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The nerves to conduct a multiple sclerosis crime investigation

Sameeksha Chopra[1,2,3], Zoë Myers[1,2,3], Henna Sekhon[1,2,3] and Antoine Dufour[1,2,3]

[1] McCaig Institute for Bone and Joint Health, Calgary, Alberta, Canada
[2] Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada
[3] Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada

Abstract

Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative autoimmune disease characterized by aberrant infiltration of immune cells into the central nervous system (CNS) and by the loss of myelin. Sclerotic lesions and various inhibitory factors hamper remyelination processes within the CNS. MS patients typically experience gradual cognitive and physical disabilities as the disease progresses. The etiology of MS is still unclear and emerging evidence suggests that microbiome composition could play a much more significant role in disease pathogenesis than was initially thought. Initially believed to be isolated to the gut microenvironment, we now know that the microbiome plays a much broader role in various tissues and is essential in the development of the immune system. Here, we present some of the unexpected roles that the microbiome plays in MS and discuss approaches for the development of next-generation treatment strategies.

Keywords: Multiple sclerosis (MS), microbiome, bacteria, virus, central nervous system (CNS), immunity.

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Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease characterized by aberrant infiltration of immune cells into the central nervous system (CNS) parenchyma[1]. Immune cell infiltration is accompanied by the loss of myelin (demyelination) and neurodegeneration of the CNS[1,2]. The accumulation of sclerotic lesions and inhibitory factors can significantly hamper remyelination processes within the CNS, causing MS patients to experience debilitating cognitive and physical disabilities as the disease progresses[1,2]. While the etiology of MS is unknown, emerging evidence continues to suggest that the microbiome may play a significant role in disease pathogenesis[1,3,4]. Autoimmune diseases, including MS, are more prevalent in countries that have a higher standard for sanitation and a greater use of antibiotics[5]. This phenomenon is described as the *hygiene hypothesis*[6], which suggests that overly hygienic westernized lifestyles may result in gut dysbiosis and dysregulated immune responses. Several environmental factors such as diet and vitamin D exposure have been linked to an increased risk of MS and may help explain the distinct geographical distribution of the disease[6]. People moving from countries with low prevalence of MS to those with high prevalence of MS adopt a higher risk of developing the disease[6–9], perhaps facilitated by the changes in lifestyle and diet that drive a shift in the composition of the intestinal flora. People with MS[10–12] and animal models of the disease[13] have altered gut microbiota composition, which brings us to question whether the microbiome could be a driver of MS pathogenesis. There is an imminent need to better characterize changes in the microbiome composition of individuals living with MS and study the complex mechanisms by which microbiota can influence host biology and affect disease pathogenesis.

Box 1: What if the CNS of MS patients was compared to a crime scene where a detective would arrive at the crime scene to investigate the *damage* done and gather forensic evidence.

The victim: Central Nervous System

- Damage myelin coating on nerves
- Evidence of an activated immune response in the CNS that resulted in clearance of myelin debris and promotion of remyelination
- Bacterial contribution that impacted resident and peripheral immune cells in addition to modulation of CNS myelination

Investigating the Multiple sclerosis *Crime scene*

MS patients are monitored and diagnosed after presenting with lesions visible on an MRI that are disseminated in time and space. However, there is little known about the initiating cellular and molecular events that contribute to dysregulated inflammation prior to formation of MS lesions. There are likely drastic changes to the CNS tissue microenvironment that accompany neuroinflammation and the subsequent development of MS plaques. Many of the inhibitory factors that accumulate within plaques impair remyelination in the CNS and thus limit the organs endogenous tissue repair processes.

***Breaking and Entering* the Blood brain barrier**

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Homeostasis is maintained in a healthy CNS through the regulated entry of substances from peripheral circulation. Tight-junction proteins create a selective blood brain barrier (BBB) surrounding the delicate CNS tissue. However, BBB breakdown in MS patients is accompanied by increased peripheral immune cell infiltration into the CNS. There are many mechanisms proposed to cause a dysfunctional BBB including inflammatory cytokines, activated peripheral immune cells recognizing CNS antigens, secretion of proteases and microbial derived factors[14,15].

Alterations in the gut microbiome could affect the permeability of the BBB and have indications in neurological diseases such as MS. In a study that investigated the effects of antibiotics on BBB integrity in a germ-free mouse model of MS, a decrease gut microbiome diversity was positively correlated with an increase in BBB permeability and a downregulation of short chain fatty acids (SCFAs)-producing microbes was observed[16]. Several studies have reported the effect of gut microbes and their metabolites on BBB permeability[17]. For example, germ-free mice colonized with bacterial strains that produce SCFAs reinforced the integrity of the BBB by upregulating the expression of tight junction proteins[15]. Furthermore, resident microglial cells in the brain can also modulate BBB permeability[18]. As described next, the microbiome can influence the function of cell types resident to the CNS, highlighting the complex interplay between the gut and brain. In MS patients, a deficit of SCFAs-producing bacteria likely contributes to increased BBB permeability and subsequent entry of neuroinflammatory factors into the CNS. People with MS also often have elevated serum zonulin concentrations[19], which is a protein known to be released in response to gut dysbiosis[20] capable of increasing intestinal permeability and breaking down the BBB through the regulation of tight junction proteins[19,21]. Altering the microbiome or delivering BBB-reinforcing microbial metabolites may be a strategy to repair a damaged BBB, stop peripheral immune cell access into the CNS and thereby prevent the exacerbation of disease.

Is the Peripheral Immune System an accomplice?

The increase of immune cell infiltration into the CNS during MS relapses supports the notion that MS is an immune-mediated disease. However, whether neuroinflammation during the early stages of MS is a result of infiltrating immune cells activated in the periphery or is a process initiated within the CNS itself remains to be characterized. Gut bacteria transferred from MS patients into mice exacerbate the development of MS-like disease in various mouse models[22,23], showing that the intestinal microbiome is capable of influencing disease progression. Despite the less obvious connection between the brain and gut, growing evidence suggests that intestinal immune responses and neuroinflammation are interconnected (**Figure 1**).

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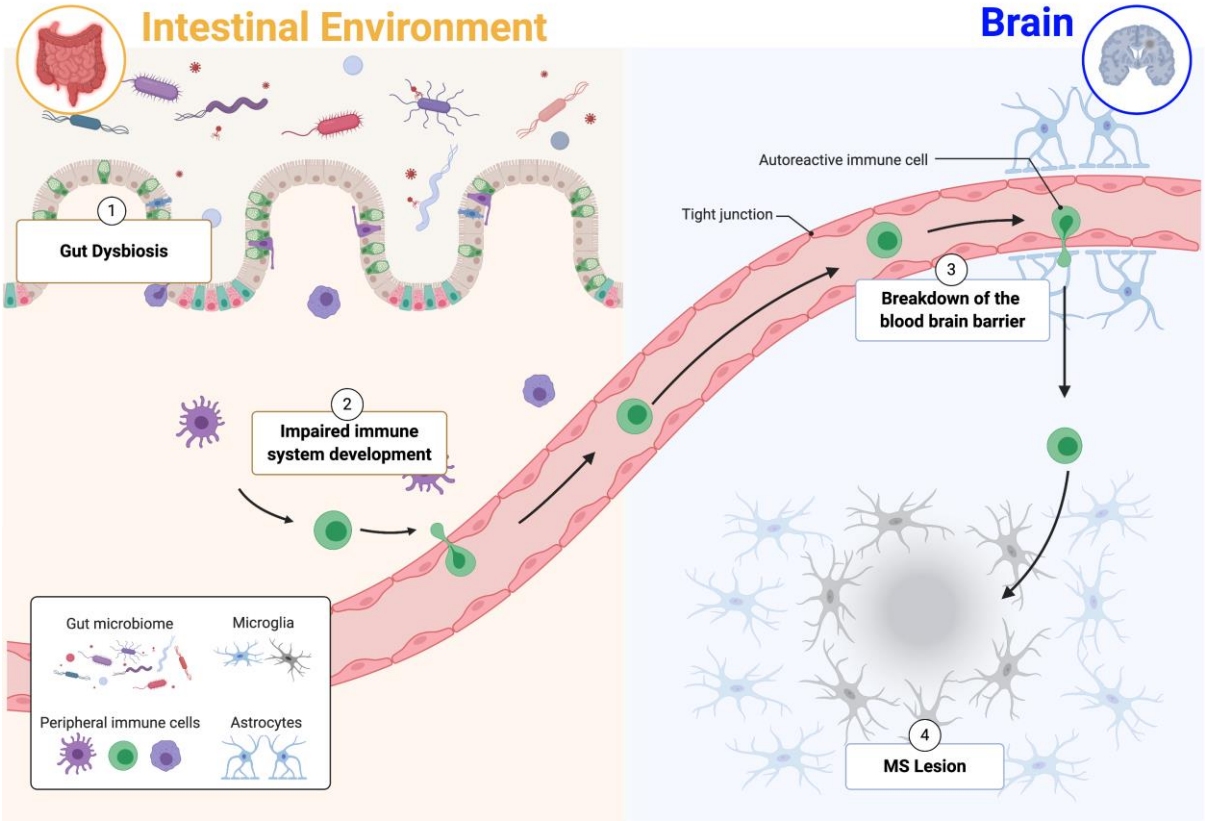

Figure 1: Gut-immune-brain connection. 1) Gut dysbiosis can impair mucosal immunity homeostasis. 2) Changes in immune system development are linked to multiple autoimmune diseases. 3) Blood brain barrier breakdown is a hallmark of multiple sclerosis (MS) and the gut microbiome is a key regulator of blood brain barrier permeability. 4) Peripheral immune cell infiltration and subsequent autoimmune responses contribute to the development and exacerbation of MS lesions.

Maternal and in utero effects on immune system development

The maternal microbiome has been shown to affect fetal immune system development[24], which may confer lifelong consequences on the offspring by modulating their risk of developing autoimmune diseases such as MS. Interestingly, fetal thymic CD4+ T cell and regulatory T cell (Treg) development is compromised in germ-free mice[25]. Maternal supplementation of the intestinal bacterial metabolite acetate significantly rescued CD4+ T cell and Treg development by upregulating the autoimmune regulator (AIRE)[25], which is essential for self-tolerance induction and Treg development early in life. In humans, low maternal serum acetate levels are mirrored in the fetus, which may predispose the offspring towards impaired self-tolerance. The specific connection between *in utero* effects of bacterial-derived factors on immune development in offspring later in life, particularly in the context of multiple sclerosis, has yet to be explored in depth. A case-control study analyzed maternal vitamin D concentrations in the serum of pregnant women in the Finish Maternity Cohort whose children had developed MS[26]. Maternal vitamin D deficiency during early pregnancy was associated with a 2-fold increase of MS risk in the offspring[26]. Low concentrations of neonatal vitamin D are also associated with increased risk of MS[27]. Interestingly, birth month influences the risk of developing MS later in life, with the risk of MS peaking in May and dropping in November

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which may be linked to maternal vitamin D levels[28]. Additionally, bacterial/viral infections during pregnancy may also contribute to the unique birth month pattern of MS risk. Cohort studies that assess bacterial metabolites using unbiased metabolomics may be insightful to understand the contribution of the maternal microbiome in modulating the risk of offspring developing MS.

Immune system development

Epidemiological data support the idea that exposure to environmental factors in childhood may be strong determinants of MS risk[27,29–31]. As migration studies suggest, the incidence rate of MS in migrants tends to be somewhere between the incidence rate associated with their birthplace and the incidence rate associated with their final residence. If this migration happens in childhood, then the incidence rate tends to be closer to that associated with their final residence[27,31,32]. For example, natives of the Caribbean islands and Asia do not experience a significant increased risk of developing MS upon migrating to the United Kingdom (UK), however high rates of MS are documented in their UK-born children[31]. This may suggest that individuals spending early life in low-risk areas tend to benefit from long-lasting protection against MS that is not transferred to their children[27,31], hinting at the possibility that these factors may include bacteria and viruses that shape the development of their immune system. Perhaps there are vulnerable periods in one's early life where environmental factors are particularly critical for immune system development affecting one's future risk of developing MS and other autoimmune diseases.

The gut microbiome plays an essential role in the development of the immune system[33]. The gut microbes and the host exist in a symbiotic relationship and when this relationship becomes dysregulated, dysbiosis can occur, resulting in defects in the immune response, immune system disorders and triggering of autoimmune diseases. During fetal and infant development, the gut microbiome is developing alongside the immune system[34]. As demonstrated in germ-free mice, the immune system requires the colonization of the gut bacteria to properly mature. Initial colonization of gut microbes occurs *in utero*, indicating a possible transfer of bacteria from the blood stream of the mother to the fetus, and this continues after birth as the infant breast feeds and begins eating solid foods[35]. The method of birth delivery may affect the development of the gut microbiota as it is suggested that Caesarian delivered babies have low bacterial diversity within their gut[35]. This makes these infants more susceptible to immune disorders like asthma and Celiac disease[35,36]. Similar results were seen in infants who use baby formula rather than breastfeeding indicating that there are various environmental factors that play a role in the development of the gut microbiome and that a slightly impaired gut microbiota (decrease in diversity) may lead to immune disorders later on in life. Additionally, the immunogenicity of early colonizing intestinal bacteria may have profound impacts on the repertoire of immune responses later in life. Vatanen *et al*. followed gut microbiome development in 222 infants in Northern Europe from birth until the age of three[37]. Bacteroides species that inhibit innate immune signalling were lowly abundant in infants from Russia, where early-onset autoimmune diseases are less prevalent as compared to Finland and Estonia[37]. In line with the hygiene hypothesis, early colonization with immunologically silencing microbiota may impair immune education and maturation.

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Short chain fatty acids (SCFAs) can modulate immunity

Gut bacteria play a crucial role in extracting key nutritional factors from a healthy diet. Non-digestible carbohydrates can be fermented by specific bacterial species to make metabolites that modulate host biology[38,39]. SCFAs produced by the intestinal microbiota are critical in the development and maintenance of a healthy immune system[38,40]. Importantly, SCFAs, such as acetate, propionate, butyrate and pentanoate, can exert anti-inflammatory effects on the host and are often downregulated in chronic inflammatory conditions[41]. In MS patients, serum concentrations of butyric acid (BA) are reduced and medium chain fatty acids (MCFAs), such as caproic acid (CA), are increased[42]. The altered BA/CA ratio correlates with the immunological profile of MS patients, as they present with an increase in Th1 and Th17 cells as well as a decrease in Treg lymphocytes[42]. Concurrently, the microbiota of MS patients is depleted of BA producers[42]. Decreased serum butyric acid levels correlate with increased intestinal permeability as measured by serum intestinal fatty acid binding protein (I-FABP) and bacterial lipopolysaccharide (LPS) in MS patients compared to healthy controls. The presence of these factors in the blood suggests impaired gut barrier integrity in MS. Pentanoate is a potent regulator of immunometabolism as it can reprogram the metabolic activity of lymphocytes. In experimental mouse models of multiple sclerosis, pentanoate-induced regulatory B cells protect against autoimmunity[43]. Additionally, pentanoate induces IL-10 production in lymphocytes and reduces IL-17A production by epigenetically modifying CD4 T cells[43]. Germ-free mice are highly resistant to the development of experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. However, segmented filamentous bacteria (SFB) induce pathogenic Th17 immune response and promote CNS inflammation in SFB mono-colonized germ-free animals[43]. Interestingly, administering pentanoate in SFB-colonized germ-free mice ameliorates EAE and decreases Th17 cells in the CNS[43].

The gut, an unexpected suspect in MS, modifies the autoimmune response

Macrophages and Microglia

Microglia are tissue resident immune cells in the CNS that can influence inflammation and neurodegeneration by secreting factors and coordinating with other cell types, such as astrocytes, oligodendrocytes, neurons and peripheral immune cells that have infiltrated into the CNS[44]. A thorough characterization of how the microbiota influences the function of neural cell types in MS remains to be done. Gut microbiota can metabolize dietary tryptophan to produce aryl hydrocarbon receptor (AHR) agonists that can limit CNS inflammation by acting on AHR receptors expressed by microglial cells[45]. Tryptophan metabolism has been of interest in autoimmunity research as metabolic products of the kynurenine pathway used to metabolize tryptophan are known to exert several effects on the immune system, including modulating immunotolerance[46]. Several studies have investigated the levels of kynurenines in MS patients or in the EAE mouse model, demonstrating that aberrant kynurenine pathway activity is associated with increased severity of disease[46–49]. Tryptophan-derived metabolites, such as indole-3-propinonic acid, are produced by the microbiota and are associated with MS relapses[46]. However, further research is required to understand the specific pathophysiological role of tryptophan-derived microbial metabolites in MS. Bacterial products from members of the gut microbiota, such as SCFAs, play a substantial role in microglia homeostasis as temporal

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eradication of the host microbiota or limited microbial complexity can significantly change microglia properties[50]. Duscha *et al.* showed that supplementing MS patients with the SCFA propionic acid increases Tregs and can be a promising immunomodulatory supplement to MS drugs[51]. While the study did not assess the impact of SCFA on tissue resident immune cells in the CNS, it is likely that SCFA treatment modulates the maturation and activation of microglia as well[50]. Ultimately, applying our understanding of microbiome-host interactions specifically pertaining to the CNS may help to design better therapeutic approaches for neurological illnesses.

T cells

The gut-brain axis plays a key role in neuroimmunology, with emerging evidence suggesting that encephalitogenic immune responses may be driven by intestinal dysbiosis in MS[52,53]. Th17 cells are key mediators of CNS autoimmunity and their role in MS has been established in both humans and animal models[54]. Specific microbiota modifications in the human intestinal microenvironment, including a higher *Firmicutes*/*Bacteroidetes* ratio, increased relative abundance of *Streptococcus*, and decreased *Prevotella* strains, have been implicated in promoting Th17 cell expansion and correlating with brain autoimmunity in MS patients with high disease activity[52]. In an adoptive transfer mouse model of MS, CNS-specific Th17 cells first migrate to the intestine to proliferate during disease pathogenesis prior to reaching the CNS and inducing neurological symptoms[54]. The pro-inflammatory properties of encephalitogenic Th17 cells are strengthened in part by gut microbiota changes in mice. Blocking α4β7-integrin and its cognate ligand mucosal addressin cell adhesion molecule 1 (MAdCAM-1) disrupts Th17 cell intestinal homing and attenuates EAE severity[54,55]. In another study, eradication of the gut microbiome using antibiotics prevented the induction of CNS inflammation by gut derived T cells and abolished the development of EAE[56]. These studies implicate the intestine as a regulatory checkpoint for regulating autoimmune T cells. The gut microbiota may also modulate the disease course of MS. In the EAE model, differentially abundant intestinal bacteria determine susceptibility to chronic progressive versus relapse remitting forms of disease[57]. The contribution of intestinal T cell subsets, including the presence of mucosal associated invariant T (MAIT) cells in brain lesions[58] and peripheral circulation of MS patients[59], is starting to be recognized in the pathophysiology of MS. Ultimately, studies in animal models and patient data suggest that the microbial composition of the gut may play a critical role in catalyzing CNS-specific autoimmune responses in MS.

B cells

Gut-associated B cells, most of which differentiate into IgA-secreting plasma cells (PC), are predominantly found in Peyer's patch[60]. IgA antibodies are a critical first line of defence against antigens in the intestinal microenvironment. During EAE, there is a downregulation of IgA PCs in the gut and an upregulation of IgA PCs in the CNS[61]. PCs that egress from the gut into the CNS suppress the symptoms of EAE via the interleukin (IL)-10 pathway[61]. Therefore, intestinal derived IgA B cells may represent a population of regulatory cells that can be recruited to tissues to mediate inflammation independent of their receptor specificities. Also, intestinal PCs may be neuroprotective by migrating to and limiting neuroinflammation of the damaged CNS. Future studies should test the efficacy of treatments that mobilize IgA PCs in

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MS. Overall, the presence and the functions of immunosuppressive IgA PCs in the CNS of MS patients needs to be further characterized.

Immune activation: What is the motive for the crime?

Autoimmune responses often exacerbate MS pathogenesis, however the specific triggers that initiate the activation of pathogenic self-reactive immune cells and lead to the onset of MS remain unidentified. One possible trigger is the cross-reactivity of immune cells to microbiota-derived peptides[62]. Genetic and environmental risk factors are likely both implicated in MS pathogenesis[2]. Some of the most common environmental risk factors potentially involved in MS are viral infections[2]. Viral models of MS, such as the Theiler's murine encephalomyelitis virus (TMEV) model[63], are commonly used to study MS pathology. The need to study viral contributions to disease is emerging as pathogenic human endogenous retroviruses (HERVs), normally dormant and comprising 8% of the human genome, are upregulated in response to environmental factors in MS[64–66]. Another virus implicated in MS pathogenesis is the Epstein-Barr virus (EBV), a member of the herpes virus family that causes infectious mononucleosis[67,68]. While more than 95% of healthy individuals demonstrate an immune response to EBV, virtually all MS patients are seropositive for EBV[69,70]. Libbey *et al.*[71] hypothesize that MS could be triggered via molecular mimicry, whereby specific peptides secreted by pathogens have sequence and/or structural similarities to self-antigens. Previous studies have suggested the role of molecular mimicry in the pathogenesis of MS through Haemophilus influenzae viral infections[72] and a potentially similar mechanism has been observed for EBV infection[73]. The proposed cascade of events leading to CNS autoimmunity following EBV infection involve the proliferation and maturation of T-cells against Epstein-Barr virus nuclear antigen 1 (EBNA1)[74]. Tengvall *et al.*[74] hypothesized that T cell responses against EBNA1 may cross-react with a protein known as anoctamin 2 (ANO2) expressed in the CNS. Interestingly, autoantibody levels against ANO2 have been shown to be increased in the cerebral spinal fluid (CSF) of MS patients[74]. ANO2 is a calcium activated chloride channel important for ion transport and control of neuronal excitability that is expressed in hippocampal and cortical regions, and specifically detected near and inside of MS plaques[74]. EBV remains a *priority suspect* as: 1) MS does not occur until people have developed an immune response to EBV, and 2) a history of mononucleosis doubles the risk for MS[74]. Molecular mimicry remains the primary explanation for the link between EBV and MS.

Other viruses may have similar indications in the pathogenesis of MS. Human herpesvirus 6 (HHV6) is a member of the herpesvirus family and has two subtypes: HHV6A and HHV6B[75]. Both types preferentially attack and/or affect the nervous system, but HHV6A has greater neurotropism which may implicate a closer link to MS[75]. Opsahl and Kennedy detected significantly higher levels of HHV6 in post-mortem lesioned brain tissue from MS patients compared to the healthy controls[76]. Another study reported that a high antibody response against the HHV6A-specific antigen immediate-early protein 1A (IE1A) positively correlates with MS[77]. Individuals with strong humoral immunity against IE1A, particularly those younger than 20 years old, have a higher risk of developing MS later in life[77]. The positive association between the IE1A antigen from HHV6-A and MS is intriguing considering that HHV6A can impede the migration of oligodendrocyte progenitor cells and establish latent infection in

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oligodendrocytes[78], the myelin-producing cells presumed to be a target of the MS-related autoimmune responses. However, the mechanistic role of HHV6 in the pathology of disease remains unknown. Human endogenous retroviruses (HERV) are usually epigenetically silenced within our genome but certain triggers, including transactivation by exogenous viral infections such as EBV or other viruses, may lead to the re-expression of HERVs. HERV type W (HERV-W), formerly known as multiple sclerosis-associated retrovirus (MSRV), was discovered in the CSF of MS patients and is frequently detected in the brains of MS patients[79]. In MS lesions, myeloid cells contain the envelope protein ENV from HERV-W, which has recently been shown to polarize microglia and contribute to neurodegeneration[79]. A Phase IIb clinical trial (NCT02782858) tested the efficacy of GNbAC1 treatment, a humanized anti-ENV IgG_4 monoclonal antibody[80]. GNbAC1 exerted significant neuroprotective effects in MS patients within 1 year of treatment including a 1) 31% reduction of cortical atrophy, 2) 72% reduction of thalamic atrophy and, 3) 63% reduction of T1 hypointense lesions correlating with permanent brain tissue damage[79,80]. Several studies suggest an association between viral infections and MS, however there is still a need to delineate if there is a causal relationship between viruses and the onset of MS.

Another area of research that remains underexplored is the contribution of bacteriophages, viruses that infect bacteria, in MS. It is plausible that bacteriophages are the underlying cause of intestinal dysbiosis in MS patients, however they have also been implicated in mimicry of MS-relevant autoantigens. The Synechococcus phage is suggested to be a major contributor to the molecular mimicry phenomenon[81], whereby antibodies raised to viral proteins cross react with self-antigens leading to autoimmune responses. Bacteriophages may also induce chronic inflammation by changing gut microbiome composition and in turn increasing intestinal permeability[82,83]. Zhao *et al.* found less diversity in the intestinal virome of children who subsequently developed serum autoantibodies associated with progression to type 1 diabetes (cases) as compared to healthy controls[84]. Populations of bacteriophages differed significantly between cases and controls[84]. The disease-discriminating bacteriophage sequences identified also significantly correlated with different bacterial OTUs in both cases and controls, suggesting unique virome-bacterial microbiome interactions were present prior to development of autoantibodies[84]. Ultimately, epidemiological evidence, genetic association and mechanistic data in humans is needed to elucidate the role of viruses in the pathogenesis of MS. To this end, a broader characterizing of the MS patient virome[85] will be valuable.

Deception in the interrogation room: Crosstalk between host and bacteria, not two distinct species, but one intermixed system

The investigation of the complex crosstalk between host and bacteria is still in its infancy. Bacteria derived factors have been shown to influence host proteases[86] and host proteins were shown to modulate the activity of bacterial proteases[87]. In MS patients, the human protease inhibitor, cystatin C, is significantly upregulated in brain biopsies[88]. A bacterial protease, IdeS, produced by the human pathogen Streptococcus pyogenes (Group A Streptococcus [GAS]) hijacks mechanisms deployed by the host to inhibit proteases by using cystatin C as a cofactor to increase its own activity[87]. The intriguing finding that a host protease inhibitor may increase the activity of a bacterial protease further supports our lack of understanding of the complexity

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of host-microbial interactions. Cystatin C is also highly expressed in the brains of EAE mice[88]. Whether it interacts with bacterial proteases to influence disease pathogenesis in MS is currently unknown, however, it does seem to have a detrimental function in myelin oligodendrocyte glycoprotein 35-55-induced EAE in female mice, but not in male demonstrating a sex-specific role[88]. An analysis of the fecal proteome of EAE mice during disease latency demonstrated a transient increase in host protease inhibitors that inversely correlated with disease severity[89]. The administration of the antibiotic vancomycin attenuated EAE symptoms and elevated protease inhibitors[89] further supporting the idea that the microbiota modulates host biology.

Guo *et al*.[86] identified microbial metabolites with protease inhibitory activity produced by the gut bacteria of most healthy people. These metabolites impact cathepsins' activity in human cell proteomes[86], demonstrating new biological roles in interspecies signalling. Compared to healthy controls, MS patients present with a significant decrease in the abundance of certain bacteria, including species belonging to the genus Clostridium[90], which may produce microbial metabolites that inhibit host cell cathepsins[86]. Accordingly, MS patients have increased cathepsin activity which may contribute to disease pathogenesis (**Figure 2**). Hypomethylation of the cathepsin Z locus and increased cathepsin Z transcripts were detected in the pathology-free regions of MS brains as compared to healthy controls[91]. EAE mice deficient in cathepsin Z exhibit attenuated neuroinflammation and demyelination[92]. Cathepsin Z deficiency dramatically reduced circulating interleukin 1 beta levels and comprised the ability of mice to develop Th17 responses critical for the development of EAE[92]. Perhaps restoring the balance of the gut microbiome may help to regulate the aberrant activity of host proteases such as cathepsins seen in chronic inflammatory diseases such as MS.

Metabolites produced by gut bacteria are also capable of influencing immune homeostasis[93], therefore alterations in gut bacteria composition may have drastic effects on immune responses (**Figure 2**). $CD4^+Foxp3^+$ Tregs are critical mediators of immune homeostasis and the development of this cell population is directly affected by interactions with gut microbiota[93]. The spore-forming components of intestinal microbiota, specifically *Clostridia* clusters IV and XIVa, was shown to promote Tregs by providing an environment rich in transforming growth factor (TGF)-β to drive Treg development[94]. The microbiome of MS patients has a reduction of bacteria belonging to *Clostridia* clusters IV and XIVa - potent producers of SCFAs that regulate inflammation[90,95]. Mizuno et al.[95] demonstrated that administration of SCFAs suppresses lymphocyte-mediated systemic inflammation in EAE, thereby reducing EAE severity. Ultimately, further research characterizing changes in bacteria-host interactions will contribute to a stronger understanding of the molecular mechanisms affecting MS pathogenesis.

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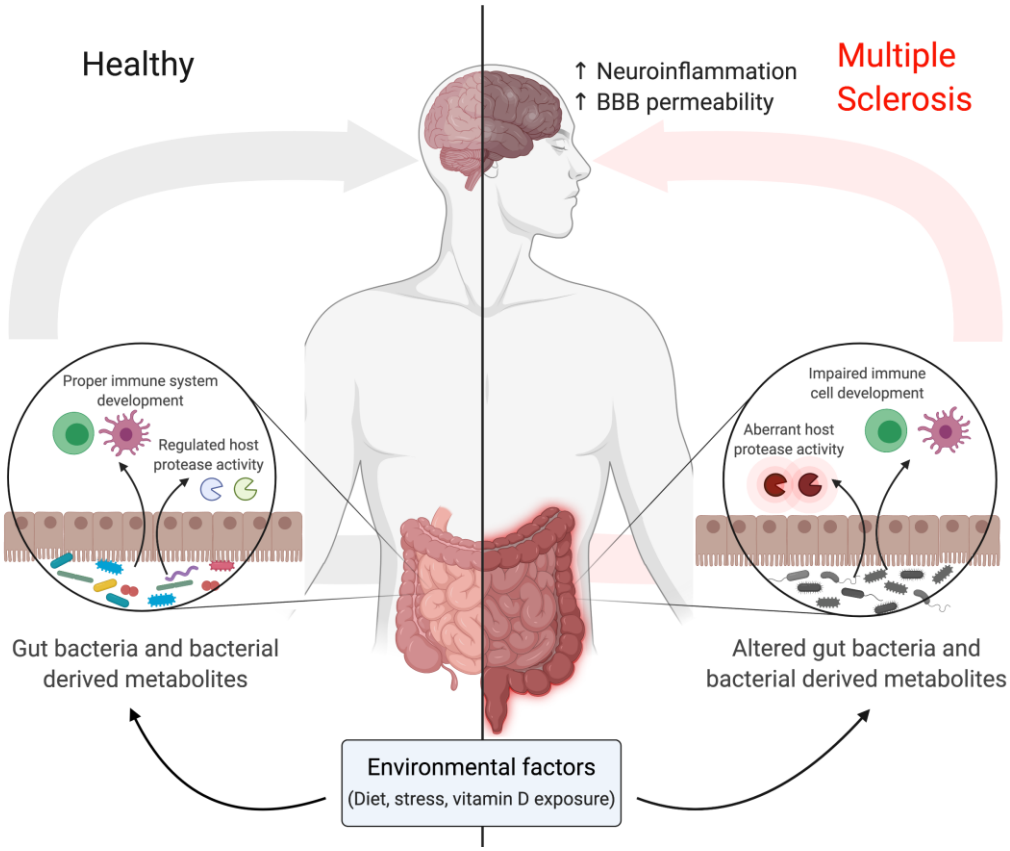

Figure 2: Gut microbiome can modulate host biology. There are known environmental factors such as diet, stress and vitamin D levels that can impact exacerbation of MS.

Interrogating the suspect: Is the gut behind the increased crime rate in the CNS?

Animal models of MS have provided novel insights into the effect of the gut microbiota on MS pathogenesis. In the EAE murine model of MS, perturbing the gut microbiota has been demonstrated to affect disease susceptibility via modulation of immune responses[96]. Interestingly, germ-free (GF) mice are resistant to the development of EAE, and show attenuated signs of disease, reduced clinical scores and shorter duration of EAE symptoms[97], which is likely a result of broadly impaired immune system development in mice that lack an intestinal microbiome[98]. While the molecular interactions between intestinal microbial organisms and the host was shown to affect the balance between pro- and anti-inflammatory immune responses, the immune system in return may further impact the composition of the microbiota[99]. However, changing the gut microbiome composition through the administration of probiotics does not significantly improve remyelination potential in mice afflicted with EAE[99]. Additional research is needed to delineate the cause-and-effect relationship between the role of the microbiome in MS. In cancer immunotherapy, intestinal bacteria have been associated with enhanced efficacy of immune checkpoint inhibitors[100]. Future studies should consider assessing the efficacy of probiotic supplementation in conjunction with pharmacological inhibitors and lifestyle changes to elucidate potential synergistic effects of probiotic therapy on maximizing benefit afforded to patients living with MS. Additionally, the human gut commensal *Prevotella histicola* (*P. histicola*) was demonstrated to be as effective as the immunomodulatory drug glatiramer acetate in supressing disease severity in a preclinical mouse model of MS as demonstrated by a lower cumulative EAE score[101]. While combination

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of *P. histicola* and glatiramer acetate did not result in an additive effect on EAE disease severity[101], it would be interesting to investigate whether *P. histicola* works synergistically with a different pharmacological drug approved for MS.

Profiling other tissues beyond the gut

While the gut has been implicating in the initial priming and proliferation of autoreactive CNS-specific immune cells, the lungs are another site potentially important for the reactivation of immune cells prior to disease relapse[102]. MS patients have elevated breath methane[10] which is often attributed to the presence of methanogens such as *Methanobrevibacter* in the gut. Methanogens are also found in the respiratory tract[103,104], however, the lung microbiome is currently under characterized in health and disease. To better characterize the role that the microbiota plays in MS, it is important to study the microbiome of MS patients at sites other than the gut, such as the microbiota of the skin and lungs. Besides altered intestinal bacteria composition in chronic inflammatory diseases, the influence of other environmental factors, such as changes in the virome, fungome or parasitome, remain underexplored. Importantly, certain microbes and their products have also been implicated to exert protective effects in MS[105–107]. Infection of MS patients or animal models of the disease with helminth parasites has been shown to be protective due to beneficial immune modulation and improvements in clinical symptoms[105]. Unbiased *Omics* technologies such as genomics, transcriptomics, proteomics and metabolomics, are valuable tools to enhance our understanding of the human microbiome as a whole[108].

Chasing a cure: MedXercise.

Exercise is known to exert a wide range of health benefits. A review by Lozinski and Yong presented the impact of exercise on structural and functional changes in the CNS[109]. The therapeutic effects of exercise in MS may, in part, be mediated by direct immunomodulatory effects since exercise is known to promote anti-inflammatory immune responses[110]. However, exercise may also induce beneficial changes for patients with neuroimmune illnesses by shifting the composition of their microbiota[111–116]. Whether these effects are solely attributed to physical activity or are influenced by other factors associated with healthier lifestyle, such as an altered diet, needs to be investigated[117]. It is important to note that the benefits of exercise may be dependent on the composition of the microbiota[118], thus combined treatment targeting the microbiota and engaging in exercise may maximize therapeutic effects. Interestingly, a physically active lifestyle is associated with beneficial changes to the microbiome[119] and it may prove to be useful in managing MS. Exercise has been shown to work in combination with pharmacological interventions such as clemastine[120], also known as meclastin, a drug that promotes remyelination and is a H1 histamine antagonist. In a toxin-induced model of focal demyelination, exercise promotes changes in the lesion microenvironment and makes the CNS more conducive to repair[120]. Exercise was shown to be as equally efficacious as the pharmacological treatment clemastine at promoting remyelination of lysolecithin-induced lesions in mice[120]. Furthermore, exercise and clemastine used in combination work synergistically to additively enhance remyelination[120]. While exercise exerts direct neuroprotective effects by promoting anti-inflammatory innate immune responses and increasing phagocytosis of myelin debris[120], it would be interesting to explore whether these

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changes are correlated with a shift in the microbiome. Nonetheless, the potential to couple pharmacological interventions with an adjunctive therapy like exercise, a concept termed MedXercise[109], is exciting as it provides a new means by which maximal benefit of treatments can be afforded.

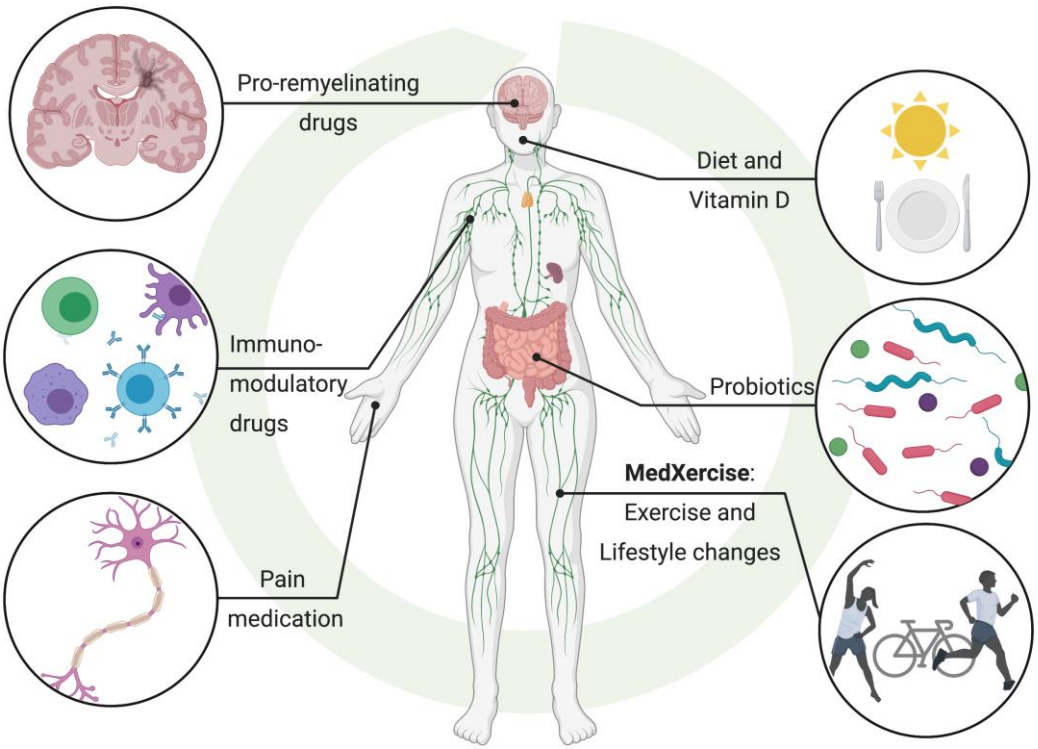

Figure 3: Multimodal approach to managing and treating MS and other chronic inflammatory diseases

The evidence speaks: Multifactorial diseases must be treated by multimodal means

For complex diseases, such as MS, individual drugs have proven to be ineffective at managing and treating MS and have failed to inhibit its progression. A multimodal approach may be more effective at addressing complex chronic diseases including MS and its comorbidities[121]. Administering a pro-remyelinating drug may not be as efficacious for long-term usage without reducing recurring chronic inflammation. Importantly, the immunomodulatory roles of the microbiota and other environmental or lifestyle factors should be considered. McMurran *et al.* showed that inflammatory responses during remyelination depend on the composition of the microbiota, but interventions that modify the microbiota have minimal impact on endogenous CNS remyelination[99]. Here, a multimodal approach of modulating the microbiota along with taking a pro-remyelinating drug is an optimal treatment strategy, as shown by Jensen and colleagues[120]. Additionally, monoclonal antibodies, such as natalizumab approved for MS[122,123], have been shown to be efficacious at preventing leukocyte trafficking into the CNS[124], but whether this therapy can be combined with exercise or other means to modulate the microbiome to reduce inflammatory immune cells warrants investigation. While pharmacological treatment may focus on one aspect of disease, an effective “cure” will need

to address all dysregulated features of MS. It is likely that microbiota composition plays an influential role in MS pathogenesis. Correcting intestinal dysbiosis may also improve cognitive behaviour and depression[125], which is a common symptom of MS[121,126]. Therefore, future treatments should consider how disease modifying treatments (DMTs) can be combined with therapies targeted at the microbiota, including lifestyle changes[127] such as exercise and/or modified diets, to maximize tissue repair and clinical benefit for MS patients (**Figure 3**). This may be of particular importance for MS patients residing in long-term care facilities as nursing home residents microbiomes are associated with decreased SCFA-producing organisms and increased intestinal dysbiosis[128] which may exacerbate MS pathogenesis. Multicenter studies across the world may help identify microbial strains common to MS patients independent of vastly different lifestyle and diets of patients from different countries. However, it is important to consider the effects of treatment and disease duration on the microbiome of MS patients, as several current/promising DMTs have antimicrobial effects including glatiramer acetate[129], minocycline[130], fingolimod[131], teriflunomide[132] and dimethyl fumarate[132]. It is likely that *the crime of MS* is committed by more than one criminal. Therefore, solving the cold case of MS will require the combination of multiple experts from various disciplines including microbiology, virology, neurology and immunology.

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