

The chicken or the egg dilemma: intestinal dysbiosis in multiple sclerosis

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Provenance: This is a Guest Editorial commissioned by section editor Dr. Mingzhu Gao (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Comment on: Jangi S, Gandhi R, Cox LM, *et al.* Alterations of the human gut microbiome in multiple sclerosis. *Nat Commun* 2016;7:12015.

Abstract: Recent findings suggest that the intestinal microbiota of patients suffering from relapsing remitting multiple sclerosis (MS) shows changes on the relative abundances of archaeal and bacterial genera. Although the richness and overall structure of the microbiota may be similar compared to the intestinal microbiota of healthy controls, elevated and reduced frequencies suggest a dysbiotic microbiota in MS. Over the past decade experimental evidence obtained in murine models of the disease highlighted the important relevance of the microbiota in the regulation of the immune system and in the severity of the disease. More recent findings on peripheral immune cells derived from human MS patients support the initial observations that changes in the microbiota may affect immunological pathways that could exacerbate disease. However, important questions remain to be answered. For instance, it is unclear whether dysbiosis precedes disease or, if in the contrary, an autoimmune disease such as MS can lead to gut dysbiosis. In this brief discussion, we speculate about this later possibility based on findings observed in murine models of disease. Further human studies are needed to answer the dilemma and determine specific immunomodulatory pathways that could have an impact on the therapeutic approaches to treat MS.

Keywords: Microbiota; intestine; dysbiosis; multiple sclerosis (MS)

Submitted Dec 20, 2016. Accepted for publication Dec 29, 2016.

doi: 10.21037/atm.2017.01.18

View this article at: <http://dx.doi.org/10.21037/atm.2017.01.18>

The microbiota is the complete population of microbes that include species from the three domains that include cellular microbes, *Bacteria*, *Archaea*, *Eukarya*, and acellular infectious organisms such as viruses, that inhabit a biogeographical location (1). In the intestine of experimental animal models, the microbiota plays a significant role shaping the immune system, regulating the host's metabolism, providing a biological defense mechanism against infections, as well as modulating the appropriate neuronal development. Despite the experimental evidences obtained using gnotobiotic animals, treating mice with antibiotics or by performing monocolonization studies, the direct link between the microbiota and human diseases has not been elucidated.

Recent advances in DNA sequencing and management of large amount of data implemented have allowed investigators to compare the composition of the intestinal microbiota in the context of human diseases. Experimental evidence suggests that changes in the composition of the heterogeneous intestinal ecosystem may be associated with alterations in the severity in different models of human autoimmune diseases such as inflammatory bowel disease (2), asthma (3), allergies (4), diabetes (5), rheumatoid arthritis (6), or systemic lupus erythematosus (SLE) (7). Furthermore, results obtained in the multiple sclerosis (MS) animal model, experimental autoimmune encephalomyelitis (EAE), suggest an association between the gut and the central nervous system (CNS)

Table 1 Peer reviewed studies comparing the Intestinal microbiota of MS patients with healthy individuals

MS type diagnosis	Specifics of study	Major findings	Ref.
Relapsing remitting	HC (n=31) vs. MS (n=30)	Type A <i>Clostridium perfringens</i> reduced presence in MS vs. HC Type B <i>Clostridium perfringens</i> found in one MS patient	(12)
Relapsing remitting	HC (n=8) vs. MS (n=7); 4 MS patients with pre- and post-vitamin D supplementation, 2 treated with GA Total of 5 patients treated with GA	Prior vitamin D supplementation, ↓ <i>Bacteroidaceae</i> and <i>Faecalibacterium</i> , and ↑ <i>Ruminococcus</i> in MS when compared to HCs Vitamin D supplementation: ↑ frequencies of <i>Faecalibacterium</i> and <i>Enterobacteriaceae</i> , ↓ <i>Ruminococcus</i> Untreated MS ↑ frequencies of <i>Akkermansia</i> , <i>Faecalibacterium</i> , and <i>Coprococcus</i> when compared to HC and GA-treated MS	(13)
Relapsing remitting	HC (n=50) vs. MS (n=20)	↓ frequencies of specific clusters of <i>Clostridium</i> and species of <i>Bacterioides</i> in MS vs. HC	(14)
Relapsing remitting, children	HC (n=17) vs. MS (n=18); immunomodulators- (n=9) or corticosteroids-treated (n=9)	No differences in alpha diversity and beta diversity, beta diversity different for immunomodulators vs. corticosteroids Early microbiota alterations in MS found at specific taxonomic levels: ↓ <i>Faecalibacterium</i> , <i>Enterobacteriaceae</i> , <i>Clostridiales</i> , <i>Bacteroides fragilis</i> ; ↑ <i>Methanobrevibacter</i>	(15)
Relapsing remitting, children	HC (n=9) vs. MS (n=15)	Found microbiota dysfunction in MS Uneven microbiota (species overgrowth) associated with ↑ markers of Th17 and Th2 populations; associated microbiota composition with immunological markers in blood	(16)
Relapsing remitting	HC (n=36) vs. MS (n=59)	MS has distinct intestinal microbiota structure Active vs. remission in MS show different microbiota structure In MS, ↑ <i>Pedobacter</i> , <i>Flavobacterium</i> , <i>Blautia</i> , <i>Dorea</i> , <i>Pseudomonas</i> and <i>Mycoplana</i> and ↓ <i>Adlercreutzia</i> , <i>Collinsella</i> , <i>Parabacteroides</i> , <i>Erysipelotrichaceae</i> , <i>Lachnospiraceae</i> , <i>Veillonellaceae</i> , <i>Lactobacillus</i> , <i>Coprobacillus</i> , and <i>Haemophilus</i>	(17)
Relapsing remitting	HC (n=43) vs. MS (n=60) Untreated vs. treated with immunomodulators	↑ <i>Methanobrevibacter</i> , <i>Akkermansia</i> and ↓ <i>Butyricimonas</i> in MS; levels restored in MS treated with GA or beta-interferon Positive association between ↑ frequencies of <i>Methanobrevibacter</i> , <i>Akkermansia</i> and proinflammatory immune pathways Potential use of exhaled methane as indicator of ↑ frequencies of <i>Methanobrevibacter</i>	(18)

↓, reduction in relative abundance; ↑, increase in relative abundance. MS, multiple sclerosis; HC, healthy controls; GA, glatiramer acetate; Ref., reference number.

inflammatory demyelination (8). The gut-brain connection is currently being experimentally explored in the context of different human CNS disorders, such as autism (9,10), and Parkinson's Disease (PD) (11).

The alteration of the intestinal microbial community that might lead to either animal or human diseases is termed intestinal or gut dysbiosis. The number of studies

comparing the composition of the intestinal microbiota of MS patients with healthy individuals is gradually increasing over the recent years (Table 1). Many of the studies shown in Table 1 have been recently reviewed by Newland *et al.* (19). The studies conducted so far suggest the presence of a disrupted or altered microbiota in MS patients when compared to healthy controls (12-18). All studies were

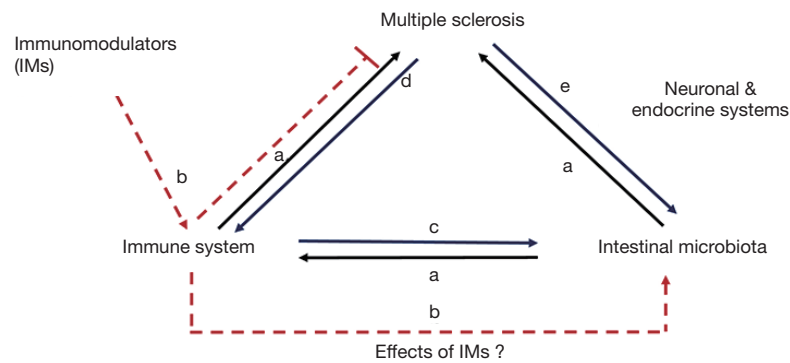


Figure 1 The bidirectional nature of the gut/disease axis in the context of MS. To date, the studies performed using samples obtained from MS patients and healthy individuals suggest that relative abundances of specific gut microbes are significantly altered (*Table 1*). Experimental evidence suggests that changes in the microbiota might affect immune, endocrine and neuronal function (a). However, it is not known whether the intestinal disruption precedes the onset of disease or the changes occur once the immunological dysfunction that results in disease is already ongoing. Therapies that target the immune system and modulate the function of key immune cells (b) could affect their interaction with the gut microbiota (c). Disease-associated immunological responses could also affect the intestinal ecosystem, by alterations in the intestinal barrier permeability for example (d). That could also result in concomitant effects on the function of immune cells. Additionally, experimental data suggest a direct effect of the microbiota on the endocrine and the neuronal system. On the other hand, changes in mood, stress, depression and other behavioral factors that occur in MS could ultimately affect the composition of the microbiota (e). MS, multiple sclerosis; IMS, immunomodulators.

performed in patients diagnosed with relapsing remitting MS, and in some the effects of immunomodulatory therapies were evaluated (13,18). Overall, the analysis suggest that significant differences in specific genera or even species are found, including elevated relative abundances in *Methanobrevibacter* and *Akkermansia*, as well as reduced *Butyrivimonas* found in the stool samples of MS patients (18). A recent study showed that the overall structure of the MS microbiota is significantly different to healthy controls and interestingly also significantly different between active and remission MS (17). Genera *Adlercreutzia*, *Collinsella*, *Parabacteroides*, *Erysipelotrichaceae*, *Lachnospiraceae*, *Veillonellaceae*, *Lactobacillus*, *Coprobacillus*, and *Haemophilus* relative abundances were reduced in the intestines of MS when compared to healthy controls, whereas genera *Pedobacter*, *Flavobacterium*, *Blautia*, *Dorea*, *Pseudomonas* and *Mycoplana* relative abundances were elevated. Other significant observations have been reported, such as significant reductions in specific clusters of *Clostridium* and species of *Bacteroides* (14,15). The changes in the abundances of these microbes could have direct effects on the immune system. As it will be discussed later, we suggest that the interaction between the microbiota and disease is multifactorial and bidirectional. The complex array

of interactions is described in *Figure 1*. Both *Clostridium* and *Bacteroides* species present in the colon are known to produce short chain fatty acids (SCFA) as a product of non-digestible carbohydrates fermentation. SCFAs regulate immune cells gene expression, promotion of a regulatory phenotype, decrease of oxidative stress, and reinforce the intestinal barrier and blood-brain barrier (BBB) (20,21). *Bacteroides fragilis*, an inhabitant of the human gut, is known to induce strong immunomodulatory effects via the expression of the capsular polysaccharide A (PSA) (22-25). Our laboratory has previously shown that oral treatment with PSA protects mice against EAE through the induction of regulatory CD39⁺ T cells and Foxp3⁺ T regulatory cells (Tregs) (24,26,27). *B. fragilis* colonization also improves the integrity of the barrier (9). The complete absence of microbes in gnotobiotic mice C57BL/6 mice subjected to the induction of active EAE (28) and in gnotobiotic transgenic mice (29) that develop the disease spontaneously significantly affects the development of disease. Furthermore, treatment with oral antibiotics also reduce significantly the severity of EAE (30,31). The experimental evidence gathered using animal model studies indicate the effects of the microbiota in the regulation of EAE in animals (24,26-28,32-36). Furthermore, gut microbes, such

as *B. fragilis* and *Lactobacillus rhamnosus* have been shown to interact with neurons in the enteric nervous system (ENS) directly and affect their function (37). The gut microbiota can directly influence hormonal levels, thus modulating the effects of the endocrine system in the autonomous nervous system (38). For example, the effects of the microbiota on MS maybe associated with direct interactions and functional changes on the immune, endocrine and neuronal systems (Figure 1a), as previously reviewed (39-41).

In a recent work published by Jangi and collaborators, the gut microbiota of 60 MS patients was compared with the microbiota of 43 healthy donors (18). Both male and female relapsing-remitting MS patients were selected for the study. A cohort of patients that received beta-interferon or glatiramer acetate for at least half-year were also included in the study, although individuals under treatment with immunosuppressive drugs as well as individuals suffering from other autoimmune diseases were excluded. Microbial 16S ribosomal RNA profiles were obtained from stool samples using two distinct sequencing platforms. The comparison of the intestinal microbial profile showed no significant differences in the diversity of the microbial populations of all samples analyzed. The overall structure of the microbiota determined by all sequences of both healthy and MS populations were found to be not significantly different. Of note, we have obtained preliminary data in EAE mice that indicates no significant changes in alpha diversity and time-dependent changes in the overall composition of the intestinal microbiota (42).

At the genus level the analysis showed an increased relative abundance of the archaeal genus *Methanobrevibacter* and bacterial genus *Akkermansia* and reduced relative abundance of *Butyricimonas* in the stool samples of MS patients, either untreated or treated with interferon-beta or glatiramer acetate and untreated when compared to healthy individuals. Reduced abundances of *Prevotella* and *Sutterella* were also reported in samples obtained from untreated MS patients when compared with healthy controls. Interestingly, the treatment with these two immunomodulatory therapies increased the relative abundances of genera, such as *Prevotella* and *Sutterella*, that were shown to be reduced in untreated MS patients versus treated. No significant differences were observed between those treated with either interferon-beta or glatiramer acetate, possibly due to the reduced size sample available for the comparison, as argued by the authors. However, despite the potential differences in the mechanism of action between these two drugs, the analysis performed suggests that immunomodulatory drugs

might have the capacity to alter the composition of the microbiota, and reverse the apparent dysbiosis that was observed in those with relapsing MS (Figure 1b).

Teriflunomide, a more recently FDA approved treatment for relapsing MS may also have an impact on the dysbiosis in those with MS. We have recently shown that the oral treatment of non-EAE C57BL/6 mice with teriflunomide promotes the acquisition of a CD39 phenotype on gut-associated lymphoid tissues (GALT) T cells with enhanced protective ability when transferred into EAE mice (43). The TERI-DYNAMIC study on the immunomodulatory effects of teriflunomide on relapsing-remitting patients recently reported an increased frequency of circulating inducible Tregs in treated patients when compared with controls (44). Together, the human and murine immune studies suggest that teriflunomide may have immunomodulatory effects that could potentially affect gut dysbiosis.

In their study, Jangi and colleagues demonstrate an association between the changes in the intestinal microbiota and the peripheral immunological responses observed in relapsing remitting MS patients (Figure 1a). The gene expression in circulating T cells and in monocytes of patients and controls was used to define an immune-related gene profile. The gene expression analysis was performed after screening patients based on the intestinal relative abundances of *Methanobrevibacter*, increased in samples obtained from MS patients when compared to healthy individuals. Correlation studies demonstrated a positive association between increased intestinal relative abundances of *Methanobrevibacter* and *Akkermansia* and the upregulation of genes of T cells related to proinflammatory pathways of interferon and IL-2 signaling, as well as activation of peroxisome proliferator-activated receptor (PPAR) and LXR/RXR. Similar comparison was performed in monocytes isolated from blood samples of MS patients. When the expression genes of monocytes were compared, a positive correlation was observed between the presence of increased relative abundances of *Methanobrevibacter* and *Akkermansia*, and proliferative pathways including interferon signaling, dendritic cell maturation and TREM signaling. Conversely, the increased relative abundances of *Butyricimonas*, a genus that belong to the phylum *Bacteroidetes*, showed a negative correlation with the pathways described for both T cells and monocytes. The results reported are of great interest since they might define an immunological effect of the dysbiotic state of the intestinal microbiota in those with relapsing MS. Thus, these results obtained might suggest that specific changes

on relative abundances could drive opposite immunological responses that could regulate disease progression and severity (Figure 1a). Valuable therapeutic information might be culled from similar studies conducted in MS patients under treatment with other immunomodulators (Figure 1b). Of great relevance would be to discern whether there are differences in the microbiota and immune pathways of MS patients that respond or not to therapy.

The search for novel MS biomarkers has been exhaustively explored with little success. When considering intestinal dysbiosis, it would be of great interest to utilize an inexpensive, noninvasive and cheap parameter to determine potentially pathogenic changes in the gut microbiota. Based on the intestinal abundances of *Methanobrevibacter smithii*, exhaled methane was compared in patients and healthy individuals. Archaeal microbes are classified based on metabolic and environmental adaptations that define them as extremophiles. In the gut, *Methanobrevibacter* use end-products of the sugars fermentation by other microbiota members for their own benefit, producing methane. Using gas chromatography, Jangi *et al.* detected enhanced amounts of methane exhaled by MS patients. Although preliminary, expanded studies would determine whether the detection of exhaled methane could potentially be considered a marker for MS-associated intestinal dysbiosis.

One important question that remains to be answered is whether disease can affect the intestinal ecosystem and the gut microbiota, as hypothesized in Figure 1 (Figure 1c,d). The induction of experimental CNS inflammatory demyelination increases intestinal permeability in mice as early as 7 days after active induction of the disease, with an associated increase in the frequencies of Th1, Th17, and IL-17⁺ gamma-delta ($\gamma\delta$ T) cells in the GALT of EAE mice (45). The intestinal permeability increases when EAE is induced. Of note, there is a similar pattern in mice that were adoptively transferred with autoreactive CD4⁺ T cells, a widely used protocol for the passive induction of EAE. The adoptive transfer findings are important due to the absence of adjuvants used for passive EAE. It could be postulated that the inclusion of adjuvants to induce disease can perhaps be associated with the increased intestinal permeability observed in mice developing EAE. An increase in the intestinal permeability was also observed in a murine model of autism spectrum disorders (ASD) that exhibit GI dysfunction and intestinal dysbiosis. These effects can be reversed with the oral treatment with *B. fragilis* (9). In germ-

free mice gut epithelial integrity is also affected and is restored by colonization with a probiotic therapy consisted in *Lactobacillus rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919, improving the intestinal morphology and barrier integrity (46). Experimental findings suggest a change in the intestinal permeability that occurs even before disease onset may imply that the disease itself affects the integrity of the intestinal interaction between microbes and our cells. In humans, a recent pilot study showed that the average levels of mannitol in urine were increased in 22 relapsing-remitting MS patients when compared to 18 healthy individuals (47). Authors hypothesized that the observation was the result of an increased intestinal permeability associated with MS. However, whether intestinal barrier disruption precedes or not disease is a question that remains to be answered.

The studies performed using human stool samples obtained from relapsing MS patients suggest a dysbiotic gut (Table 1). Two of the studies that compared the gut microbiota of MS patients associated the changes in the relative abundances of intestinal microbes with phenotypic modifications in the immune homeostasis and balance between pro- and anti-inflammatory or regulatory cell populations in blood (15) and genotypic changes (18). These studies advance our understanding on the interaction between the gut microbiota and the immune system. This interaction may be in one direction, by microbes regulating the type and function of the responsive immune system and immune cells controlling immunopathology of MS (Figure 1a). However, could the MS-specific immune status (Figure 1c) and disease-modifying immunomodulatory therapies also affect the composition of the intestinal microbiota (Figure 1e)? Jangi and colleagues discussed potential limitations in their study, including the effects that MS-physiological changes might have on the composition of the microbiota. The effects of EAE induction on the intestinal barrier disruption as well as on the intestinal microbiota raise the question whether on the animal models of MS, dysbiosis is cause or effect.

In summary, the experimental observations obtained from MS patients suggest that intestinal dysbiosis occurs during the course of the disease (Figure 1c-e). The association made by Jangi and collaborators between patients with elevated relative abundances of the archaeal methanogen *Methanobrevibacter* and increased expression of genes related to immunopathologic pathways in MS is intriguing and constitutes a step forward on the understanding of the gut/

disease axis. More and larger efforts are now necessary to discern whether the differences observed in the gut microbiome are translated into a functionally biological relevance. Whether dysbiosis precedes MS (*Figure 1a*) or if it is the disease that affects the composition and function of the microbiota (*Figure 1c-e*), is a question that remains to be answered. Future directions might have a significant impact on MS patients since these will likely determine whether the effects that are now observed in the gut microbial populations and function can be used as potential target for novel therapies against the disease.

Acknowledgements

Funding: Studies referenced in the manuscript were funded by the National MS Society (NMSS) (award #PP3423) and Sanofi Genzyme Corporation (Genzyme/IST Concept Award GF-2014-11153).

Footnote

Conflicts of Interest: J Ochoa-Repáraz received funding from the National Multiple Sclerosis; K Magori reports no disclosures; LH Kasper received honoraria from a speakers bureau.

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Cite this article as: Ochoa-Repáraz J, Magori K, Kasper LH. The chicken or the egg dilemma: intestinal dysbiosis in multiple sclerosis. *Ann Transl Med* 2017;5(6):145. doi: 10.21037/atm.2017.01.18