months), history of mononucleosis, low sun exposure at age 13-18 years

Comment

No association was detected between allergies overall and MS in both populations, which is consistent with most literature data (**Table 1.1**). When considering instead respiratory and food allergy separately, a significant inverse association was found between MS and food allergy in both populations, especially for Italian women even if the significance was only detectable for both genders together after adjustment for confounders.

In the Norwegian population instead, the association was significant for women before adjusting and in men without adjusting. These results were quite in accordance with those of an Iranian study [Sahraian 2013] and an American study [Bourne 2017], the latter reporting an inverse association only in the unadjusted analyses. On the other hand, taking into account the age of allergy onset and the type of allergen, a different pattern of association appeared between Italian and Norwegian populations. To note, our crude and adjusted ORs were quite variable due to the correction for several confounding factors. We found indeed a strong interference exerted by some demographic and environmental factors, especially IM and breastfeeding, which appeared to be associated with allergy. In particular, respiratory allergy was directly or inversely associated with age at the study time, at age MS onset, disease duration, female sex, IM, breastfeeding and smoking habit. Food allergy, as well, was associated with age at the study time, at age MS onset, female sex, IM and breastfeeding. When investigating on the association between allergies and MS onset, we therefore had to consider a number of potential confounders and effect modifiers which could have altered the results, so as to increase its validity.

The Italian female MS patients showed a tendency towards higher prevalence of allergies to food and dust with onset between 0-10 years (*p* for trend= 0.043 and 0.035, respectively), although the sex-specific risk was not significantly higher as compared to controls and after adjusting for confounders in the analysis in both sexes.

Interestingly, we found a strong inversed adjusted OR for food allergy with onset 0-10 years, which was 7 times greater for Italian cases (both sexes) compared to controls. The rise in the risk was observed especially after including breastfeeding in the multivariate analysis, although a clear interaction effect with other variables could not be detected.

In the Norwegian sample, the analyses yielded instead a significantly higher prevalence of pollen, dust, animal hair and food allergies among MS patients, even if with a different distribution. The risk for MS was significantly higher among female cases with pollen allergy onset between 11 and 20 years old and male cases with animal hair allergy onset between 11 and 20 years old. Male cases with dust allergy onset between 11 and 20 years old showed instead a non-significant positive association with MS. Food allergies had a significant trend towards a greater prevalence among cases during adolescence and early adulthood even if the risk was not statistically increased comparing the age ranges 0-10 and 11-20 with 21-30 years old.

The variability of our results when considering the whole population vs. stratification by sex,

allergens and age of exposure, may account for the conflicting results reported in literature over the years. The strength of the EnvIMS study was the investigation of the same exposure on different populations, in addition with the collection of the time and type of exposure. To summarize, our findings imply that allergies (mostly pollen and animal hair allergies for Norwegians, food and dust allergies for Italians) might be directly associated to MS onset in the general population, statistically behaving as 'risk factors'. In accordance with our findings in the Norwegian sample, the evidence from an English cohort study and an Australian case-control study suggests a positive association between respiratory allergy or asthma and MS [Edwards 2004, Ponsonby 2006]. Of note, the Australian authors also described an enhanced positive association between asthma and MS in lack of an exposure to younger siblings. Conversely, different results were obtained from other case-control studies, supporting an inverse association between MS and respiratory allergy [Evans 2000, Tremlett 2002, Pedotti 2009, Bergamaschi 2009, Ren 2017]. In our populations this trend was only detectable when considering the whole population, even if significantly lower risks for MS were only associated with food allergies. Our results could be explained with the inverse risk we have found above the specific age range of susceptibility that is subjects with the onset of allergy after the period of susceptibility might be "protected" against MS.

The only evidence suggesting a positive interaction between MS and food allergy comes from the cohort study by Fakih and colleagues. The authors highlighted a potential influence of food allergy on promoting MS inflammatory activity (e.g., relapses, gadolinium-enhancing lesions) in a large sample of patients (1349) [Fakih 2019], with opposite results to those of a case-control study on pediatric MS including 193 cases and 418 controls [Bourne 2017] (**Table 1.2**). The different type of allergy, as we have seen, have been associated with different age of susceptibility for the increased risk for MS. The susceptibility age was earlier for the Italian population, during early childhood until 10 years of age, while for the Norwegians the most affected age was during adolescence, between 11 and 20 years old mostly, and up to 25 years old for animal hair allergy. This type of evaluation has never been done before.

Unlike what has been traditionally proposed according to the Th1/Th2 paradigm, our work suggests that allergy and MS are not inversely associated. Immunological acquisitions support a common mechanism for immune-mediated inflammatory diseases, underpinned by T-helper cell subsets and a new model has been proposed which goes beyond the Th1/Th2 paradigm [Hirahara 2016]. Considering both the Italian and Norwegian populations, our most consistent finding was the positive association between allergy and MS risk, depending on sex, type of allergen and age at allergy onset. Even so, we do not account our results as conflictual with the "hygiene hypothesis" as autoimmune and allergic diseases should be considered mutual interferers rather than mutual protective, thus explaining the literature conflictual findings. Furthermore, in consideration of the large plethora of factors potentially affecting the risk for allergy in the general population, homogeneity in the type of observed associations over the years would be been highly unlikely.

Moreover, what we consider interference between MS and allergy, does not actually mean a direct influence of one condition over the other, rather a dependency upon a common immunological mechanism. The immune system is the real target of the exposures influencing the diseases risk, and allergy can never causatively associate to MS or *vice versa*.

We suggest that certain environmental exposures, belonging to the "old friends" pool, during a specific age range of life (form childbirth to adulthood) affect the risk for both diseases. Some kind of underlying environmental (infectious?) exposure has already been suggested in the past by two space-time clustering studies. These researches actually described a clustering in MS risk in the Italian (Sardinian) and Norwegian populations. In the Sardinian population, a specific space-time clustering pattern at ages 1-3 years was described [Pugliatti 2006] while, in the Norwegian population, space-time clustering was identified for the age range between 13 and 20 years, peaking at age 15-19 years [Riise 1991]. This clustering with the increasing incidence of MS in Sardinia, can be attributable to some change in environmental exposure. According to the similarity between ours and their age ranges of susceptibility for the risk for MS, which were specific for each population, these findings increase the consistence of our results.

In accordance with the "hygiene hypothesis", we then suggest that "old friends", including the gut microbiota, could represent the specific environmental factors capable of affecting the risk for the disease with a geographical distribution pattern. Moreover, the specific "influence" of food allergy on MS risk in our Italian population further points to a potential role of the gut microbiota, also belonging to the "old friends". The impact of the gut microbiota on MS pathogenesis and neuroinflammation has been widely studied but some kind of interaction also exists between the microbiota and allergy [Penders 2007]. In children with atopic disease a microbial signature as even been suggested, which could let to discriminate the presence or absence of food allergy [Fieten 2018]. Moreover, interesting inference can be made from recent evidence regarding the development of atopic dermatitis modulated by the skin microbiota [Li 2020]. The gut microbiota could be then specifically involved in food allergies development. This hypothesis could explain the increased risk for MS observed when accounting for breastfeeding. Breastfeeding during the first months of life strongly affect the microbiota composition, e.g., according to Davis and colleagues, formula fed infants had a decreased pathogen exposure and their gut microbiota showed a lower abundance of Proteobacteria, to which belong some potentially pathogen genera (e.g., Escherichia, Helicobacter, Salmonella, Shigella), than those exclusively breastfed [Davis 2020]. The interaction between low breastfeeding and food allergies in strongly increasing the risk for MS that we have proposed, could be mediated by the lack of action of breastfeeding on the gut microbiota, which in turn modulates the allergy and MS risk development.

It could be even suggested that the gut microbiota may be a link between MS and allergies. Thus, a certain gut microbiota profile, mainly influenced by early life exposures, could translate the exposure in the risk for the immune disease. A dysbiotic condition could potentially promote both MS and allergy development, explaining the positive association we found between these two

conditions. Even so, this is possible only when others predisposing conditions are satisfied (e.g., genetic, demographic predispositions, timing of the exposure, etc).

2.4 Study B. Multiple sclerosis and the sibling effect

Results

This substudy was conducted in the same populations as in Study A. Demographic and clinical characteristics are shown in **Table 2.1**.

Most of the study participants of both populations had siblings and being an only child was not found to be associated with MS. Although the percentages of only children were similar between cases and controls among each population, there was a difference between Italy and Norway: 6.8% of the Italian subjects was an only child as compared to the 4.5% of the Norwegians. We ruled out participants who had both older and younger siblings to avoid confounding, we then compared 'having only younger siblings' and 'having only older siblings' with 'being only child': we did not find any difference between cases and controls. When comparing 'having only older siblings' to 'having only younger siblings' we found only in the Norwegian population that having only younger siblings was associated to a lower risk of MS (OR 0.81, 95%CIs 0.66, 0.98, p=0.032); however the association lost significant after adjusting for confounders (**Table 2.5**).

Table 2.5 Crude (OR) and adjusted odds ratio (ORadj) with 95% confidence interval (95%CIs) for the association between MS and being an only child or having older or younger siblings.

	Cases	Controls	OR (95% CIs)	p	OR$_{adj}$ (95% CIs)	p
ITALY						
Only child, N (%)	49 (7.1)	89 (6.7)	1.0	*0.753*	1.0	*0.244*
Subjects with siblings, N (%)	639 (92.9)	1230 (93.3)	0.94 (0.66, 1.36)		0.78 (0.51, 1.19)	
Only child, N (%)	47 (17.8)	87 (16.8)	1.0	*0.732*	1.0	*0.143*
Only older siblings, N (%)	217 (82.2)	430 (83.2)	0.93 (0.63, 1.38)		0.71 (0.44, 1.13)	
Only child, N (%)	47 (19.2)	87 (18.6)	1.0	*0.857*	1.0	*0.614*
Only younger siblings, N (%)	198 (80.8)	380 (81.4)	0.97 (0.65, 1.43)		0.89 (0.56, 1.42)	
Only older siblings, N (%)	217 (52.3)	430 (53.1)	1.0	*0.791*	1.0	*0.187*
Only younger siblings, N (%)	198 (47.7)	380 (46.9)	1.03 (0.82, 1.31)		1.22 (0.91, 1.65)	

NORWAY

Only child, N (%)	46 (4.9)	74 (4.3)	1.0	0.559[a]	1.0	0.317
Subjects with siblings, N (%)	897 (95.1)	1629 (95.7)	0.89 (0.61, 1.29)		0.79 (0.51, 1.25)	
Only child, N (%)	46 (12.3)	74 (12.6)	1.0	0.920[a]	1.0	0.638
Only older siblings, N (%)	329 (87.7)	511 (87.4)	1.04 (0.70, 1.54)		1.12 (0.70, 1.80)	
Only child, N (%)	46 (13.3)	74 (11.3)	1.0	0.413[a]	1.0	0.267
Only younger siblings, N (%)	301 (31.9)	580 (34.1)	0.84 (0.56, 1.24)		1.31 (0.82, 2.09)	
Only older siblings, N (%)	329 (52.2)	511 (46.8)	1.0	**p=0.032[a]**	1.0	0.220
Only younger siblings, N (%)	301 (47.8)	580 (53.2)	0.81 (0.66, 0.98)		1.16 (0.92, 1.47)	

p for trend = 0.031

OR=odds ratio;
ORadj: adjusted for smoking habit at age 15 (smokers vs. non smokers at age 15), level of education (see text), low sun exposure at age 0-15 years, history of mononucleosis (yes vs. no), exposure to breastfeeding (no/less than 4 months vs. 4+months)
[a] Fisher test

Evaluating the sibship size, we detected in the Norwegian population a linear association between the total number of siblings and the risk of MS: increasing the sibship size (from 0 to 6 or more) decreased the disease risk (*p* for trend=0.021) (**Table 2.6**).

Focusing on younger siblings, a potentially protective condition for the Norwegian population, we identified 3 interbirth intervals between the index child and the younger sibling: 0-2 years, 3-6 years and over 6 years. The classification of each subject into these ranges, depended on the sibling closest in age to the index child (e.g., the oldest among the younger siblings). Norwegian people with at least one sibling 0-2 years younger than them had a markedly reduced risk of MS compared to subjects with no younger siblings (OR 0.58, 95%CIs 0.47, 0.72, *p*<0.000001), the significance was still high after the correction for confounders (OR$_{adj}$ 0.63, 95%CIs 0.49, 0.81, *p*=0.00002). Having 3-6 years younger siblings or siblings younger more than 6 years was not protective against MS. We even found a statistically significant trend towards a reduction of the risk with lower interbirth interval between the index child and the younger sibling (*p*=0.000023).

Conversely, in the Italian population we did not detect any association between MS risk and the interbirth interval with younger siblings (**Table 2.7**).

Table 2.6 Association between MS and the sibship size.

	Cases	Controls	*p*
ITALY			
Siblings, N (%)			
0	49 (7.1)	89 (6.8)	*0.721*
1	199 (28.9)	400 (30.3)	
2	196 (28.5)	336 (25.5)	
3	93 (13.5)	192 (14.6)	
4	60 (8.7)	118 (9.0)	
5	30 (4.4)	73 (5.5)	
6+	61 (8.9)	110 (8.3)	
p for trend (only child is excluded)= 0.852			
NORWAY			
Siblings, N (%)			
0	46 (4.9)	74 (4.3)	*0.021*
1	276 (29.3)	475 (27.9)	
2	312 (33.1)	551 (32.4)	
3	183 (19.4)	321 (18.8)	
4	77 (8.2)	140 (8.2)	
5	27 (2.9)	82 (4.8)	
6+	22 (2.3)	60 (3.5)	
p for trend (only child is excluded)= 0.025 [a]			

Once analyzed the effect of a specific interbirth interval among the Norwegians, we analyzed the effect of the sibship size for this category of siblings and found a strong linear association. The Norwegian cases had more frequently only one sibling 0-2 years younger while their controls had a greater sibship size of close in age younger siblings ($p<0.000001$). We also calculated the sibship size for 1-2 years older siblings to understand whether a protective effect could also be exerted by the closest in age siblings, but we did not find any trend in the MS risk from 0 to 2 older siblings. In the Italian population, once again, we confirmed the absence of any detectable sibling effect (**Table 2.8**).

Table 2.7 Crude and adjusted odds ratio (OR_{adj}) with 95% confidence interval (95%CIs) for the association between MS and interbirth interval between index child and younger sibling.

	MS cases N (%)	Controls N (%)	OR (95%CI)	*p*	OR_{adj} (95% CI)	*p*
ITALY						
No younger siblings	257 (37.4)	499 (37.9)	1.0		1.0	
0-2 years interval	179 (26.1)	359 (27.2)	0.97 (0.77, 1.22)	*0.786*	1.08 (0.79, 1.46)	*0.642*
3-6 years interval	188 (27.4)	369 (28.0)	0.99 (0.79, 1.25)	*0.927*	1.14 (0.85, 1.52)	*0.379*
>6 years interval	63 (9.2)	91 (6.9)	1.34 (0.94, 1.92)	*0.102*	1.33 (0.85, 2.09)	*0.212*
p for trend = 0.345						
NORWAY						
No younger siblings	375 (39.8)	585 (34.4)	1.0		1.0	
0-2 years interval	202 (21.4)	540 (31.7)	0.58 (0.47, 0.72)	*<0.000001[a]*	0.63 (0.49, 0.81)	*0.00002*

3-6 years interval	286 (30.3)	445 (26.1)	1.00 (0.82, 1.22)	*1.00[a]*	1.00 (0.79, 1.27)	*0.990*
>6 years interval	80 (8.5)	133 (7.8)	0.94 (0.69, 1.27)	*0.698[a]*	0.97 (0.67, 1.40)	*0.863*

p for trend = 0.000023

OR_{adj}: adjusted for smoking habit at age 15 (smoker vs. non smoker at age 15), level of education (see text), low sun exposure at age 0-15 years of age, history of mononucleosis (yes vs. no), exposure to breastfeeding (no/less than 4 months vs. 4+months)

[a] Fisher test

Table 2.8 Association between MS and the sibship size for younger siblings with interbirth interval of 0-2 years.

	Cases	Controls	*p* [a]
ITALY			
Sibship size for younger siblings with interbirth interval of 0-2 years from the index child, N (%):			
0 siblings*	523 (74.5)	973 (73)	*0.762*
1 sibling	165 (23.5)	329 (24.7)	
2 or more siblings	14 (2.0)	30 (2.3)	
Sibship size for older siblings with interbirth interval of 1-2 years from the index child, N (%):			
0 siblings*	526 (74.4)	999 (74.9)	*0.783*
1 sibling	175 (24.8)	319 (23.9)	
2 siblings**	6 (0.8)	15 (1.1)	
NORWAY			
Sibship size for younger siblings with interbirth interval of 0-2 years from the index child, N (%):			
0 siblings*	743 (78.6)	1167 (68.4)	*<0.000001*
1 sibling	194 (20.5)	498 (29.2)	
2 or more siblings	8 (0.8)	42 (2.5)	
Sibship size for older siblings with interbirth interval of 1-2 years from the index child, N (%):			
0 siblings*	770 (80.5)	1411 (82.2)	*0.244*
1 sibling	184 (19.2)	303 (17.6)	
2 siblings**	3 (0.3)	3 (0.2)	

*the number of siblings refers only to those born with the above specified birth interval from the index child.
**no one had more than 2 older siblings with interbirth interval of 1-2 years.
[a] chi square

Comment

Our investigation of sibship characteristics in two different populations, the Italian and the Norwegian, produced different results. In the Norwegian sample, we found that having siblings

(any) and the sibship size were inversely associated with MS risk. In particular, this protective effect was due mainly to younger siblings while having older siblings did not affect the risk of the disease at all. We interestingly found that the having younger siblings with interbirth interval of 0-2 years was strongly protective for MS, even after adjusting for confounders. Siblings with interbirth interval of 0 from the index child have been included in the pool of younger siblings for the analyses; they could be twins, homozygous or heterozygous, or siblings born within the same calendar year as the index child. When considering instead younger siblings with a greater interbirth interval (over 2 years), the sibship lost its protective role.

Only two more Australian studies reported the protective effect of having younger siblings against the risk of MS or first clinical diagnosis of CNS demyelination [Ponsonby 2005a, Hughes 2013]. Similarly to the Australian group of Ponsonby et al, we also found a linear association between the risk of MS and the exposure to younger siblings: a greater sibship size of close-in-age younger siblings was the most protective sibship profile. This assumption has previously been suggested by two older studies on birth order performed on the French population and on the Israelis MS patients; the latter described a greater birth order of the MS Israelis patients born abroad compared to the natives, meaning that they were more frequently younger than their siblings [Alperovitch 1981, Zilber 1988]. A Swedish study found instead a positive association between smaller sibship size and MS risk regardless of the birth order [Montgomery 2004]. Conversely, in the Italian population we found no change in the risk for MS dependent on having siblings of any number, age and order. Our results for the Italian subjects were in accordance to that of a large population-based Danish cohort study [Bager 2006]. Moreover, the American and the Canadian searches found no association between birth order and MS, as did another Swedish study [Visscher 1982, Gaudet 1995, Sadovnik 2005, Ahlgren 2005]. Finally, evidence exists even supporting the protective role of older siblings against MS in Danish, native Israelis and German populations [Isager 1980, Zilber 1988, Conradi 2011]. Methodological differences have been suggested to be responsible for those wide range of results. Although the oldest studies have used only birth order as sibship measure, some other studies were of high quality. Furthermore, some methodological differences have been overcome in our study in which, using the same methodology, two different populations were investigated, the Norwegian and the Italian, which have never been studied before in relation to the association between sibship and MS. How was then possible to explain our results dichotomy? The first thinking is that some kind of risk factors may affect differently cases and controls of the two populations. To avoid this bias, we adjusted all the ORs for many environmental confounders including some of the well-known risk or protective factors for MS. The only major risk factor that could not be considered is the genetic predisposition. Therefore, the most probable explanation to our opposite results between Italy and Norway, and also to the conflicting findings of the previously published studies over years, is that the sibling effect varies depending on the geographical location. The geographical variability is indeed the most consistent finding and could be explained by some differences in the early life exposures, measured through the sibship, which

are related to the geographic location. Among the factors characterizing a specific geographic region (e.g., latitude, culture, diet), we suggest to consider a specific pool of microorganisms colonizing the environment and the humans. The circulating microbial pattern determines the quality of the exposure to the so called "old friends" which, on the other side, affect the immune system development during early phases of life influencing the subsequent risk of MS.

2.5 Discussion

All these apparently chaotic data regarding the variable "effect" of allergies and sibship on MS risk could find they order considering the intervention of some factors which differ depending mainly on early life exposure which will in turn affect the future risk for MS.

The most susceptible age range to the modulation of immune system is probably form birth to up to 6 years with also potential consequences later in life. Nevertheless, from our and previous evidence, it appears that the susceptibility age for the risk for MS widely differs between Italy and Norway: it is set between 0-10 years for Italian people and 11-20 years for the Norwegians. At the same time, our epidemiologic study on sibship yielded for the Norwegian population a crucial period of susceptibility in early childhood, linked to the exposure to siblings younger than 0-2 years, and related to a reduced risk for MS. This variability could be probably due to both genetic and environmental factors, and, among the latter, the "old friends" and the gut microbiota.

The potential explanatory theory we propose makes use of the "hygiene hypothesis" itself. The variable and confused association of MS with allergies and the opposite pattern of protective exposure to siblings (younger vs older siblings) in different countries and between populations could be due to different timing and type of influencing exposures during life. With our results we demonstrated the relevance of the timing of the exposure and the existence of different timing of susceptibility, typical for specific populations. To better understand this concept, we will provide some examples exploiting studies on sibship, a likely vehicle of exposure. It is possible that in Germany, France or Denmark, the crucial exposures associated with reduced MS risk occur during earlier phases of life and are therefore triggered by older siblings. On the contrary, in countries where the 'younger siblings' protective effect was prominent, such exposures do occur during the first years of life but at a later stage, and are triggered by younger siblings.

Space-time clustering studies provided a clear age range for risk exposures, but we do not know if the susceptible age range for protection is the same or not. For the Norwegians we could identify both age ranges for risk (11-20 years) and for protection (0-2 years); the concordance of our results with space-time clustering suggests that this age range is not highly susceptible of variation over time. However, these results are only applicable to Norwegian subjects, as for Italian the ranges were different.

The difference in the type of exposure is difficult to study, mostly it is possible to make indirect hypothesis on the basis what evident.

"Old friends" quality changes across geographical areas due to multiple condition and at the same time, the gut microbiota also widely varies across countries and regions. The microbiota geographical variability between children living in Italy and Burkina Faso has been attributed to dietary differences [De Filippo 2017], even so, microbiota differences have also been demonstrated among people living in the same country: as stated by a Chinese study including 2164 participants from 15 province-level divisions in China, the geographic regions explained the largest proportion of the variance (17.9%) of the gut microbiota, the authors also suggest the distinct provincial microbial structures may respond differently to diet, lifestyle and other host factors and health outcomes linked to the microbiota are likely different in different regions [Sun 2020]. Beside the microbiota profile, a lot of microorganisms, with different distribution across the world, have been postulated to be involved in influencing the immune system and MS risk. Mycobacteria, Helicobacter Pylori, malaria parasite, Toxoplasma gondii and helminths are among the pathogens proven protective for MS [Sotgiu 2008, Jaruvongvanich 2016, Cicero 2021]. Conversely, EBV, HHV6, varicella-zoster virus, cytomegalovirus, John Cunningham virus and human endogenous retroviruses may be a risk factor and even predispose to a younger onset of the disease [Villoslada 2003, Tarlinton 2020]; recent evidence also suggest Helicobacter Pylori to be a risk rather than a protective factor for MS [Kountouras 2020]. Ponsonby and colleagues described an association between the exposure to infant siblings and lower IgG levels against EBV and lower risk of IM, even if the protective effect of siblings' exposure on the risk for MS was independent from the EBV IgG titers [Ponsonby 2005a]. This finding supports the role of siblings as a measure of the exposure. It has also been proposed that early sensitization to pathogens and the consequent boosting of immune responses against latent infections could confer protection against the autoimmunity trigger of late infections such as that of EBV [Ponsonby 2005a].

It is known that different geographical locations underlie different patterns of exposure to microorganisms: some protective environmental "old friends" may be endemic in some locations and rare in others. According to what previously suggested, a different local exposure to "old friends" may affect the disease risk with a space-time cluster. The potential extreme exposure variability between geographical areas should be taken into account when studying the risk of an exposure into a defined population, moreover, each population should be studied singularly to enhance the possibility of finding a measurable results. It follows that the wider the origin of the sample, the higher the probability of not measuring an effect due to confounding effect of different exposures.

In addition, we suggest that the effect of the exposure, that is the risk or protection, also depends on its size: the effect could be stronger if the exposure to the specific microorganism or allergen is repetitive and persistent across the susceptible years of childhood. A higher exposure could be due to higher sibship size and child-to-child contact or to the exposure to the countryside rather than the city.

Finally, different timing and type of exposures during life are the principal factors influencing the outcome of such exposure and this may explain our apparently contradictory results.

The EnvIMS study is one of the largest epidemiological studies allowing researchers to assess the association between allergy and sibship with MS. Its adjunctive strength is the inclusion of different populations by latitude and cultural background. In addition, the nature of the EnvIMS study allowed us to adjust our results for several environmental confounders: smoking habit (ever smokers or smoking at age 15), level of education, low sun exposure in infancy or adolescence, history of IM and breastfeeding. The possibility to adjust for many variables, which had been specifically selected for each study, increases the validity of our results.

Considering the retrospective nature of the study, which is the main limit of case-control studies, we had to exclude those exposures occurred before the age of MS onset. In our specific sub-studies, the measured exposures (allergy and sibship) occurred mostly during early phases of life and adding the assignment of an index age to matched controls and the fact that all subjects were at least 18 years old at the moment of the enrollment, this bias is unlikely to have occurred. Furthermore, having siblings is not susceptible to recall bias. Allergy, on the other hand, especially if with onset early in life and then resolved, could be forgotten and not registered unless the subject has received help from parents in completing the questionnaire. Furthermore, we cannot exclude that food intolerance has been misinterpreted and reported as food allergy, which is a common misunderstanding among the population. In order to avoid this kind of mistake, some examples of allergic reactions were reported by the questionnaire. These potential biases, however, would affect both cases and controls and we have no reason to believe that misclassification bias could have occurred in filling out the questionnaire.

The When-What-How much-Old Friends (WWHOLF) methodological approach

Considering the potential protective effect of the exposure to "old friends" during childhood on the risk of allergic and autoimmune diseases, in particular MS, with our work we attempted to shed light and provide a contribution to select a most sound methodology when investigating such matter.

In a hypothetical such conceptual framework a baseline assumption is that not only is important "*When*" the exposure to the "old friends" takes place, but also "*What*", that is to which microorganism, and "*How much*" in relation to the strength of effect of such exposure on the MS risk. Ideally, the effect of any exposure should be framed within these three conditions, which, far from being novel concepts, to our knowledge were never applied to the "hygiene hypothesis" and the "old friends". We will call this kind of analysis, aimed to characterize the early life exposures, the "*WWH* ("*When*" "*What*" "*How much*") *OLd Friends* (*WWHOLF*) methodological approach". The concept of "*When*" applied to the "hygiene hypothesis" was first proposed by Kramer with the "early immune challenge hypothesis" [Kramer 2013]; nevertheless, what has become clear now is that "*When*" is not standardized across populations but is strictly dependent on the "*What*". The "*What*" factor in turn is deeply related to another variable, the "*Where*", because different geographic areas may be inhabited by a different spectrum of microorganisms and also the gut microbiota differs according to geographical regions. The "*How much*" instead reflects strength and repetitiveness of the exposure which influences the immune system response and adaptation to it. This concept can be easily applied to atopic manifestations: children living in the countryside and who are exposed to a high number of allergens tend to have less allergic disorders than those living in the cities and the protective effect is higher for long-term and early-life exposures [Riedler 2001]. Thus, the "*How much*" factor may be influenced by the environment ("*Where*", e.g., countryside) and by the time ("*When*", e.g., early/long-term exposure).

As for sibship, the "*How much*" is instead better expressed by the sibship size: a greater sibship size is associated to higher exposure. Nevertheless, as we have seen, the size of exposure is not sufficient to modulate the immune system and needs to occur during the most susceptible period ("*When*") and to involve the right source of exposure ("*What*"). This implies that no single variable is sufficient, but all must be satisfied in order to measure an effect. To specifically evaluate the extent of the effect of these variables in the context of a population-based study, it would be preferable to investigate at least two of them. This is the reason why the birth order per se, which is a poor measure of the "*When*" and "*How much*", may not be sufficient to obtain a reliable result.

Ideally, the WWHOLF approach could be applied to all exposures beyond that of "old friends" in the context of the "hygiene hypothesis". Nevertheless, while for the measurement of other factors, such as smoking, the "*What*" dimension is predominant and the variable rather easy to measure, to investigate the "hygiene hypothesis" is much more complicated and we have to find indirect but easily measurable indicators of the "old friends" exposure. For this purpose, siblings and,

traditionally allergic diseases, intended as a counterpart to MS, have been proposed. However, while sibship could be a direct measure of the exposure, the role of allergy is more difficult to comprehend.

Applying the *WWHOLF* approach to our work will lead to the following considerations and interpretation of results.

The results of the EnvIMS study on allergy yielded two different "*When*" variables for the Italian and Norwegian populations, that is early childhood for Italy and adolescence for Norway. Unlike sibship, allergy does not constitute a real exposure, but it is an effect of the exposure. Studying the effect does not exactly coincide with studying the sum of exposures. It has been proposed that MS and allergy share protective factors (e.g., the "old friends" exposure, sibship), but not necessarily the same "*What*" is sufficient to produce both effects (MS and allergy). Moreover, the "*What*" here is likely a mixture of multiple exposures, rather than one. Anyway, the aim of such a study is not measuring the "*What*" but the effect, meaning searching for the sum of the three variables, without knowing them. This is the reason why evaluating the association between two different diseases, such as MS and allergies, even if supposed associated, is much trying than directly measure the claimed exposure and the chance of mistake is high. If we then add the fact that some exposures might have an inverse impact on the two diseases or vice versa the cumulative effect might be exponential, we completely understand the conflicting results of literature and also of our analysis which indeed could have given rise to misunderstandings without using correction methods.

With regard to sibship, in our study we were able to adequately measure two variables: "*When*" and "*How much*". We were therefore able to conclude that in the Norwegian population having younger siblings aged 0-2 years younger than the index child was protective against the MS risk. Here, the "*When*" was 0-2 years while the linear association between the sibship size and the protective effect, that was present only when the "*When*" factor was satisfied, constitutes a measure of the "*How much*" variable. Considering the strength of this association even after the adjustment for confounders, we have no reason to think it should not be real. Once clarified the "*When*" and "*How much*", the "*Where*" may suggest us some candidates to solve the "*What*" question, even if the answer is unlikely to be unique. In fact, it is certain that several microorganisms are involved in determining the risk for the disease and not just one, even if some major players can be suggested.

The absence of a measurable effect of the exposure could be due to the selection of a too heterogeneous population with people coming from different geographical areas: the same timing ("*When*") and quantity ("*How much*") of exposure is referred to different unidentified actors ("*What*"). Measuring altogether the effect of the exposure to different pools of "old friends" belonging to distant regions and with potential different actions, generates confounding and it could mask the exposure effect, which is therefore unquantifiable. Another possible explanation is the intervention of other risk or protective factors, which level and cover the effect of the exposure. In our study we corrected our results for confounding including environmental risk factors (smoking, sun exposure, level of education, breastfeeding and IM) which influence can be then excluded.

Another possible confounder is the genetic predisposition which might exert a greater role in some regions (e.g., Sardinia) than others [Sotgiu 2003].

The WWHOLF approach could also help explaining the finding by Zilber and colleagues that MS patients who developed the disease in Israel but were born abroad had a different birth order trend compared to the natives: the former had indeed a higher birth order than expected while MS patients had a lower birth order than controls [Zilber 1988]. The age at migration and the country of origin were not mentioned, probably they varied, yet the teaching of this unique but ancient analysis is that sibship may be capable, even within the same population, of influencing MS development in function of early life exposures. In this specific case, the difference between exposures lays probably in the "*What*". Unfortunately, the birth order alone does not allow any further consideration. Anyway, this result is consistent with our finding, supporting the idea that sibship might be one of the early life factors influencing the risk of MS during the early stages of life and it is indirectly dependent on the geographic area. Further migrants' study would be helpful in better understanding the difference in sibling effect across populations.

A different strategy is to start from the "*What*", that is to investigate the exposure to one or more specific microorganisms proposed to have a pathogenic role in the disease (e.g., EBV). Anyway, considering that it is impossible to globally measure the exposure over time and the interactions between the different "old friends", the effect of the single microorganism will be measurable only when sufficiently strong. Some evidence exists regarding a potential contradictory effect of specific pathogens, such as Helicobacter Pylori which has been proposed both as a protective and a risk factor for MS. Even if the final effect is unknown, we may suppose that its effect on the immune system and MS risk is not sufficient to determinate a consistent modification of the risk. Otherwise, its effect is only measurable when the other variables are fulfilled, that is when the exposure occurs with a specific timing and for a sufficient period of time. Another possibility is that the same microorganism is capable of exerting different effect depending on the "*When*" and "*How much*". We have to mention that for H. Pylori nor the "*When*" or the "*How much*" variables have been identified yet, it is therefore reckless to make conclusions.

We have seen how difficult could be trying to study this kind of exposure starting from the supposed causal factor, mostly because it is probably a mixture of multiple environment stimuli which could also induce a subjective response dependent on host interaction, as for example the gut microbiota. An adjunctive possible limitation for this type of studies lies in the measurement method which can vary from serology to histological detection.

In conclusion, the *WWHOLF* conceptual framework provides support to a simplification of complex mechanisms underlying the risk for MS as well as other polyfactorial diseases and albeit resembling a mathematical thinking, far from the biological world, may indeed reflect interpreting real-world evidence in such conditions. The identification of specific exposures modifying the risk for disease, with the help of the WWHOLF methodological approach, could allow in the future to implement individualized prevention strategies.

Chapter 3. – Interactions between the gut microbiota, oral disease modifying treatments and environmental factors in multiple sclerosis patients

3.1 Rationale and Objective

A dysbiotic condition can influence the pathogenesis of neuroinflammatory diseases: the gut microbiota has been related to the development of EAE in experimental animal models and MS in humans. Microbiota derangement may result in a reduction of some bacterial species involved in the synthesis of immunomodulatory molecules and, on the other hand, an increase in other species with immunostimulant or neurotoxic properties which could promote an immune response against the CNS. Strachan's "hygiene hypothesis" and the following theories have invested the microbiota of a crucial role in determining the predisposition to develop MS, starting from the early phases of life. Furthermore, it has also been suggested that gut microbiota is able to influence MS prognosis, directly or indirectly. Recent evidence has related the gut commensal flora to MS activity and bacterial networks have been associated to different MS outcomes [Tremlett 2016a, Horton 2021]. Although only sporadic case reports and one longitudinal single-subject study have described the therapeutical effect of faecal microbiota transplantation in the treatment of MS [Borody 2011, Makkawi 2018, Engen 2020], this could be a valid strategy according to the results of studies on animal models; clinical trials in humans are still on-going [Schepici 2019]. We must also consider the potential mediation role of the gut microbiota between MS and environment: several environmental factors indeed influence the microbiota composition affecting consequently the immune pathways. This role can be seen as a strength but also a weakness meaning that the microbiota permeates almost all the pathogenetic aspects of MS that are affected by the environment but, at the same time, this makes it very difficult to study it properly due to the multiple confounders.

Given its immunomodulatory properties, in both pathogenetic and prognostic sense, the gut microbiota has now become a potential target for disease-modifying treatments (DMTs). Emerging evidence suggests that some DMTs could normalize the composition of the gut microbiota [Cantarel 2015, Jangi 2016, Tremlett 2016a, Katz Send 2018, Storm-Larsen 2019], which may underlie a therapeutic effect mediated by the microbiota itself. At the same time, DMTs action on the gut microbiota could be responsible for their side effects [Storm-Larsen 2019]. Nevertheless, wide longitudinal studies are still lacking and most of the researchers did not focus on a single DMTs but considered together different therapies. Moreover, it lacks an evaluation of if and how environmental factors affect the microbiota response to the DMT.

With the present study we wanted to shed some light on this complex question trying to clarify the longitudinal impact of the oral DMTs dimethyl fumarate (DMF) and teriflunomide (TFN) on the gut microbiota in RRMS patients assessing also the influence of environmental factors and lifestyle habits. Starting from the hypothesis that DMTs could normalize the altered gut microbiota of MS patients, we evaluated the microbiota composition in a cohort of MS patients without therapy, candidates to start DMF or TFN, which were followed with serial fecal samples over 6 months. The primary aim of this exploratory study was to capture potential microbiota modifications after oral DMTs start. Additional endpoints were i) to investigate the influence of lifestyle habits and diet on the gut microbiota composition and biodiversity before and during DMTs intake, ii) to disclose an association between DMTs side effects and the microbiota composition.

The final objective of the study is therefore to increase knowledge on the role of intestinal bacterial flora in the pathogenesis of MS and in the response to immunomodulating drugs. Identifying the major action of DMTs on the microbiota may contribute to add information on their action on neuroinflammatory processes and secondarily on the pathogenetic mechanisms of MS.

3.2 Materials and methods

Patients' enrollment and data collection

We enrolled consecutives patients with diagnosis of relapsing-remitting MS (RRMS) according to McDonald criteria of 2010 or McDonald criteria of 2017 (for patients with more recent diagnosis). Patients were recruited at the Multiple Sclerosis Center and at the Unit of Neurological Clinic of the Hospital of Ferrara between January 2018 and January 2020. We included patients aged 18 to 65 years and candidate to start DMF or TFN. Past treatment with other DMTs was not an exclusion criterion. Exclusion criteria were antibiotic treatment or high-dose corticosteroids in the last 30 days before enrollment, previous GI surgery, pregnancy, current or recent treatment with immunosuppressive drugs (in the previous 12 months). An informed consent was acquired for each subject. We collected information regarding the history of the disease (year of onset, clinical data, previous treatments, neuroimaging), life-style (smoking habit, physical activity, sun exposure, diet), delivery mode, breastfeeding, comorbidities and concomitant medications. All the patients underwent a clinical evaluation at baseline, before starting the DMT, and during the follow-up after 1, 3, and 6 months from the enrollment. Follow-up visits included EDSS score calculation, registration of any modification of lifestyle habits (smoking, sun exposure, physical activity, diet) and of DMT side effects especially focusing on the presence of flushing for the subjects assuming DMF and of gastro-intestinal (GI) side effects in both DMF and TFN group (e.g., gastralgia, diarrhea, abdominal pain). Each patient during the visit also provided a stool sample for a total of 4 samples per subject.

Eating habits were collected using a self-administered questionnaire; these data were entered on an "open access" online platform (Grana Padano Nutritional Education) which allowed us to calculate

the relative percentage and the estimated amount of macro and micronutrients in the diet. Basing on the known proinflammatory properties of dietary factors [Riccio 2015, Riccio 2018], we defined the proinflammatory diet as the intake of a high percentage of daily Kcal of lipid derivation (> 35%) and/or the consumption of more than two portions of red meat per week. None of the patients received specific dietary advice; we just recommended taking the therapy after meals for those about to start DMF.

Gut microbiota analysis

Stool specimens have been collected before starting the DMT and after one, three and six months. Each patient has been provided with tubes for the collection and storage of fecal nucleic acids. The presence of storage liquid inside the tube makes the immediate processing or freezing of the fecal sample unnecessary, allowing storage at room temperature for up to 2 years for DNA and up to 7 days for RNA preservation. After the collection, the samples have been stored at 4°C and then transferred within a week to the Neurochemistry Laboratory of the Unit of Neurological Clinic and stored at -20°C until the analysis.

The microbiota analysis was performed by GenProbio s.r.l. (Probiogenomics Lab, University of Parma). After extracting bacterial DNA from stool samples, the region V4 of 16S rRNA has been amplified through PCR and sequenced using the platform Illumina MiSeq with the protocol 2x150bp paired-end. The generated "reads", after filtering, were grouped into taxonomic units (OTUs) through the QIIME 2 (Quantitative Insights Into Microbial Ecology) software. QIIME is an open-source software for comparison and analysis of microbial communities; it allows the taxonomic assignment and the construction of phylogenetic trees. Bifidobacteria subspecies have been analyzed using the internal transcribed spacer bifidobacterial profiling (ITSbp).

The database Silva v. 132 and a customized database containing the sequences of the bifidobacteria currently known were used respectively to obtain the results of the 16S rRNA analysis and of the ITS analysis of bifidobacterial.

The analysis of biodiversity α was carried out calculating the Chao 1 index.

Statistical method

We used descriptive statistics to present the demographic and clinical characteristics of the patients; continuous variables were shown as average or median and standard deviation.

The statistical approach included a cross-sectionally analysis to measure the influence of demographic, lifestyle, early life factors and clinical variables (sex, month of enrollment, smoking habit, physical activity, sun exposure, diet, breastfeeding, childbirth, EDSS score, previous treatment with DMTs and the presence of new lesions or contrast-enhancing lesions) on the microbiota composition at baseline for which we used the non-parametric tests Mann-Whitney U test and Kruskal Wallis for independent variables. Depending on the enrollment month, corresponding to baseline data and first samples collection, we have distinguished 4 periods: 1=

between January and March, 2= between April and June, 3= between July and September, 4= between October and December. In order to measure the effect of the lifestyle, we created a unique variable including physical activity and sun exposure ad we noticed that these kinds of exposure were associated with each other in our patients. We therefore defined a sedentary lifestyle when the sun exposure during the weekend was less than 3 hours and the patient used to practice intense physical activity less than once a week. The opposite lifestyle according to sun exposure and intense physical activity was defined as active. For the analysis of the microbiota in relation to the breastfeeding we considered ≤3 months as cut-off between breastfed and non-breastfed patients.

The overall MS population was then grouped by DMT, the demographic and clinical characteristics of the two were compared with Mann-Whitney U test.

The non-parametric Wilcoxon signed-rank test was used to longitudinally evaluate the microbiota composition, at phylum, family and genus level, comparing the baseline samples with those obtained at first, third and sixth month. We analyzed separately the patients treated with DMF and TFN. The analysis was then repeated stratifying for dichotomous variables related to environmental factors: diet (proinflammatory diet vs balanced diet), lifestyle (physical activity and sun exposure) and smoking habit.

We considered p less than 0.05 to be significant; as for Wilcoxon signed-rank test we showed all non-adjusted p-values as previously done by other researchers' group [Storm Larsen 2019], considering the experimental nature of the study and in order to capture also slight variations. If we had to correct the exposed results, considering three longitudinal comparisons (baseline-first month, baseline-third month and baseline-sixth month), the significant p-value would be inferior to 0.0167. We applied the analysis to Chao 1 index and to the relative abundance of each phylum (n=7), family (n=21), genus (n=33) and Bifidobacterial subspecies (n=9). We excluded from the statistical analysis the phylum Fusobacteria and some genera because absent in almost all the patients. The two main phyla were also measured as Bacteroidetes/Firmicutes (B/F) ratio.

As counter-proof we also cross-sectionally compared the microbiota composition, with Mann-Whitney U test, of the DMF and TFN groups at baseline and after 6 months of treatment.

Statistical analysis was performed with SPSS Statistics for Windows, version 27 (IBM Corporation, Armonk, New York, USA).

Ethical approval

The study was approved by the local Ethics Committee. Every patient gave written informed consent.

3.3 Results

Population's demographic and clinic characteristics

We enrolled 26 patients with diagnosis of relapsing-remitting MS between January 2018 and January 2020. One patient withdrew the consent before starting the study and one stopped

assuming TFN after the first month cause of side effects, therefore, we performed the analysis including 24 patients, those for whom the microbiota analysis was available. Nineteen subjects started DMF (13 females and 6 males) and 5 TFN (3 females and 2 males). These two groups were not significantly different except for disease duration (average 6,8 ± 6,2 vs 9,8 ± 2,6 for DMF and TFN patients respectively) and EDSS (median 1,5 vs 1 for DMF and TFN patients respectively). Demographic, clinical and life-style characteristics of the two subgroups of the population are shown in **Table 3.1**. Some patients had autoimmune comorbidities: 4 patients suffered from thyroiditis, 2 from psoriasis, one from undifferentiated connective tissue disease, one from lichen planus and one from vitiligo. Moreover, one patient referred a history of Henoch Schonlein purpura, another one had platelet disease due to the JAK2 mutation, one had hyperinsulinism and 3 patients suffered from psychiatric disorders (depression, bipolar disorder and anxiety). Only one patient was taking PPI at the moment of enrollment while 5 women were under oral contraceptives. Four more subjects were integrating vitamin D. Most of the patients (N=18; 75%) had a proinflammatory diet. The estimated amount of carbohydrates, lipids, sugars, proteins, fibers and micronutrients was similar between the DMF and TFN groups (**Table 3.2**). The overall diet composition and other lifestyle factors (physical activity, sun exposure, smoking) did not change significantly between the baseline and the follow-up period (data not shown).

DMTs side effects

The most frequent side effects in DMF population were flushing and GI disorders (52,6% for both disorders during the first 6 months of treatment). GI side effect were more common soon after DMF start, they were referred by the 42% of patients after the first month, the 37% after the third months and only by the 18% after the sixth month of treatment. Females were more likely to have GI side effects measured during the first 3 months of treatment with DMF ($p=0.028$) compared to males. As for flushing, it was reported by the 42% during the first month follow-up and its prevalence remained quite stable up to the end of the study. In the group taking DMF we found an association between GI side effects during the first month of the treatment and the diet, specifically the percentage of lipids and carbohydrates assumed: the presence of GI disorders was positively associated with a lipid-rich diet ($p=0.007$) and negatively with a carbohydrate-rich one ($p=0.006$). The presence of GI side effects during the first month of treatment was also associated to oral contraceptives intake in DMF group ($p=0.003$). The flushing showed a trend toward an inverse association with carbohydrates intake during the first month of treatment ($p=0.053$); conversely, patients with proinflammatory diet had more frequently flushing in the same period ($p=0.031$). No further association was found between DMF side effects and diet and other lifestyle factors. From routine analyses we also detected, as expected, a significant decreased in lymphocyte count. In the TFN group, the most prevalent side effects were GI symptoms which were frequent (40%) and persistent during the observation period. We found no association between TFN side effects and other variables. Nobody had to stop the treatment because of side effects or serious adverse events.

Table 3.1 Demographic, clinical and life-style characteristics of the study population divided by treatment started.

	DMF N=19	TFN N=5	p
Demographic characteristics			
Age (years), average (SD)	38.5 (8.7)	41.8 (4.4)	*0.476*
range	27-55	37-47	
Females, N (%)	13 (68.4)	3 (60)	*0.728*
Clinical characteristics			
Age at MS onset (years), average (SD)	34.1 (9.1)	33.6 (6.4)	*0.943*
range	16-53	27-43	
MS duration (years), average (SD)	6.79 (6.2)	9.8 (2.6)	***0.046***
range	3-25	6-13	
Previous treatment with DMT, N (%)	7 (36.8)	3 (60)	*0.360*
EDSS score, median (range)	1.5 (1-4)	1	***0.045***
Number of relapses in the previous 2 years, average (range)	1.2 (0-3)	0.4 (0-1)	*0.458*
New lesions on the MRI, N (%)	12 (63.2)	2 (40)	*0.360*
Contrast enhancing lesions on the MRI, N (%)	3 (15.8)	1 (20)	*0.507*
BMI, average (SD)	24.5 (4.7)	24.7 (4)	*0.972*
Lifestyle characteristics			
Smoking			
Ever smokers, N (%)	11 (57.9)	3 (60)	*0.934*
Sun exposure			
Work mainly outdoors, N (%)	4 (21,1)	2 (40)	*0.394*
Sun exposure during the weekend over 2 hours, N (%)	14 (73.7)	3 (60)	*0.558*
Physical activity			
Intense physical activity ≥ 1 per week, N (%)	8 (42.1)	2 (40)	*0.934*
Modest physical activity ≥ 1 per week, N (%)	16 (84.2)	4 (80)	*0.826*
Early life factors			
Breastfeeding			
More than 3 months, N (%)	7 (36.8)	0 (0)	*0.114*
Childbirth			
Natural, N (%)	18 (94.7)	4 (80)	*0.299*
Month of enrollment			
Period 1, N (%)	11 (57.9)	1 (20)	*0.235*
Period 2, N (%)	3 (15.8)	2 (40)	
Period 3, N (%)	2 (10.5)	1 (20)	
Period 4, N (%)	3 (15.8)	1 (20)	

Table 3.2 Diet characteristics of the study population divided by treatment started

	DMF N=19	TFN N=5	*p*
Diet			
Proinflammatory diet*, N (%)	14 (73.7)	4 (80)	*0.776*
Stimated daily macronutrients assumption, g (SD)			
Proteins	66 (21.4)	64.1 (20.3)	*0.881*
Carbohydrates	230.1 (64.4)	193.8 (100.1)	*0.333*
Sugar	97.1 (30.4)	75.6 (38.3)	*0.233*
Lipids	61.7 (24.8)	69.3 (48.1)	*0.766*
SFA	21.4 (8.3)	21.5 (12.1)	*0.823*
PUFA	9.1 (3.8)	9.6 (4.3)	*0.766*
Fibers	22.8 (8.5)	17.7 (6.6)	*0.180*
Alcohol	5.7 (8.6)	3.9 (4.6)	*0.755*
Stimated daily oligoelements assumption, (SD)			
Calcium, mg	863.4 (289.8)	732.6 (293.7)	*0.264*
Iron, mg	10.6 (3.3)	9 (2)	*0.248*
Zinc, mg	9.4 (2.9)	8.8 (2.6)	*0.628*
Vitamin A, mcg	1131.3 (577.7)	861 (371.6)	*0.264*
Vitamin D, mcg	1.7 (1)	1.9 (0.7)	*0.247*
Vitamin E, mg	10.5 (4.8)	10.9 (7.5)	*0.709*
Folic acid, mcg	338.4 (125.6)	284.8 (89.1)	*0.434*
Vitamin B12, mcg	3.5 (1.6)	3.8 (1.2)	*0.314*
Vitamin C, mg	174.8 (83.9)	123.4 (44.6)	*0.157*
Percentage of daily Kcal, (SD)			
Carbohydrates	50.2 (5.2)	44.2 (7.3)	*0.050*
Sugar	21.5 (5.9)	17.2 (3.7)	*0.101*
Lipid	32.1 (5.4)	36.6 (6.7)	*0.224*
SFA	10.9 (2.3)	11.8 (1.9)	*0.472*
PUFA	4.8 (1.5)	5.6 (1.1)	*0.230*

*Proinflammatory diet was defined as a high daily Kcal intake (> 35%) of lipid derivation and/or the consumption of more than two portions of red meat per week.

Microbiota characteristics

Stool specimens of 24 patients (8 males and 16 females) were analyzed. At baseline we found a high interindividual variability in microbiota composition, the main phyla were Bacteroidetes and Firmicutes (**Figure 3.1**). We evaluated the microbiota characteristics by sex, month of enrollment, smoking habit, diet, physical activity, sun exposure, BMI, breastfeeding, childbirth, EDSS score, previous treatment with DMTs and the presence of new lesions or contrast-enhancing lesions on the last brain MRI.

We found between males (n=8) and females (n=16) no difference in the alpha diversity and phyla relative abundance, as for genera, Bifidobacterium (phylum Actinobacteria) was more abundant in males (p=0.032) while the genus Blautia (phylum Firmicutes) in females (p=0.027).
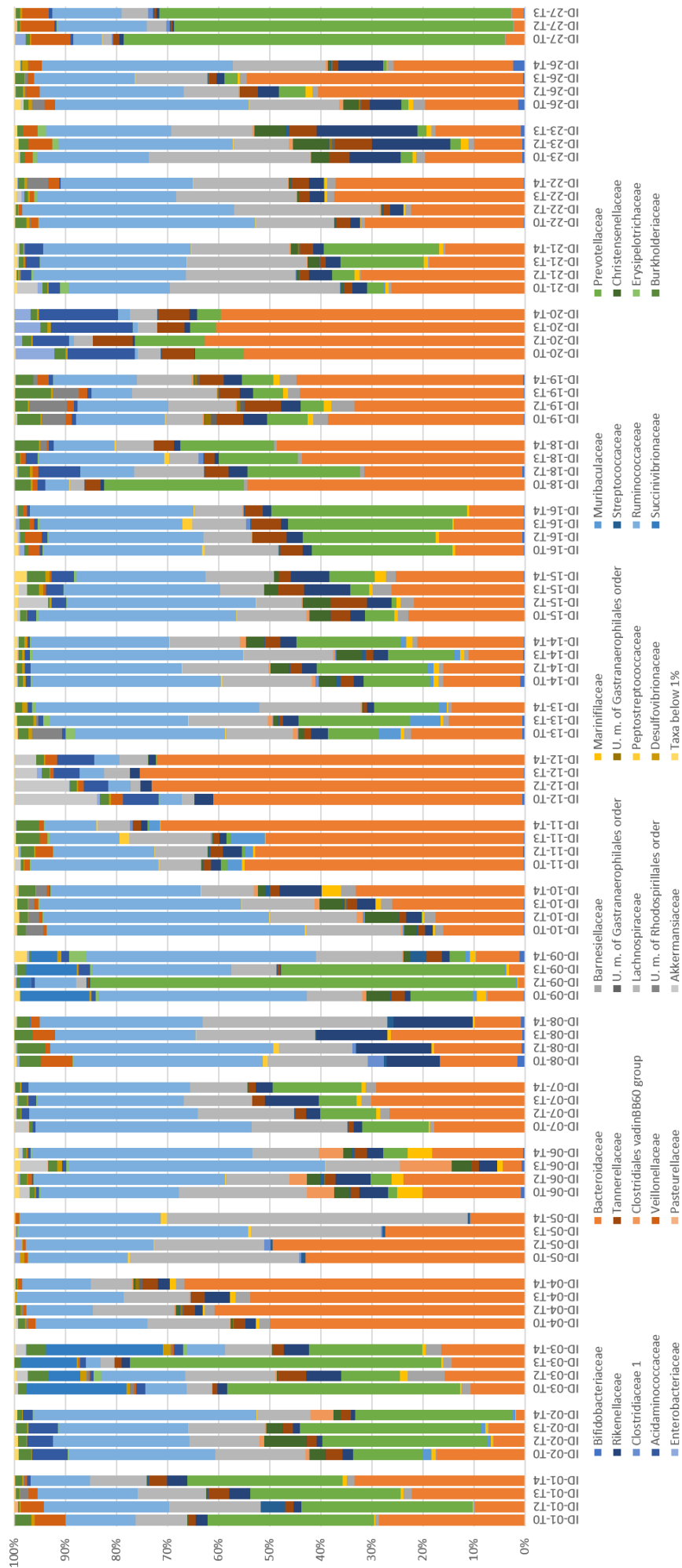
Figure 3.1: Relative abundance of the bacteria families for each patient at each time-point.

Depending on the enrollment month, we detected differences in 3 main phyla, 2 genera and one subspecies of Bifidobacterium. In patients enrolled during the period 1 the phylum Actinobacteria was less represented while it was more abundant in those enrolled in the 4th (p=0.038). The phylum Bacteroidetes was predominant for subjects enrolled in the period 1 and 3 while it was significantly lower for those of the period 2 (p=0.004). On the contrary, the phylum Firmicutes was more abundant in patients enrolled in the period 2 and less in the period 1 and 3 (p=0.025). This seasonal difference in the phyla abundance has also been shown by the greater B/F ratio for the subjects enrolled during period 1 or 3 (p=0.005) (**Figure 3.2**). As for genera, we found a higher relative abundance of Bifidobacterium in the period 4 and lower in the 1st (p=0.013); the subspecies Bifidobacterium Breve was conversely more abundant for those subjects enrolled during the period 1 and 2, and less abundant in the period 4 (p=0.033). Finally, the relative abundance of the genus Ruminococcus 2 (phylum Firmicutes) was greater during the period 4 and lower in the 2nd (p=0.038).
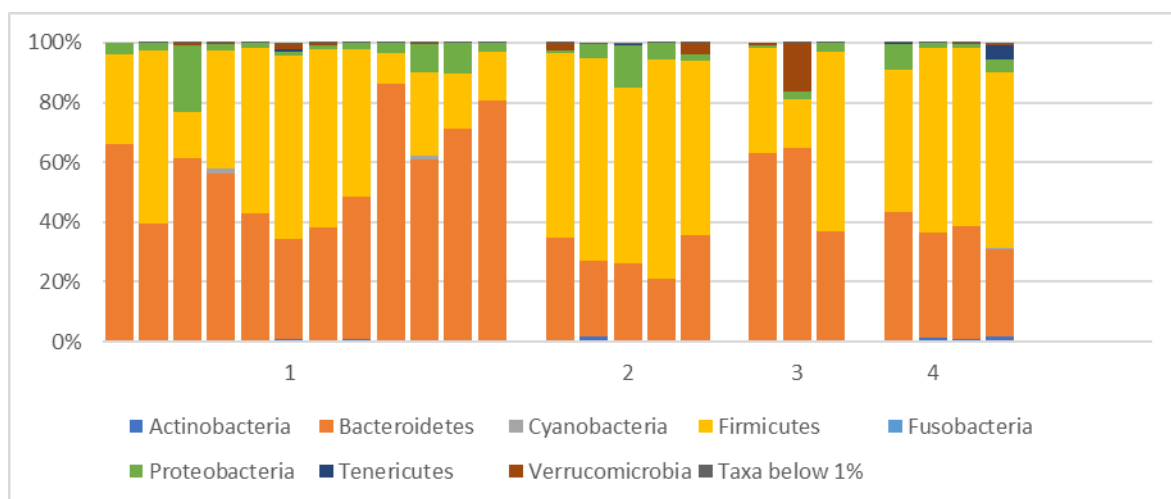

Figure 3.2 Relative abundance of bacterial phyla for each patient at baseline by month of enrollment.
1 Januray-March, 2 April-June, 3 July-September, 4 October-December.

At baseline the biodiversity tended to be higher in never smokers (n=10) compared to ever smokers (n=14) but without statistically significance (181.2 ± 55.4 vs 137.1 ± 54.4; p=0.053) (**Figure 3.3**). Ever smokers microbiota showed a lesser relative abundance of the phylum Tenericutes (p=0.011) and of the genera Butyricimonas (p=0.041) and Odoribacter (p=0.040), belonging to the phylum Bacteroidetes, of Christensenellaceae R-7 group (p=0.020), Coprococcus1 (p=0.037), Eubacterium coprostanoligenes (p=0.030), Ruminococcaceae NKA214 group (p=0.040) and Ruminococcaceae UCG-002 (p=0.011), belonging to the phylum Firmicutes. Roseburia (phylum Firmicutes) was the only genus tending to increase in ever smokers compared to never smokers (p=0.046). Some of these mentioned genera were poorly represented within the microbiota and could even be absent especially in ever smokers, albeit the difference in their prevalence between ever and never smokers was significant only for Butyricimonas (p=0.021) and Akkermansia (phylum Verrucomicrobia) (p=0.045).

No difference in the microbiota composition was found between people with proinflammatory diet (n=18) and those following a more balanced diet (n=6). Comparing instead the microbiota of subjects with overweight or obesity (n=7) to that of the individuals with BMI<25 (n=17), we detected a lesser abundance of the genus Streptococcus (*p*=0.024) (phylum Firmicutes) and of Bifidobacterium adolescentis (*p*=0.008) with a greater abundance of Bifidobacterium pseudocatenulatum (*p*=0.005) in overweight people.
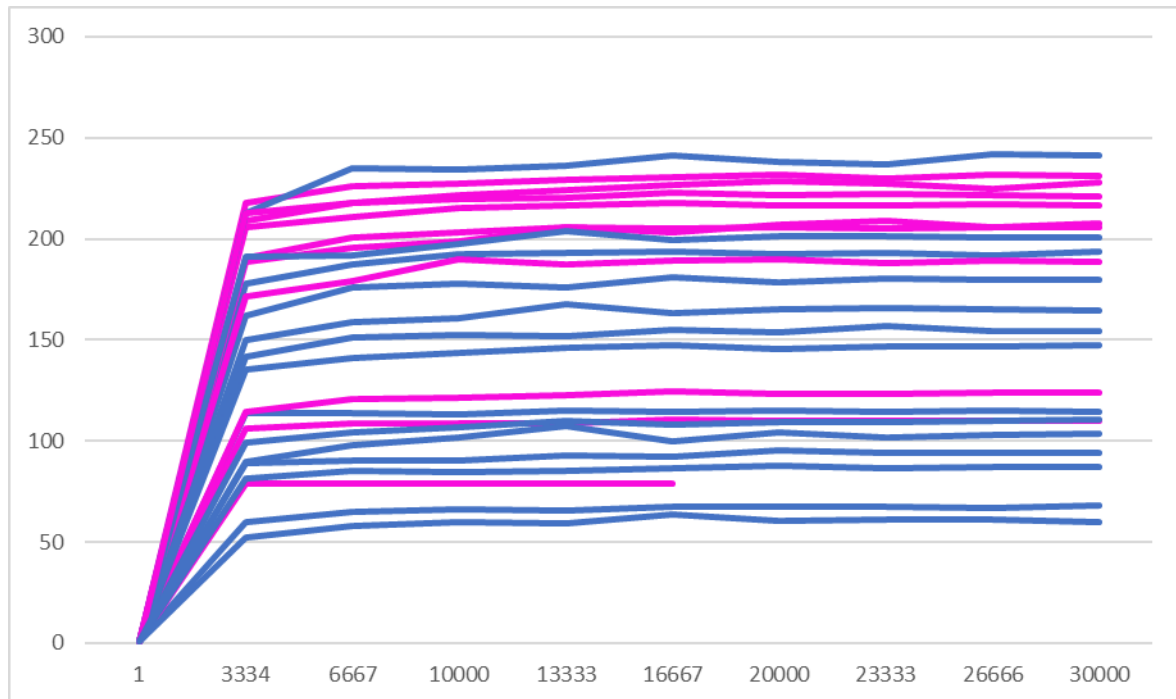
Figure 3.3 Rarefaction curves of each patient at baseline divided by ever smokers (blue lines) and never smokers (fuchsia lines).

To measure the potential effect of a healthy and active life, without considering diet, on the microbiota, we evaluated sun exposure and physical activity, these two variables were associated in our populations: those subjects practicing more physical activity also were those more exposed to the sun during the weekend days. Therefore, we created only one variable on "lifestyle" (see "statistical methods"). The subjects with an active lifestyle (n=17) showed a greater abundance of the genus Barnesiella belonging to the phylum Bacteroidetes (p=0.042) and of Bifidobacterium pseudocatenulatum (p=0.007) than those with a sedentary life (n=7), which in turn had a higher representation of Streptococcus (p=0.014) and Parabacteroides (p=0.045), belonging to the phylum Bacteroidetes. The genus Dorea (phylum Firmicutes) also tended to be greater in sedentary people (p=0.05).

We then investigated the microbiota characteristics by early life factors: mode of childbirth and breastfeeding. Although only 2 over 24 subjects were born by caesarean section, we found some differences between the 2 groups: higher abundance of Bifidobacterium (*p*=0.047) and of the genera Parabacteroides (*p*=0.032) (phylum Bacteroidetes) and Anaerostipes (*p*=0.036) (phylum Firmicutes) in those born by cesarean section; on the other side Eubacterium coprostanoligenes

(*p*=0.036), Ruminococcaceae NKA214 group (*p*=0.046) and Ruminococcaceae UCG-002 (*p*=0.046) were more abundant in those with natural birth. As for breastfeeding, patients breastfed for over 3 months (n=17) showed higher levels of the phylum Proteobacteria (*p*=0.006) and the subspecies dentium of the genus Bifidobacterium (*p*=0.009); Bifidobacterium animalis tended to be higher but without significance (*p*=0.057). Non-breastfed patients (n=7) showed a greater abundance of the genera Clostridium sensu strictu 1 (*p*=0.019), Agathobacter (*p*=0.045), Faecalibacterium (*p*=0.012), Subdoligranulum (*p*=0.007), all belonging to the order Clostridiales of the phylum Firmicutes.

We then investigated potential microbiota alterations related to EDSS score, MRI activity and the use of previous DMTs. The analysis on EDSS was affected by the high prevalence of very low EDSS (n=22). Patients with EDSS between 0 and 2 had greater abundance of the genus Alistipes (p=0.047), conversely Streptococcus was higher between subjects with EDSS >2 (*p*=0.037). Naïve patients (n=14) compared to subjects that came from other treatments (n=10) had a greater abundance of the genus Bifidobacterium (*p*=0.020) while the genera Bacteroides (phylum Bacteroidetes) and Lachnoclostridium (phylum Firmicutes) were higher in non-naïve individuals (*p*=0.040 for both genera). Comparing instead patients with MRI activity (new lesions or contrast-enhancing lesion at the last MRI performed before the treatment starting) (n=15) to those with stable exams (n=9), we found that Ruminococcus1 (*p*=0.022) and Ruminococcus2 (*p*=0.045) were more abundant in subjects with MRI activity, while Streptococcus (*p*=0.04) and Lachnoclostridium (*p*=0.022) were lesser abundant.

Gut microbiota and DMTs

The microbiota analysis was performed for all 24 subjects (19 taking DMF and 5 taking TFN). In the DMF group, we excluded from the analysis the sample after the first month of treatment for one patient due to the recent assumption of antibiotic therapy and two other patients didn't reach the sixth month of follow-up at the time of the last microbiota analysis. Therefore, the numbers of samples analyzed for DMF group were 19 at baseline, 18 at the first month, 19 at the third month and 17 at the sixth month; 20 samples were instead analyzed from the TFN-treated patients for a total of 93 samples. After starting the DMTs, the biodiversity did not significantly change in both groups. The taxonomic analysis showed the absence of major changes in the microbiota composition. Nevertheless, some phyla and genera had minor fluctuations in the relative abundance, most of which transient. In the group assuming DMF, we found transient reduction of the phylum Proteobacteria (*p*=0.014) and transient increase of the genus Anaerostipes (*p*=0.021) during the first month of treatment. Ruminococcaceae UCG002 after 3 months of treatment was increased (*p*=0.027) while Ruminococcaceae NK4A214 group tended to be reduced (*p*=0.047). Finally, the genus Clostridium showed a significant late decrease after 6 months of treatment (*p*=0.006). The subspecies of the genus Bifidobacterium did not significantly change during follow-up compared to baseline (**Table 3.3**).

Table 3.3 Biodiversity and relative abundance of the microbiota bacteria by phyla and genera during the treatment with DMF.

Microbiota	Baseline N=19	1st month N=18	p	3rd month N=19	p	6th month N=17	p
Chao1	154,335 (55,8504)	154,0416 (56,02970)	0.420	157,33958 (61,726292)	0.573	157,66045 (58,866644)	0.246
Phylum, RA (SD)							
Actinobacteria	0,00373563 (0,003461430)	0,00257725 (0,002086759)	0.170	0,00297034 (0,003069419)	0.355	0,00327850 (0,004308527)	0.687
Bacteroidetes	0,49090616 (0,18682434)	0,52128211 (0,179531660)	0.744	0,51944713 (0,182979141)	0.295	0,50388571 (0,199028218)	0.463
Cyanobacteria	0,00166496 (0,004473048)	0,00400170 (0,012971676)	0.445	0,00057293 (0,001137753)	0.575	0,00127436 (0,002611528)	0.374
Firmicutes	0,43729664 (0,206868391)	0,42945373 (0,190399620)	0.879	0,42817809 (0,192106268)	0.494	0,43764172 (0,211515643)	0.795
Proteobacteria	0,05220132 (0,056037417)	0,02949878 (0,031325947)	**0.014**	0,03956061 (0,040967824)	0.059	0,04658518 (0,065482081)	0.332
Tenericutes	0,00102377 (0,001891263)	0,00128817 (0,002529497)	0.515	0,00145432 (0,003484704)	0.721	0,00090008 (0,001704735)	0.241
Verrucomicrobia	0,01246812 (0,036769127)	0,01101696 (0,028079550)	0.300	0,00677065 (0,015512495)	0.496	0,00462997 (0,010871109)	0.158
Genus, RA (SD)							
Bifidobacterium	0,00214948 (0,002507520)	0,00138870 (0,001543573)	0.554	0,00179515 (0,002102478)	0.711	0,00163823 (0,002652432)	0.438
Bacteroides	0,27904360 (0,172573324)	0,26533345 (0,211651037)	0.586	0,27251184 (0,206458463)	0.748	0,31685547 (0,207862931)	0.287
Barnesiella	0,00652153 (0,006959379)	0,01108311 (0,018334354)	0.177	0,00929071 (0,010282656)	0.124	0,00917369 (0,010222423)	0.388
Butyricimonas	0,00200047 (0,002098778)	0,00277504 (0,003744152)	0.388	0,00218513 (0,002214054)	0.311	0,00331871 (0,003814996)	0.131
Odoribacter	0,00500893 (0,010136124)	0,00354444 (0,004370456)	0.433	0,00337675 (0,002821850)	0.551	0,00782052 (0,011469540)	0.133
Paraprevotella	0,01004944 (0,022090937)	0,01249551 (0,032086653)	0.767	0,00668032 (0,012524872)	0.059	0,00670671 (0,012017649)	0.314
Prevotella 9	0,09030973 (0,198171689)	0,10743169 (0,237181858)	1.00	0,11097050 (0,212110929)	0.331	0,04286386 (0,083277475)	1.00
Alistipes	0,02540245 (0,023086256)	0,03321800 (0,036347851)	0.124	0,03877369 (0,046779422)	0.260	0,02971507 (0,022099585)	0.326
Parabacteroides	0,02376887	0,03068478	0.227	0,02244796	0.528	0,02568697	0.278

	(0,016636131)	(0,027503015)		(0,017363636)		(0,016077866)	
Streptococcus	0,00175219 (0,002484343)	0,00422192 (0,010793044)	*0.647*	0,00190306 (0,002174836)	*0.872*	0,00338098 (0,007492506)	*0.831*
Christensenellaceae R7	0,01343398 (0,016763027)	0,01882119 (0,028457622)	*0.173*	0,01397836 (0,020541837)	*0.756*	0,00675700 (0,009160528)	*0.255*
Clostridium s.s. 1	0,00097060 (0,001771992)	0,00132653 (0,003120542)	*0.917*	0,00151798 (0,002923143)	*0.875*	0,00011240 (0,000212310)	***0.006***
Eubacterium eligens group	0,01375835 (0,020500500)	0,00711349 (0,008681274)	*0.088*	0,00793111 (0,006320180)	*0.196*	0,00974080 (0,011178326)	*0.326*
Agathobacter	0,01433723 (0,027037950)	0,01039490 (0,015559830)	*0.730*	0,01209919 (0,014679033)	*0.469*	0,00862396 (0,015257220)	*0.975*
Anaerostipes	0,00074423 (0,001353722)	0,00356675 (0,005512016)	***0.021***	0,00109881 (0,002487705)	*0.401*	0,00127885 (0,002147223)	*0.208*
Blautia	0,00392001 (0,003669322)	0,00511949 (0,006075472)	*0.586*	0,00303244 (0,002859506)	*0.277*	0,00366620 (0,002691528)	*0.981*
Coprococcus 1	0,00544236 (0,008745599)	0,00300269 (0,002220306)	*0.981*	0,00277229 (0,002841478)	*0.085*	0,00380201 (0,004303078)	*0.865*
Dorea	0,00229060 (0,004843860)	0,00263253 (0,005322491)	*0.397*	0,00122181 (0,002266068)	*0.470*	0,00089977 (0,001203002)	*0.530*
Lachnoclostridium	0,00849676 (0,009485584)	0,01201636 (0,015785382)	*0.744*	0,00859355 (0,008056122)	*0.629*	0,01060833 (0,009061479)	*0.332*
Lachnospira	0,01190578 (0,013287153)	0,00728682 (0,007211213)	*0.109*	0,00832775 (0,007946441)	*0.227*	0,00804174 (0,010289914)	*0.088*
Lachnospir.NK4A136	0,01146827 (0,013840453)	0,01128591 (0,015193145)	*0.523*	0,00982296 (0,009234111)	*0.523*	0,01125765 (0,010996093)	*0.981*
Roseburia	0,02637117 (0,031041991)	0,02285502 (0,024627812)	*0.981*	0,03498424 (0,043213878)	*0.093*	0,04576899 (0,100478030)	*0.363*
Eubacterium_coprostanoligenes	0,00647080 (0,008668712)	0,00896066 (0,013433105)	*0.868*	0,00715591 (0,008681680)	*0.653*	0,00561382 (0,009990043)	*0.836*
Faecalibacterium	0,12528009 (0,107921702)	0,12392285 (0,089460958)	*0.845*	0,12929615 (0,107356006)	*0.841*	0,13351621 (0,099490924)	*0.868*
Ruminococcaceae NKA214	0,00295456 (0,005078750)	0,00175132 (0,002232046)	*1.00*	0,00093059 (0,000953027)	***0.047***	0,00179396 (0,002413138)	*0.221*
Ruminococc.UCG002	0,01773434 (0,023868025)	0,02340332 (0,030784359)	*0.064*	0,02170348 (0,025077344)	***0.027***	0,02346318 (0,034270461)	*0.196*
Ruminococc. UCG014	0,00775457 (0,015723888)	0,00538409 (0,007976210)	*0.807*	0,00439020 (0,007301412)	*0.955*	0,00370523 (0,004788650)	*0.311*
Ruminococcus 1	0,01851663 (0,052000898)	0,01158018 (0,028058874)	*0.492*	0,01408803 (0,039237779)	*0.084*	0,00969445 (0,020517664)	*0.363*
Ruminococcus 2	0,00466483 (0,007823411)	0,00333681 (0,003720978)	*0.820*	0,00407847 (0,005654307)	*0.594*	0,00662845 (0,012887429)	*0.730*
Subdoligranulum	0,01423315	0,01524195	*0.586*	0,01396594	*0.758*	0,01497829	*0.955*

	(0,018357155)	(0,019086182)		(0,020950692)		(0,020449464)	
Sutterella	0,01410994 (0,011532375)	0,01212726 (0,010257546)	0.233	0,01416267 (0,015691509)	0.918	0,01430322 (0,014585053)	0.730
Haemophilus	0,00029369 (0,000516026)	0,00097094 (0,001697074)	0.099	0,00051855 (0,000926438)	0.382	0,00046497 (0,001106225)	0.790
Akkermansia	0,01236705 (0,036801788)	0,01076375 (0,028168552)	0.245	0,00664352 (0,015550331)	0.460	0,00429040 (0,010912683)	0.075
Bifidobacteria (RA (SD)							
B adolescentis	0,47678196 (0,306721145)	0,42554148 (0,240353978)	0.157	0,42750554 (0,255919597)	0.629	0,40737818 (0,317212411)	0.868
B animalis	0,02099287 (0,042715600)	0,03651788 (0,090415646)	0.272	0,05105949 (0,145787709)	0.125	0,04424180 (0,126915525)	0.552
B asteroides	0,01291550 (0,020704177)	0,01075352 (0,014353747)	0.929	0,01563825 (0,020413285)	0.445	0,00884782 (0,014292040)	0.508
B bifidum	0,02174961 (0,025978833)	0,04263239 (0,070240133)	0.408	0,02020020 (0,028942614)	0.352	0,03155854 (0,045401002)	0.198
B breve	0,03711894 (0,055211414)	0,04029022 (0,041673122)	0.875	0,05561245 (0,076665033)	0.125	0,03617707 (0,051669218)	0.382
B dentium	0,04787519 (0,073482547)	0,05965765 (0,102063457)	0.826	0,04595015 (0,062768742)	0.717	0,08774106 (0,185822868)	0.925
B longum subspp.	0,23073736 (0,185914226)	0,28920111 (0,217876067)	0.133	0,25308719 (0,157490527)	0.520	0,24725105 (0,158400990)	0.463
B pseudocatenulatum	0,03858645 (0,095749212)	0,02390096 (0,040115942)	0.753	0,01938056 (0,043061207)	0.554	0,03859296 (0,070546908)	0.753
B pseudolongum	0,02520292 (0,035811507)	0,03101052 (0,038189016)	0.534	0,04153004 (0,057684266)	0.074	0,032570418623189 (0,047058737496740)	0.799

RA, relative abundance. SD, standard deviation.

After stratifying for dietary habit we detected some differences in the microbiota composition according to proinflammatory versus balanced diet. The subjects with proinflammatory dietary habits (n=14) showed an increase of the genera Bacteroides (*p*=0.028), Butyricimonas (*p*=0.017), Alistipes (*p*=0.019) and Parabacteroides (*p*=0.034) after 6 months and of the genus Barnesiella (*p*=0.041) and Bifidobacterium breve (*p*=0.013) and pseudolongum (*p*=0.018) after 3 months. Clostridium sensu strictu 1 once again appeared reduced (*p*=0.028), the same trend was present among the group with balanced diet but without statistical significance due to the small sample size. In the people with balance diet (n=5), we also found a transient reduction of biodiversity measured at the third month (*p*=0.043), an increase of the phylum Firmicutes after 6 months of treatment (*p*=0.043) with a concomitant reduction of Proteobacteria (*p*=0.043) and a transient reduction of the genera Blautia (*p*=0.043) and Roseburia (*p*=0.043) after 3 months.

Stratifying for smoking habit we found instead in ever smokers (n=11) an increase of Coprococcus after one month (*p*=0.017), increased of Butyricimonas (*p*=0.028) and Ruminococcaceae UCG002 (*p*=0.018) after 3 months, increase of Odoribacter after 6 months (*p*=0.043) and reduction of Sutterella (*p*=0.021) after one month. Conversely, in never smokers (n=8) we found a decrease of Blautia (*p*=0.017) and Coprococcus (*p*=0.017) after 3 months, decrease of Clostridium (*p*=0.043) after 6 months and increase of some subspecies of Bifidobacteria which were Bifidobacterium animalis (*p*=0.043), breve (*p*=0.046) and pseudolongum (*p*=0.043) after 3 months and Bifidobacterium bifidum after 6 months (*p*=0.028) from DMF start. It is worth noting that the genus Clostridium also showed a reduction trend in ever smokers after 6 months (*p*=0.075).

Finally, evaluating the physical activity we found that those more active (n=14) during the follow-up had a transient early reduction of the phyla Proteobacteria (*p*=0.043) during the first month, an increase of the genera Anaerostipes (*p*=0.036) and Dorea (*p*=0.047) after the first month and after 3 months of treatment their levels of Barnesiella (*p*=0.041) and RuminococcaceaeUCG002 (*p*=0.016) were increased while Odoribacter gradually increased from the baseline to the 6 month (*p*=0.028 after 6 month). The sedentary subjects conversely showed a reduction of the phyla Actinobacteria and Firmicutes with increase of Bacteroidetes (*p*=0.043) after the first month; at the third month analysis the level of Parabacteroides and Streptococcus were reduced (*p*=0.043) while Faecalibacterium was increased (*p*=0.043) and so was Bifidobacterium bifidum after the sixth month (*p*=0.043).

The treatment with TFN was associated with a transient reduction of the relative abundance of the phyla Firmicutes and Actinobacteria during the first 3 months (*p*=0.043) and the genus Roseburia after the first month (*p*=0.043); the genera Odoribacter, the family Tannerellaceae and specifically the genus Parabacteroides appeared transiently increased during the first month (*p*=0.043). Finally, the Bifibacterium Adolescentis was slightly reduced after 6 months of treatment (*p*=0.043) (**Figure 3.4**). The stratification for dichotomous variables was performed but without detecting any significant modification.

Comparing cross-sectionally the microbiota composition of the two groups of patients the only significant difference that emerged was in the relative abundance of Clostridium sensu strictu 1 which appeared reduction after 6 months of treatment with DMF.
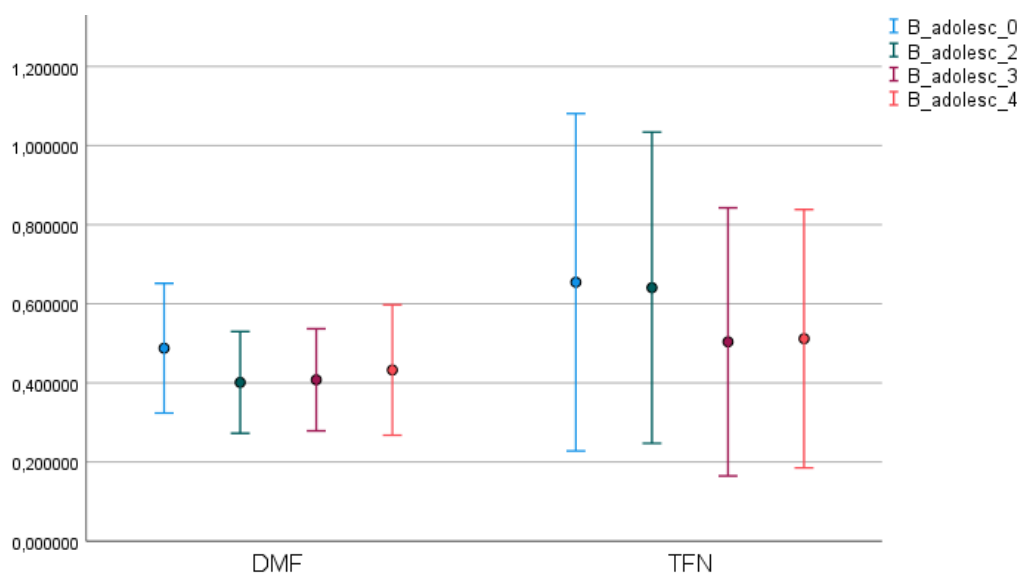

Figure 3.4 Boxplot on the distribution of the relative abundance of Bifidobacterium adolescentis during the follow-up by DMT.

Microbiota and DMTs side effects

We cross-sectionally investigated the association between side effects (GI disorders for TFN and DMF and flushing only for DMF) and the microbiota composition at the same time-point. After one month of treatment with DMF, 8 patients referred GI symptoms and their microbiota evaluation revealed a higher abundance of the phylum Tenericutes ($p=0.037$), the genera Streptococcus ($p=0.013$) and Subdoligranulum ($p=0.026$) and a lower abundance of Bacteroides ($p=0.033$). Notably, at the same time-point (first month), considering together the DMF and TFN groups (patients with symptoms=10 vs 13), the significance of the association between Streptococcus and GI symptoms increased ($p=0.004$). After 3 months of treatment with DMF, the symptomatic patients were instead 7 vs 12 and showed a lesser abundance of the genera Barnesiella ($p=0.013$) and Odoribacter ($p=0.033$) and a greater representation of Clostridium sensu strictu1 ($p=0.039$) and Blautia ($p=0.028$). After 6 months of treatment only 3 patients still referred GI disorders. Again, the microbiota analysis showed a tendency in a greater abundance of Clostridium sensu strictu1 ($p=0.051$) while the phylum Proteobacteria appeared reduced ($p=0.023$) compared to asymptomatic subjects. This comparison was not performed for the TFN group alone.

On the other hand, in patients featuring flushing after the first month of DMF assumption (n=8) higher Paraprevotella (phylum Bacteroidetes) levels ($p=0.025$) were detected and after 3 months (n=10) higher level of Streptococcus ($p=0.011$) and Haemophilus (phylum Proteobacteria) ($p=0.024$), and lower level of Paraprovetella ($p=0.047$) were detected; the genera Lachnospiraceae NK4A136 group and Faecalibacterium tended to be increased ($p=0.05$). After 6 months (n=8) we

confirmed the association between higher level of Haemophilus (*p*=0.025) and flushing, in addition we found higher level of the genus Lachnospira (*p*=0.011) and lower level of the genus Akkermansia (Phylum Verrucomicrobia) (*p*=0.036).

3.4 Discussion

The gut microbiota composition of our cohort of MS patients at baseline was not different from what expected: through the taxonomic analysis it was evident a higher interindividual variability (**Figure 3.3**) as already known from the literature evidence [Human Microbiome Project Consortium 2012].

The α biodiversity in our cohort did not significantly change after DMTs initiation, it also appeared quite resistant to environmental influences. At the same time, the relative abundance of the main phyla was not affected by DMTs treatments in a persistent and significant way which is in line with previous findings (**Table 1.5**). According to literature evidence suggesting that the stability and interindividual variability are synonymous of health, the lack of a major impact by DMF and TFN represents a positive information, which means DMTs do not disrupt the individual microbial profile. The scanty evidence existing on the effect of DMF on the microbiotic phyla suggests an increase of the phylum Bacteroidetes and decrease of the phyla Firmicutes and Fusobacteria even if these findings come from a cross-sectional study which, by definition, might be quite inappropriate in the comparison of highly variable microbiota profiles although belonging to two groups of MS patients [Katz Sand 2018].

Storm-Larsen and colleagues, who longitudinally analyzed the same MS population during DMF treatment, similarly to what was performed in this work, found only a transient reduction of the phylum Actinobacteria after 2 weeks from the start of DMF [Storm-Larsen 2019]. We performed the taxonomic analysis up to the family and genus level detecting a strong reduction of the family Clostridiaceae 1 and specifically of the genus Clostridium sensu strictu 1 after 6 months of treatment (p=0.006). A biological explanation for this kind of results exists: DMF has been found to be capable of stopping the *in vitro* growth of epsilon toxin (ETX) producer Clostridium perfringens [Rumah 2017]. Interestingly, a role of the ETX has been proposed in the etiology of MS. With our analysis we were not able to identify subspecies of the genus Clostridium and in particular those producers of ETX. Even so, the reduction of the genus Clostridium in the MS population taking DMF, acquires a potential new therapeutic meaning. Moreover, the consistency of our result is supported by the fact that cross-sectionally comparing the microbiota of DMF and TFN populations at baseline and after 6 months of treatment, the first showed a significant less abundance of Clostridium sensu strictu 1 during the follow-up which was absent at baseline. Moreover, after stratification for diet (proinflammatory vs balanced diet), smoking habit (ever vs never smoking) and lifestyle (active vs sedentary lifestyle), the trend in decrease of this genus remained. After stratification for these dichotomous variables the significance was distributed according to the size of the subgroups: the groups of patients with proinflammatory diet and active

lifestyle, which were the biggest showed significant decrease of Clostridium after 6 months of treatment. As for the stratification for smoking habit, we found that the significance was confirmed only for never smokers, the smallest subgroup (p=0.043 vs p=0.075). This finding suggests that smoking could attenuate the effect of DMF in reducing Clostridium.

Katz Sand and colleagues described a lower abundance of Clostridiales order and specifically of the families Lachnospiraceae and Veillonellaceae in the group taking DMF. In our patients no persistent modifications of genera belonging to these families has been found [Katz Sand 2018]. Other genera which were reported as decreased in MS compared to healthy controls, such as Prevotella or Faecalibacterium, did not significantly changed in our patients during the treatment with DMF. Prevotella, which appeared increased during treatment with Interferon (IFN) or Glatiramer acetate (GA) [Jangi 2016, Chen 2016], similarly to our study was not described as increased by DMF [Kats Sand 2018, Storm-Larsen 2019]. This finding supports a DMT-specific effect on the gut microbiota. Conversely Faecalibacterium was found to be increased in DFM treated patients by Storm-Larsen and colleagues [Storm-Larsen 2019].

To date there are no data available in the literature regarding the effect of TFN on the gut microbiota, thus we cannot make any comparison. After the first month of treatment, we detected an increase in the relative abundance of the family Tannerellaceae and in particular the genus Parabacteroides. This modification was transient as the Parabacteroides abundance after 3 and 6 months of treatment was not statistically different from the baseline. Interestingly, we found the genus Parabacteroides increased at baseline in patients with a sedentary lifestyle. Therefore, we its increase is supposed to represent just a transient imbalance due to the initiation of the new drug. Subsequently the start of TFN in our limited sample of patients we detected a persistently reduction of Bifidobacterium adolescentis which was conversely increased in those patients with proinflammatory diet. Unlike DMF, TFN was unable to significantly reduce the abundance of the genus Clostridium even if, in vitro could inhibit the growth of Clostridium perfringens, as demonstrated for DMF [Rumah 2017].

The subspecies analysis of Bifidobacterium was not performed by other researcher evaluating the effect of DMTs. These microorganisms are crucial during the early stages of life, they are highly abundant during childhood and then decline in adulthood. A study published in 1991 by Wagenfeld and colleagues investigated the Bifidobacterial composition in MS patients through culture analysis. They suggested a lesser abundance of Bifidobacterium adolescentis in 17 MS patients compared to controls. Bifidobacterium longum and B. bifidum were not different between the two groups. The authors proposed that B. adolescentis could be involved with a beneficial effect on the immune response. Nevertheless, its role in MS remains to be elucidated.

Of note is that in our MS cohort, the repeated sample collection during the follow-up allowed us to capture, beside persistent alterations, transient microbiota modifications which could be related to minor perturbations in the early phases of treatment. After the first month of treatment with DMF we detected a reduction of the Phylum Proteobacteria with increase of Anaerostipes. After 3

months instead, we found transient modifications of two genera belonging to the Ruminococcaceae family (Clostridiales order), Ruminococcaceae UCG-002 and Ruminococcaceae NK4A214 group, the first increased and the second reduced. These alterations may reflect the onset of side effects, in particular GI symptoms, related to the treatment and which are common in the early stages and tended to resolve over months. Our patients treated with DMF indeed showed this trend, with a significant reduction in the GI side effects prevalence from the first to the sixth month of treatment. We may hypothesize a relationship exists between early GI symptoms and these early microbiota modifications whose disappearances coincide.

With the cross-sectional evaluation of the microbiota during the follow-up we were able to find out some alterations in the taxonomic analysis between subjects with GI symptoms or flushing caused by DMF and those without side effects. We found different modifications based on the time-point considered: after the first month GI symptoms were associated with higher levels of Streptococcus, Subdoligranulum and Tenericutes phylum while Bacteroides was lower compared to asymptomatic patients. After 3 months of treatments the pattern of involved microorganisms changed with higher levels of Clostridium sensu strictu1 and Blautia and lower levels of Barnesiella and Odoribacter related to GI symptoms. After 6 months, the prevalence of GI symptoms, as expected, was lower than the earlier phases, even so, was still detectable a trend towards higher levels of Clostridium in symptomatic subjects (p=0.051). Although the group taking TFN was too small to allow us obtaining reliable results, analyzing the whole population with GI symptoms together, the significance in the association between GI disorders and Streptococcus was higher than considering the DMF group alone (p=0.004), thus underling a possible relation between higher Streptococcus levels and early GI disorders induced by DMTs.

The prevalence of flushing among DMF group, unlike that of GI symptoms, was more stable during the follow-up and was found to be associated with variable modifications of different genera. The most persistent finding was an increase in Haemophilus levels in association with flushing after 3 and 6 months of treatment, already detectable after the first month but without statistical significance.

We have also tried to correlate some microbiota profiles to a lower or higher EDSS or MRI activity at baseline or to previous treatments. Some differences in gut microbiota composition were identified although difficult to interpret. For example, Streptococcus was higher in those patients with greater EDSS (>2) but lower in those with an active MRI. Moreover, considering the short-lasting follow-up, we could not make any inference regarding microbiota and MS prognosis or treatments response.

In an ancillary analysis we stratified the sample by some environmental exposures including early life factors. Interestingly, at baseline we found a strong seasonal influence on the main phyla abundance, with a greater B/F ratio in the periods from January to March and from July to September. Previous evidences suggested that B/F unbalance, with a predominance of Firmicutes,

could be considered a dysbiotic condition and could be associated with MS [Costea 2018]. Even so, this hypothesis has not yet confirmed and the variability of B/F ratio over months is to be intended as a physiological adaptation, rather than a beneficial or dysbiotic alteration. Indeed, both phyla are crucial for SCFAs production and the immune system functioning.

Unfortunately, this seasonal variability may represent a major confounder. For the longitudinal evaluation we could not take into account the month of the year cause of the small sample size. Although this finding was unexpected, it did not invalidate our work for two main reasons: i) the greater seasonal impact affected most of all the phylum and not the genus level, where we found mostly of the microbiota alterations, ii) looking at the trend of the B/F ratio across the year, it apparently reverses every 3 months (**Figure 3.2**), therefore this variability could influence the results of the samples collected 3 months after the baseline but those after 1 and 6 months are unlikely to be affected.

Another interesting point is given the effect of smoking: the α biodiversity, as previously mentioned, remained stable during the follow-up and was not influenced by environmental factors with the exception of smoking. In particular, we detected just a trend (p=0.053) towards a lower biodiversity in ever smokers but this result was consistent with literature evidence. Moreover, this tendency was reflected in a lesser abundance of several genera belonging both to the Bacteroidetes and Firmicutes phyla. This is in line with evidence suggesting that a greater biodiversity is a healthy indicator while its reduction may accompany various pathological conditions (e.g., obesity) [Human Microbiome Project Consortium 2012, Le Chatelier 2013, Yan 2021]. Nevertheless, no literature data supported a lower biodiversity in MS patients compared to healthy individuals or a greater or lower biodiversity induced by the DMTs. Beside the lowering trend of biodiversity, smoking, which is undoubtfully a damaging habit, caused relevant microbiota modifications at genus level with reduction of the relative abundance of two members of Marinifilaceae family (phylum Bacteroidetes) with even eventual suppression of some underrepresented genera such as Butyricimonas, thus contributing to decrease the biodiversity. Ever smokers were also associated with lower levels of 5 genera belonging to the Clostridiales order (Christensenellaceae R-7 group, Coprococcus1, Eubacterium coprostanoligenes, Ruminococcaceae NKA214 group and Ruminococcaceae UCG-002) and of the phylum Tenericutes. Our findings confirmed the previous detection of a higher abundance of some species belonging to Eubacterium in non-smokers. As for the genus Akkermansia, which was found increased and capable of reducing inflammation in non-smokers by Yan and colleagues, we detected a slight trend towards lower levels in ever smokers but without reaching statistical significance (p=0.078). When instead comparing its prevalence between ever and never smokers, we found that a significant lower number of ever smokers had measurable levels of Akkermansia compared to never smokers (p=0.045), which probably affected the reliability of the statistical analysis as regard its relative abundance. The genus Roseburia, conversely, appeared higher in ever smokers compared to never smokers which was in conflict with what Yan group described [Yan 2021]. Anyway, considering the small sample of patients we

have enrolled, our findings are quite consisting with previous literature data. Interestingly, after DMF assumption, we detected in ever smokers an increase of Odoribacter and Butyricimonas levels, the same genera decreased in this category of patients at baseline. The effect of DMF on Odoribacter and Butyricimonas increasing was instead not evident for never smokers nor for patients treated with TFN which kept similar abundance level of these bacteria before and after the treatment (**Figure 3.5** and **3.6**). In addition, some changes in the microbiota composition during DMF treatment were only evident for never smokers such as the increase after 3 or 6 months of some subspecies of Bifidobacteria (bifidum, breve, pseudolongum and animalis).
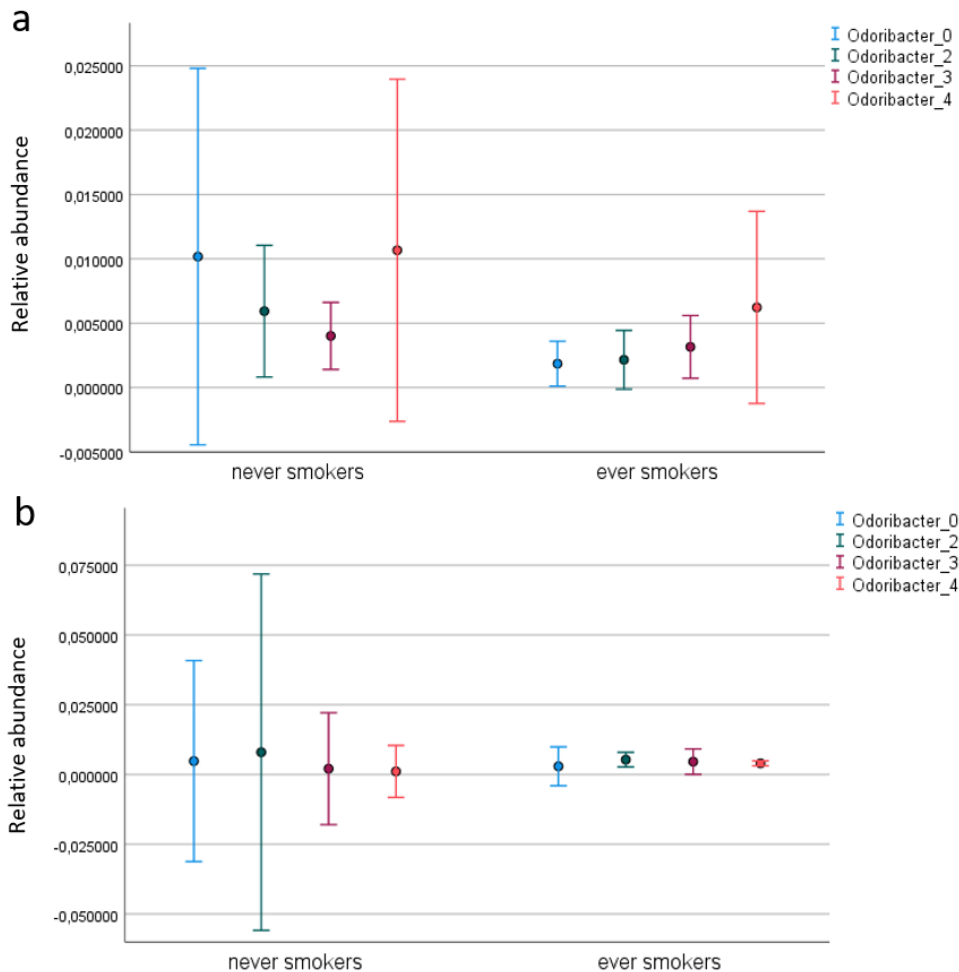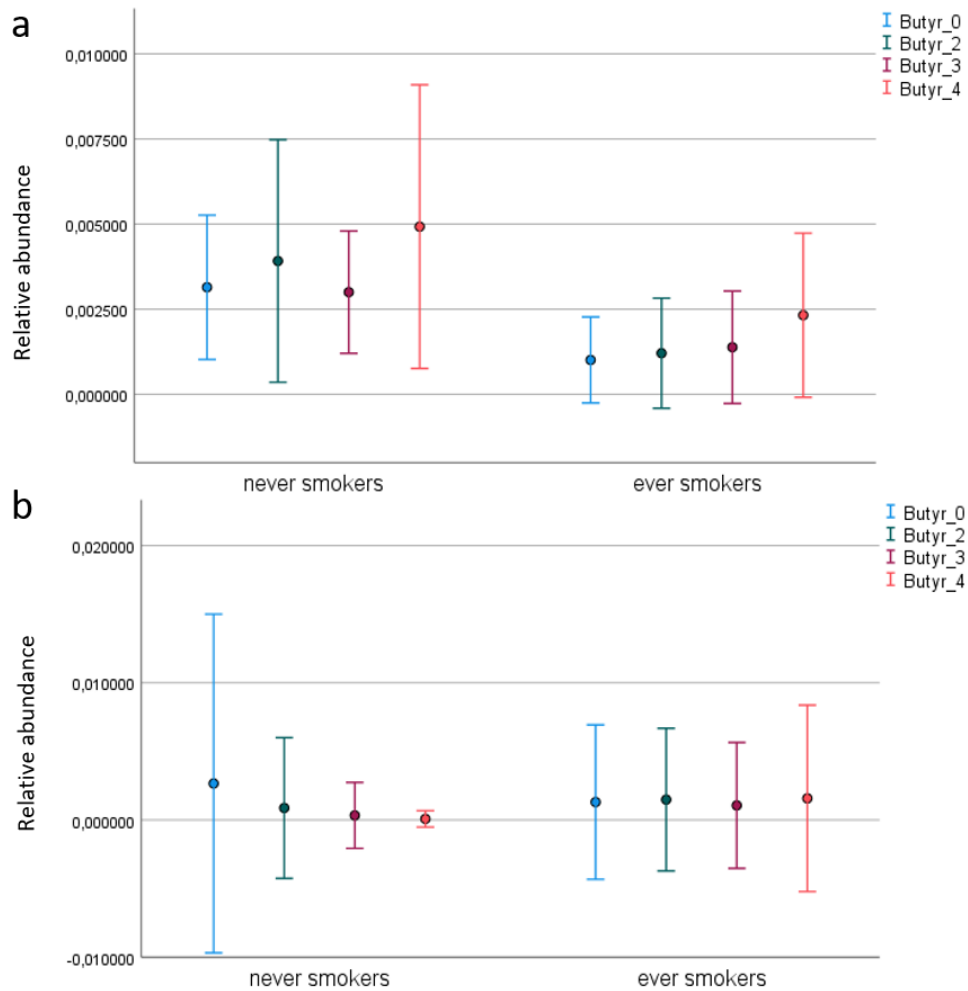

Figure 3.5 Boxplot on the distribution of the relative abundance of Odoribacter (Marinifilaceae family, phylum Bacteroidetes) during the follow-up by smoking category (never vs ever smokers) for DMF (a) and TFN (b) populations.

Some of the differences in the DMF response are probably due to the size effect (the sample never smokers was larger than ever smokers), that is for example the case of the transient increase of the genus Ruminococcaceae UCG-002 which was significant both for the whole sample of patients treated with DMF and for the subgroup of never smokers. Conversely the increase of Bifidobacterium subspecies was only detectable when considering separately the never smokers patients, thus supporting the hypothesis that smoking may alter the DMF response at the microbiota level. A recently published study suggested that smoking induces inflammation through the

reduction of SCFAs-producing bacteria and increase of proinflammatory species, that is its inflammatory action could be mediated by the gut microbiota [Yan 2021]. Therefore, we may suggest that a potential action of DMF, which modifies the microbiota towards a normalization with resettlement of depleted species by smoking, may be capable of affecting the immune system through its action on the gut microbiota. Moreover, DMF effect on the microbiota may be in part influenced by the environmental exposures of the individual which explains why some variations were evident in never smokers or ever smokers only.

Figure 3.6 Boxplot on the distribution of the relative abundance of Butyricimonas (Marinifilaceae family, phylum Bacteroidetes) during the follow-up by smoking category (never vs ever smokers) for DMF (a) and TFN (b) populations.

Another interesting finding is that the genera Streptococcus and Parabacteroides, increased in association with a sedentary lifestyle at baseline, were instead decreased after DMF start in this subset of patients.

The results support that DMF may act repairing some microbiota disfunction caused by environmental factors which, in turn, may influence the DMF effect. However, our sample seize was not large enough to assess this question.

The environmental factor analysis also pointed out the selective action exerted by different exposures.

A deep evaluation of the interactions of each environmental factor is beyond the purpose of this study which meant to explore the specific interplay between MS, DMTs and environment. However, stratifying the microbiota analysis for EDSS, MRI activity, lifestyle, smoking, BMI, diet and side effects, some microorganisms appeared to be repetitively or consistently associated with some unfavorable conditions. In particular, Clostridium sensu strictu1, Streptococcus (phylum Firmicutes), Parabacteroides (phylum Bacteroidetes) and Haemophilus (phylum Proteobacteria) were detected. On the other side, the genera Odoribacter, Butyricimonas, Barnesiella (phylum Bacteroidetes), Eubacterium Coprostanoligenes, Ruminococcaceae NKA214 group, Ruminococcaceae UCG-002, Coprococcus1 (phylum Firmicutes) and some subspecies belonging to the genus Bifidobacterium (bifidum, dentium, pseudocatenulatum) were associated with some favorable conditions (e.g., breastfeeding, non-smokers condition) or inversely related to unfavorable ones (DMTs side effects). Nevertheless, they should not be considered indisputably as "bad" or "good" microorganisms for the following reasons: i) their association with unfavorable/favorable conditions is not confirmed and further studies are needed, ii) we did not performed a taxonomic analysis which included the species and subspecies, which should be necessary to eventually identify the exact microorganisms involved (e.g., Clostridium perfringens ETX producer), iii) the same microorganisms could be "good" or "bad" depending on other factors such as the age at which the exposure occurs, the duration of the exposure, the interactions with other microorganism or external factors (e.g., environmental factors, therapies). For these reasons we may conclude that those microorganisms could be related to an unfavorable/favorable condition on this specific setting but their action in a different context, such as early life, could be different.

To our knowledge, our work is one of the few aimed to analyze the composition of the gut microbiota, including the bifidobacterial profile, in relation to the initiation of first-line immunoprophylactic therapies for RRMS. The effect of DMF on the microbiota has been previously studied, but not that of TFN. Only a little evidence is available for TFN from *in vitro* studies. The particular skill of our work is the longitudinal prospective design with four sampling time-points and a 6 months lasting follow-up. The previously published study by Storm-Larsen and colleagues, which also made use of the longitudinal observation, only focuses on 12 weeks of follow-up with only one intermediate time-point (at 2 weeks). Moreover, considering the microbiota variability and susceptibility to a wide range of factors, we measured several confounding variables belonging to the environmental exposures (month of sample collection, smoking, diet, lifestyle, child birth, breastfeeding) potentially influencing the microbiota itself. Furthermore, the influence of some selected factors on the gut microbiota composition at baseline and during follow-up through stratified analyses was investigated, and this had never been done before in such cohort of patients.

The main limit of our study was the small sample size. Unfortunately, microbiota analysis is not economic and, although the non-invasive sample collection, the study participation was not

optimal. Moreover, the design of the study did not allow to eventually correlate the microbiota to the disease activity or treatment response.

What clearly emerges from our work is the complexity in studying the gut microbiota. Several issues have to be considered planning such a study as the gut microbiota interfaces with many disease aspects and environmental factors. When considering such a complex microsystem, is mandatory to apply a methodological rigor. The optimal study design in order to evaluate the impact of some kind of exposure in general and even more on the gut microbiota is the longitudinal prospective investigation due to the high interindividual microbial variability. Moreover, using the patient as self-control reduces the influence of a lot of confounding variables (concomitant medications, diet, lifestyle, early life factors) that still need to be considered.

To summarize our results, the longitudinal analysis of the gut microbiota in a cohort of patients about to start oral DMTs with a 6 months follow-up let us to collect information on transient and persistent microbiota modifications occurring after the treatment start. The biodiversity and phyla main composition did not change according to treatment. The effect of DMF and TFN on the gut microbiota included some modification mostly occurring at genera level. The most relevant and persistent modification following the starting of DMF was a reduction of the Clostridium sensu strictu1 relative abundance, to which we could assign a therapeutic role considering the potential neurotoxic effect of some species belonging to Clostridiaceae family and to Clostridium genus which could be hypothetically mediated by ETX. Interestingly, some members of the order Clostridiales and families Lachnospiraceae (such as Blautia) and Ruminococcaceae have proven to be able to inhibit the *in vitro* oligodendrocyte differentiation through their metabolites action on gene expression, also modulating social behavior of the mice [Gacias 2016]. Therefore, Clostridiales could be involved in the mechanism of neuroinflammation and neurodegeneration and as we demonstrated, they could also constitute a potential target for DMTs such as DMF.

Another hypothesis we were able to make is that microorganisms could be selectively susceptible to the effect of environmental exposures or DMTs. For example, Clostridium was not influenced by smoking itself, but after DMF assumption, our finding suggests that the decrease of Clostridium induced by the DMT is lower in smokers than non-smokers.

The analysis on the effect of TFN were affected by the small sample of patients. Even so, we detected transient variations at genus level and a persistent decrease of Bifidobacterium adolescentis, whose meaning remains to be elucidated. We hypothesized early GI side effects by DMTs could be associated with an increased level of Streptococcus while flushing induced by DMF appeared to be associated with an increased level of Haemophilus during the whole follow-up. Through the investigation of the influence of environmental factors on the microbiota before and after the treatment start, we found a potential effect of DMF in restoring the microbiota imbalance caused by smoking, in particular in increasing some genera (Odoribacter and

Butyricimonas) which have been found to be reduced in smokers patients compared to non-smokers. DMF was also able to decrease the abundance of Parabacteroides which was instead increased in subjects with sedentary lifestyle.

Finally, our results suggest that the microbiota may actually function as a point of convergence for several factors affecting the MS prognosis, including DMTs. Nevertheless, our population sample was too small to make any conclusions; further investigations are needed to better understand the interconnection between microbiota, DMTs and environment in MS. This search line, hypothetically, may even have clinical implications by supporting the decision on the appropriate therapy for each patient basing on the gut microbiota or environmental past or present exposures. Therapeutical strategies for MS primarily involving the gut microbiota have already been proposed (e.g., probiotics intake, fecal microbiota transplantation) with encouraging results. These interventions could beneficially affect the immune system with potentially therapeutic or preventive implications in the future.

Chapter 4. CONCLUSIONS

Early life exposures significantly contribute to the risk of developing MS later in life.

According to the "hygiene hypothesis", the limited exposure to the "old friends", secondary to the increased hygienic standard and to lifestyle habits in industrialized countries, provoke a reduction of the immune system exposure to beneficial stimuli with consequent increased predisposition to allergic and autoimmune diseases. The wide spectrum of the "old friends" includes the gut microbiota and several external stimuli. The gut colonization is indeed essential for the development of the immune system during the perinatal phase and early stages of life. Evidence supports the influence of delivery mode, breastfeeding and having siblings on the microbiota composition during infancy. It is likely that gut microbiota, starting from the perinatal phase, begins to settle the immune system towards a certain self-reactive profile which early after birth and over the years can be modulated by the exposure to multiple external microorganisms (bacteria, viruses, parasites) and other environmental factors. A different microbiota composition has been described in MS patients compared to healthy controls, thus supporting its role in MS pathogenesis. The inference is that "indoor" and "outdoor" environment deeply interact with one another to determine the risk for MS. In other words MS pathogenesis can be hypothesized to depend on "outdoor exposures" and "indoor effectors". The "outdoor exposure", meaning environmental exposures also including exogenous "old friends", is probably easier to investigate through the use of surrogates such as allergy and sibship. Indeed, allergy is a counter-part of MS, because, unlikely the known Th1/Th2 paradigm, they instead derive from the same predisposing condition.

Our work showed that respiratory and food allergies may be positively associated with MS when the allergy onset occur within a specific age range and dependently on sex. This age range differed between Italian and Norwegian populations, the first occurring during early childhood and the second mainly during adolescence. Similar age ranges of susceptibility have been previously suggested by two space-time clustering studies involving specifically Italy and Norway, which suggested the influence of an environmental exposure to determine the cluster distribution of the risk for MS. The same hypothesis could also explain our findings. As for sibship, we demonstrated that the sibling effect is higher when the exposure is towards close-in-age younger siblings, with a protective effect against MS development. This is applicable to the Norwegian EnvIMS population but not to the Italian one. The role of sibship may therefore not be universal and it is probably affected by environmental exposures depending on the geographic area. Regional differences in childhood microbial exposures and microbiotic gut composition should not be overlooked when investigating such associations.

This theory allowed us to explain why the findings on birth order and sibship have been so conflicting, even when using the same methodology applied to different populations. Considering our epidemiological data as a whole, we have tried to analyze the underlying mechanisms of these crucial exposures capable of affecting the risk for MS. The "*When*" "*What*" "*How much*" Old Friends (WWHOLF) approach was proposed to measure early life exposure to the "old friends": "*When*" refers to the role of the time (age) of exposure, "*What*" to the exposure itself, and "*How much*" to the size of the exposure. The simple and known relationship between exposure and outcome, ie., the focus of clinical epidemiology, clearly also depends on the "*When*" "*What*" "*How much*", other than the presence of confounding or effect-modifiers.

Gut microbiota may ultimately represent the point of convergence of such complex interplay between 'outdoor' exposures and 'indoor' effectors. This a unique and, at the same time, dynamic system, with high variability among individuals and a composition likely to be affected by multiple factors including most of the putative environmental factors for MS etiology, such as smoking habit, proinflammatory diet, high BMI, and poor physical activity. For each such factor, evidence exists regarding their effect on the gut microbiota.

DMTs can modify the microbiota composition towards a normalization of the microbial imbalance related to MS. We have then tried to shed light on the composite effect of the oral DMTs, dimethyl fumarate (DMF) and teriflunomide (TFN) on the gut microbiota of MS patients, also accounting for environmental exposures. Through a 6-month longitudinal exploratory study, the microbiota composition was compared before and after the initiation of DMF or TFN. We found no main modifications in α biodiversity and phyla abundance during the 3-step follow-up, compared to the baseline. Conversely, at genus level some alterations were detected. The most consistent finding was a decrease of the relative abundance of Clostridium sensu strictu1 (phylum Firmicutes) at the latest follow-up after DMF start, compared to the baseline level. Accounting for the potential neurotoxic action of the genus Clostridium, this effect of DMF could be interpreted as therapeutic. Other detected changes such as those after TFN start were only transient, likely related to treatment early side effect. In addition to Clostridium, we also found some other genera variably associated with some unfavorable or favorable conditions such as GI side effect by both treatments, flushing by DMF, higher EDSS and sedentary lifestyle or conversely with non-smokers, active lifestyle and past breastfeeding. Investigating the interrelation between microbiota, DMTs and environmental exposure a potential and selective effect of DMF in limiting the microbial changes related to smoking or sedentary lifestyle. These results tend to confirm the proposed role of the gut microbiota as "indoor effector", through which environmental factors and treatments ("outdoor") interact and influence the immune system and MS prognosis.

Ultimately, our work supports the "hygiene hypothesis" and propose the gut microbiota as a potential "indoor effector" acting as a mediator between the "outdoor exposure" and the immune system.

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