

## HEALTH AND MEDICINE

# High frequency of intestinal T<sub>H</sub>17 cells correlates with microbiota alterations and disease activity in multiple sclerosis

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T helper 17 (T<sub>H</sub>17) cells are key players in multiple sclerosis (MS), and studies in animal models demonstrated that effector T<sub>H</sub>17 cells that trigger brain autoimmunity originate in the intestine. We validate in humans the crucial role of the intestinal environment in promoting T<sub>H</sub>17 cell expansion in MS patients. We found that increased frequency of T<sub>H</sub>17 cells correlates with high disease activity and with specific alterations of the gut mucosa-associated microbiota in MS patients. By using 16S ribosomal RNA sequencing, we analyzed the microbiota isolated from small intestinal tissues and found that MS patients with high disease activity and increased intestinal T<sub>H</sub>17 cell frequency showed a higher Firmicutes/Bacteroidetes ratio, increased relative abundance of *Streptococcus*, and decreased *Prevotella* strains compared to healthy controls and MS patients with no disease activity. We demonstrated that the intestinal T<sub>H</sub>17 cell frequency is inversely related to the relative abundance of *Prevotella* strains in the human small intestine. Our data demonstrate that brain autoimmunity is associated with specific microbiota modifications and excessive T<sub>H</sub>17 cell expansion in the human intestine.

## INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) caused by myelin-specific, self-reactive T lymphocytes (1). T cells with high affinity for CNS autoantigens are present in healthy individuals (2), but they normally remain dormant and do not cross the blood-brain barrier to trigger autoimmune diseases in the brain unless they are activated in peripheral tissues, such as the lung and the intestine (3, 4). Relapsing-remitting MS (RRMS), the most common disease course, is characterized by clearly defined attacks of new or increasing neurologic symptoms followed by periods of partial or complete recovery (remissions). RRMS can be classified as either active [with clinical relapses and/or evidence of new magnetic resonance imaging (MRI) activity] or inactive. Disease activity in RRMS is correlated with periodic activation of myelin-specific T cells, but the mechanisms that regulate the aggressiveness of those self-reactive T cells are still largely unknown.

T helper 17 (T<sub>H</sub>17) cells are key players in MS pathogenesis, and acquisition of interleukin-17 (IL-17)-secreting phenotype by myelin-specific T cells enhances their pathogenicity. In experimental autoimmune encephalomyelitis (EAE), the preclinical model of MS, brain autoimmunity is driven by a proinflammatory T cell response that promotes infiltration of immune cells in the CNS. Although several B and T cell subsets orchestrate disease pathogenesis, effector T<sub>H</sub>17 cells represent the first wave of pathogenic T cells infiltrating the CNS (5) because of their ability to efficiently breach the blood-brain barrier (6).

In humans, IL-17 was found in MS lesions, and whole-genome transcriptomic analysis revealed that IL-17 is the highest-ranking gene expressed in the active plaques (7).

Although T<sub>H</sub>17 cells are believed to be central in MS pathogenesis, the geography of their peripheral activation and expansion in humans is still unclear. In mice, activation of effector T<sub>H</sub>17 cells occurs mostly in the small intestine (8, 9), and acquisition of a T<sub>H</sub>17 cell phenotype by myelin-reactive T cells at the intestinal level enhances their pathogenicity and capacity to trigger brain autoimmunity (4, 10, 11). Commensal microbes residing in the small intestine, such as the segmented filamentous bacteria (SFB), induce brain autoimmunity in mice by selectively promoting T<sub>H</sub>17 cell differentiation (10). In humans, previous studies identified alterations of the microbiota composition in fecal samples of RRMS patients (12–16); however, it is still unclear how these modifications impinge on the immune system. Although two of these studies found a correlation between the microbiome modifications and specific immune markers in peripheral blood lymphocytes of RRMS patients compared to healthy controls (HCs) (14, 15), a specific analysis on how the microbiota alter immunity at the intestinal level in RRMS patients was never performed. Here, we performed analysis of immune cell subsets together with 16S ribosomal RNA (rRNA) sequencing of mucosa-associated microbiota in the small intestine of RRMS patients and HCs. Our data show that in RRMS patients, there is a selective expansion of effector T<sub>H</sub>17 cells in the small intestinal mucosa that is linked with specific microbiota modifications.

## RESULTS

### Study design

Small intestinal tissue and peripheral blood samples were collected from 36 individuals, including 19 RRMS patients and 17 HC subjects; details of the study population are illustrated in Table 1. Total lymphocytes were freshly isolated from intestinal tissue collected during esophago-gastroduodenoscopy (EGD) and from peripheral blood, stained with fluorescent monoclonal antibodies against surface markers and

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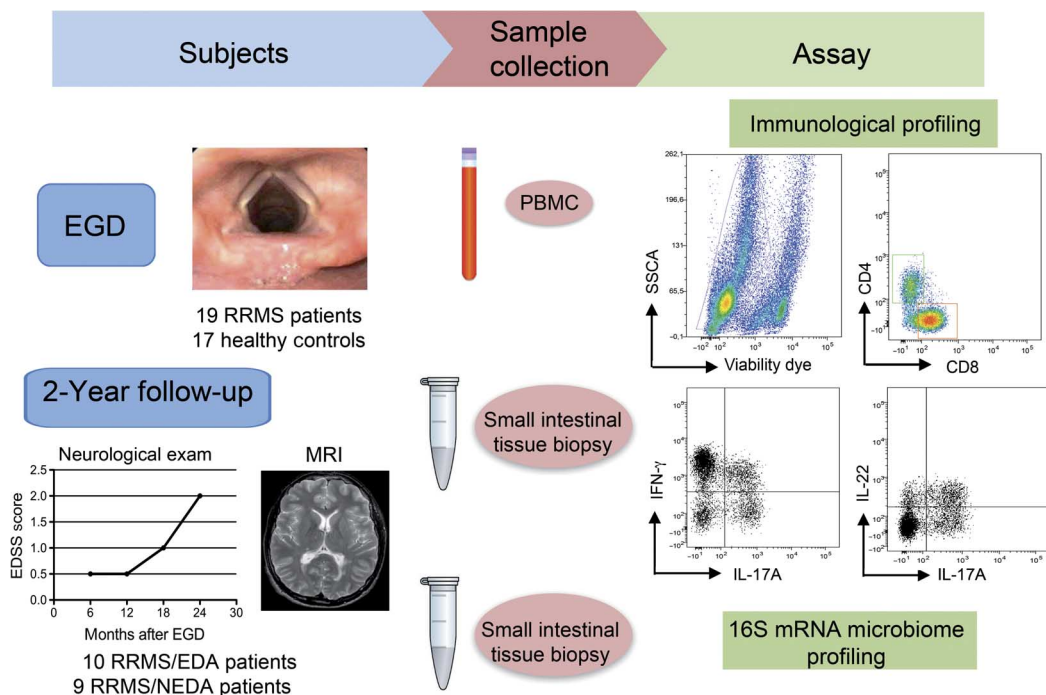
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**Table 1. Demographics of study population.** All individuals recruited for the study underwent EGD for diagnostic purposes. Exclusion criteria for both HCs and RRMS patients were treatment with antibiotics or corticosteroids in the 3 months before EGD and history of gastroenteritis, gastric ulcer, irritable bowel disease, celiac disease, inflammatory bowel disease, and gastric and colorectal cancers. All patients recruited in this study have a diagnosis of RRMS defined according to Poser's diagnostic criteria. None of the RRMS patients received corticosteroids in the 3 months before the EGD. The mean age of healthy individuals was slightly higher than that of RRMS patients, but our correlative analysis did not reveal any association between age and  $T_H17$  cell percentages and *Prevotella/Streptococcus* relative abundance. All samples analyzed were donated for research purposes, and each subject signed an informed consent. Data are means  $\pm$  SD, unless otherwise indicated. RR-NEDA, RRMS/NEDA; RR-EDA, RRMS/EDA.

| Cohort  | n  | Age (years) | Age (mean) | Sex (women/men) | Other autoimmune diseases     |
|---------|----|-------------|------------|-----------------|-------------------------------|
| Healthy | 17 | 27–74       | 48 $\pm$ 3 | 10/7            | NA                            |
| RRMS    | 19 | 25–57       | 41 $\pm$ 2 | 11/8            | Autoimmune thyroiditis (1/19) |
| RR-NEDA | 9  | 26–56       | 40 $\pm$ 4 | 7/2             | —                             |
| RR-EDA  | 10 | 25–57       | 41 $\pm$ 3 | 4/6             | —                             |



**Fig. 1. Study design.** Tissue samples from the duodenal mucosa were collected during EGD from RRMS patients ( $n = 19$ ) and HCs ( $n = 17$ ). Simultaneously, we collected 6 to 7 ml of peripheral blood from the same individuals. Total lymphocytes were isolated from one to two fragments of intestinal tissue by collagenase digestion and from peripheral blood by Ficoll gradient. Mucosal immune cells and peripheral blood mononuclear cells (PBMCs) were FACS-analyzed for immunological profiling of the following  $T_H$  cell subsets: IL-17A<sup>+</sup>CD4<sup>+</sup>  $T_H17$  cells, IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup>  $T_H1$  cells, and IL-22<sup>+</sup>  $T_H22$  cells. Microbial DNA was extracted from frozen intestinal tissue samples (seven RRMS and seven HC samples), and 16S rRNA sequencing was performed on the Roche 454 platform. At the 2-year end point, we stratified RRMS patients in two cohorts on the basis of evidence of disease activity by clinical and MRI criteria. RRMS patients with no evidence of disease activity in the 2-year follow-up were classified as RRMS/NEDA ( $n = 9$ ), whereas RRMS patients with evidence of disease activity were classified as RRMS/EDA ( $n = 10$ ). SSCA, side scatter FACS parameter.

intracellular cytokines, and analyzed by FACS (fluorescence-activated cell sorting) (Fig. 1). Microbial DNA was extracted from small intestinal samples obtained during EGD and analyzed through 16S rRNA gene sequencing on the Roche 454 platform. All patients enrolled in this study had a diagnosis of RRMS defined according to Poser's diagnostic criteria, had different degrees of disability [Expanded Disability Status Scale (EDSS) score from 1 to 5.5], and were receiving treatment with immunomodulatory drugs [interferon- $\beta$  (IFN- $\beta$ ), glatiramer acetate (GA), or fingolimod] (Table 2). Disease activity in a 2-year follow-up was evaluated by periodic neurological examina-

tions and evaluations of clinical relapses and disability progression (EDSS), and by measurement of new lesions by MRI (Fig. 1). NEDA (no evidence of disease activity) is a new concept related to the absence of disease activity in the context of MS that takes into account the following clinical and neuroradiological parameters: clinical relapses, disability progression, and active lesions evaluated by MRI (17). Our RRMS patients were classified into two subgroups on the basis of the presence or absence of disease activity at a 2-year follow-up: patients with no evidence of disease activity (RRMS/NEDA,  $n = 9$ ) and patients with evidence of disease activity (RRMS/EDA,  $n = 10$ ).

**Table 2. Clinical data.** All patients recruited in this study have a diagnosis of RRMS defined according to Poser's diagnostic criteria. None of the RRMS patients received corticosteroids in the 3 months before the EGDs (time of intestinal biopsy collection). RRMS patients were divided into two subgroups on the basis of evidence of disease activity at 2-year follow-up: RRMS patients with no evidence of disease activity in the 2-year follow-up were classified as NEDA ( $n = 9$ ), whereas RRMS patients with evidence of disease activity were classified as EDA ( $n = 10$ ). All RRMS patients recruited in this study were undergoing different therapeutic immunomodulatory regimens (IFN- $\beta$ , GA, and fingolimod) at the time of EGD and intestinal tissue sample analysis.

| MS patient ID | Sex | Age (years) | Disease activity (2-year follow-up) | EDSS | Current therapy |
|---------------|-----|-------------|-------------------------------------|------|-----------------|
| MS001         | F   | 38          | NEDA                                | 3    | Fingolimod      |
| MS002         | M   | 49          | EDA                                 | 3    | IFN- $\beta$    |
| MS003         | F   | 27          | EDA                                 | 2    | IFN- $\beta$    |
| MS004         | F   | 49          | NEDA                                | 5.5  | GA              |
| MS005         | F   | 38          | EDA                                 | 1.5  | IFN- $\beta$    |
| MS006         | M   | 42          | NEDA                                | 2    | GA              |
| MS007         | M   | 43          | EDA                                 | 1.5  | GA              |
| MS008         | M   | 37          | EDA                                 | 2    | Fingolimod      |
| MS009         | M   | 35          | EDA                                 | 4    | GA              |
| MS010         | M   | 57          | EDA                                 | 4    | GA              |
| MS011         | F   | 55          | NEDA                                | 1.5  | GA              |
| MS012         | F   | 26          | NEDA                                | 5    | GA              |
| MS013         | F   | 29          | NEDA                                | 1    | IFN- $\beta$    |
| MS016         | M   | 27          | NEDA                                | 1.5  | IFN- $\beta$    |
| MS017         | F   | 42          | NEDA                                | 1.5  | IFN- $\beta$    |
| MS018         | F   | 57          | EDA                                 | 3.5  | Fingolimod      |
| MS020         | F   | 25          | EDA                                 | 1    | GA              |
| MS021         | F   | 56          | NEDA                                | 2    | IFN- $\beta$    |
| MS022         | M   | 40          | EDA                                 | 1    | GA              |

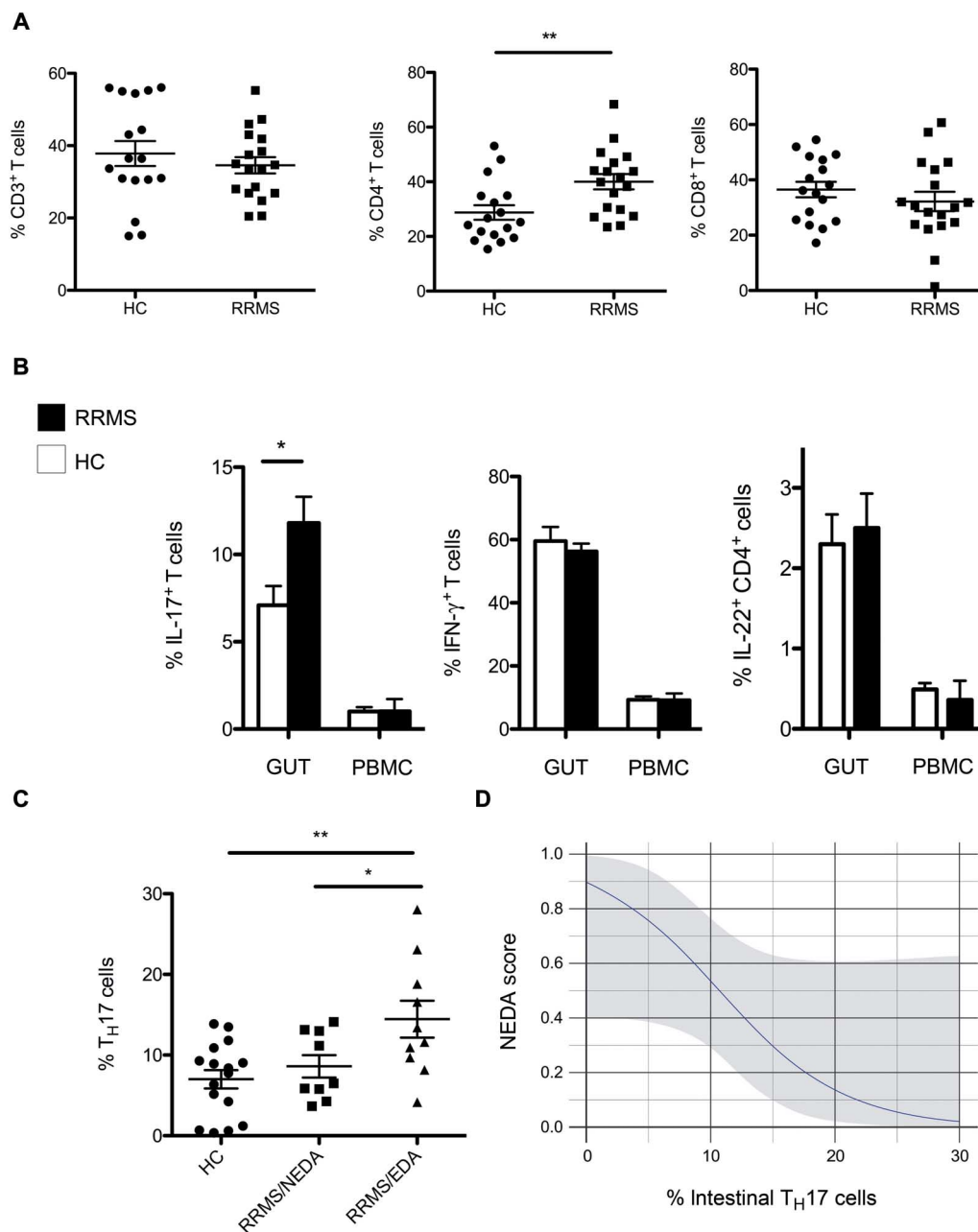
### Gut mucosal immunity in MS

Our multiparametric FACS analysis revealed a statistically significant increase in the frequencies of intestinal CD4<sup>+</sup> T cells in RRMS patients compared to those in HCs ( $P < 0.01$ ), whereas the frequency of total CD3<sup>+</sup> T cells and that of CD8<sup>+</sup> T cells were comparable between the two groups (Fig. 2A). Next, we examined the cytokine secretion profile of CD4<sup>+</sup> T<sub>H</sub> cell subsets. Our data showed a statistically significant increase in the frequency of intestinal T<sub>H</sub>17 cells in RRMS patients compared to that of HCs ( $P < 0.01$ ) (Fig. 2B). The higher frequency of T<sub>H</sub>17 cells in RRMS patients was detected in the small intestinal mucosa but not in the peripheral blood, where T<sub>H</sub>17 cells were poorly represented in both RRMS patients and HCs (Fig. 2B). The increase was limited exclusively to the T<sub>H</sub>17 cell subset, and the frequencies of other effector T<sub>H</sub> cell subsets, such as T<sub>H</sub>1 cells and T<sub>H</sub>22 cells, were comparable between the RRMS and HC groups (Fig. 2B).

Because our data revealed a bimodal trend in T<sub>H</sub>17 cell percentages in our RRMS patient cohort, we hypothesized that the T<sub>H</sub>17 cell frequency correlates with disease activity. To test this hypothesis, we divided our cohort of RRMS patients into two subgroups on the basis of the presence of disease activity at a 2-year follow-up, and we found that RRMS/NEDA patients with inactive disease had low percentages of intestinal T<sub>H</sub>17 cells, compared to those of HCs (Fig. 2C). Conversely,

RRMS/EDA patients with active disease showed T<sub>H</sub>17 cell frequencies significantly augmented compared not only to HCs ( $P < 0.01$ ) but also to RRMS/NEDA patients ( $P < 0.05$ ) (Fig. 2C). A one standard deviation increase in the frequencies of T<sub>H</sub>17 in the intestine of RRMS patients was associated with a significant reduction in the predicted probability of being disease-free at 2 years [odds ratio, 0.21; 95% confidence interval (CI), 0.02 to 0.98;  $P = 0.01$ ] (Fig. 2D). We also analyzed the percentages of double-positive IL-17<sup>+</sup>IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup>IL-22<sup>+</sup> and found a statistically significant increase in the percentage of IL-17<sup>+</sup>IL-22<sup>+</sup> T cells in the small intestine of RRMS/EDA patients with active disease compared to those in HCs, whereas the percentages of double-positive IL-17<sup>+</sup>IFN- $\gamma$ <sup>+</sup> were similar between the different groups (fig. S1).

At the time of EGD and intestinal immune profiling, all RRMS patients enrolled in this study were undergoing treatment with drugs that have strong immunomodulatory properties, such as interferon- $\beta$  (IFN- $\beta$ ), GA, and fingolimod (Table 2). These immunomodulatory drugs can differentially modulate gut mucosal immunity. For example, recent studies demonstrated that type 1 interferons (T1IFNs), such as IFN- $\beta$  play a beneficial anti-inflammatory role in the intestinal mucosa (18, 19). Hence, we determined whether administration of these immunomodulatory therapies specifically affects intestinal T<sub>H</sub>17 cell differentiation in our RRMS patients. We stratified our cohort of RRMS patients in three



**Fig. 2. Intestinal T cell subsets in RRMS patients and HCs.** (A) Relative percentages of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells out of total lymphocytes isolated from the small intestinal mucosa of individuals with RRMS and of HCs. (B) Frequencies of different T<sub>H</sub> cell subsets (T<sub>H</sub>17, T<sub>H</sub>1, and T<sub>H</sub>22) in the intestinal mucosa (GUT) and PBMCs of HCs and RRMS patients. Blood samples were collected from the same individuals at the time of intestinal tissue sample collection. Data are means ± SEM. (C) RRMS patients were divided into two cohorts on the basis of disease activity. RRMS patients with evidence of disease activity at 2-year follow-up were classified as EDA, whereas patients with no disease activity were classified as NEDA. Percentages of intestinal T<sub>H</sub>17 cells out of total CD4<sup>+</sup> T cells in HCs and RRMS/NEDA and RRMS/EDA patients are shown. (A to C) **\*\*P** < 0.01 and **\*P** < 0.05 by unpaired *t* test. (D) Logistic regression analysis showing correlation between T<sub>H</sub>17 cell percentages in the gut mucosa and predicted probability of showing disease activity at 2-year follow-up (NEDA score: RRMS/NEDA, 1; RRMS/EDA, 0). Odds ratio, 0.96; 95% CI, 0.02 to 0.98; *P* = 0.01.

subgroups according to the type of treatment (IFN-β, GA, or fingolimod). Our analysis did not reveal statistically significant differences in the percentages of intestinal immune cell subsets between the three subgroups. However, we observed that the increase of intestinal T<sub>H</sub>17 cells was particularly evident in the GA-treated subgroup in comparison with HCs (fig. S2). We also conducted a correlative statistical analysis linking intestinal T<sub>H</sub>17 cell percentage to age, EDSS score (disability score), and disease duration but found no statistically significant association between these parameters (fig. S3).

### Correlation between microbiota modifications, increased T<sub>H</sub>17 cell frequency and disease activity in RRMS

Next, we wanted to highlight the mechanism responsible for increased T<sub>H</sub>17 cell frequency in the small intestine of RRMS patients. Commensal microbiota play a crucial role in modulating intestinal T<sub>H</sub>17 cell expansion, and in EAE (the animal model of MS), gut colonization with SFB promotes brain autoimmunity by increasing intestinal T<sub>H</sub>17 cell differentiation (10). Hence, we determined whether enhanced T<sub>H</sub>17 cell

differentiation in the small intestine of RRMS patients is linked with alterations in microbiota composition. T<sub>H</sub>17 cell-inducing microbes, such as SFB, are tightly associated with the epithelium and are scarce in the intestinal lumen and in stool samples (20, 21). Therefore, we analyzed the microbiota isolated from the small intestinal mucosa rather than from luminal contents (stool samples) to characterize bacterial strains that directly regulate T<sub>H</sub>17 cell differentiation (8, 9, 22, 23). Our taxonomic, functional, and diversity microbiome profiling did not show statistically significant differences in  $\alpha$ -diversity either between HC and RRMS patients or between RRMS/EDA patients with disease activity and RRMS/NEDA patients with inactive disease (Fig. 3A). At the phylum level, again, we did not detect differences between HC and RRMS patients; but we did find a statistically significant higher abundance of Firmicutes and reduction of Bacteroidetes species in RRMS/EDA with disease activity compared to RRMS/NEDA patients (Fig. 3B). Genus-level analysis revealed that RRMS/EDA patients had higher representation of *Streptococcus* strains (Fig. 3C).

A further characterization of the *Streptococcus* strains by amplification and sequencing revealed that they were mostly *Streptococcus mitis* and *Streptococcus oralis*, two bacterial strains that normally reside in the oral cavity (fig. S4). Notably, previous studies demonstrated that *S. mitis* is capable of inducing T<sub>H</sub>17 cell differentiation in humans (24). In addition, we found that RRMS/EDA patients had a statistically significant reduction in the relative abundance of *Prevotella*, commensal bacterial strains that have beneficial anti-inflammatory properties (25), in comparison with RRMS/NEDA patients (Fig. 3C). In RRMS/NEDA patients with inactive disease, the relative abundance of the anti-inflammatory *Prevotella* strains was higher in comparison not only with RRMS/EDA patients but also with HCs. A correlative statistical analysis confirmed a negative linear relationship between the frequency of intestinal T<sub>H</sub>17 cells and the relative abundance of *Prevotella* strains in all human small intestinal samples ( $r = -0.7$ ,  $P \leq 0.01$ ), whereas no direct correlation between percentages of intestinal T<sub>H</sub>17 cells and relative abundance of *Streptococcus* strains was found (Fig. 3C). As for intestinal T<sub>H</sub>17 cell percentages, we also performed a correlative statistical analysis between the relative abundance of *Prevotella* and *Streptococcus* strains and age, EDSS score (disability score), and disease duration but found no statistically significant association among these parameters (fig. S5).

Treatment with immunomodulatory drugs in our RRMS cohort could also affect gut microbiota composition. In line with this idea, a recent study showed that treatment with GA modifies the microbiota composition in the stools of RRMS patients (12). Hence, we determined whether administration of different immunomodulatory drugs (IFN- $\beta$  or GA) specifically modifies gut mucosa-associated microbiota in our RRMS patient cohort. Although our sample size was relatively small, our analysis revealed a statistically significant increase in the relative abundance of *Prevotella* strains in the IFN- $\beta$ -treated subgroup compared with RRMS patients treated with GA (we could not perform a comparative analysis with fingolimod-treated patients because of the too-limited number of samples) (fig. S6). We detected no statistically significant difference in the relative abundance of *Streptococcus* strains (fig. S6) and no other differences at the family and genus levels between subgroups of RRMS patients stratified according to immunomodulatory treatment.

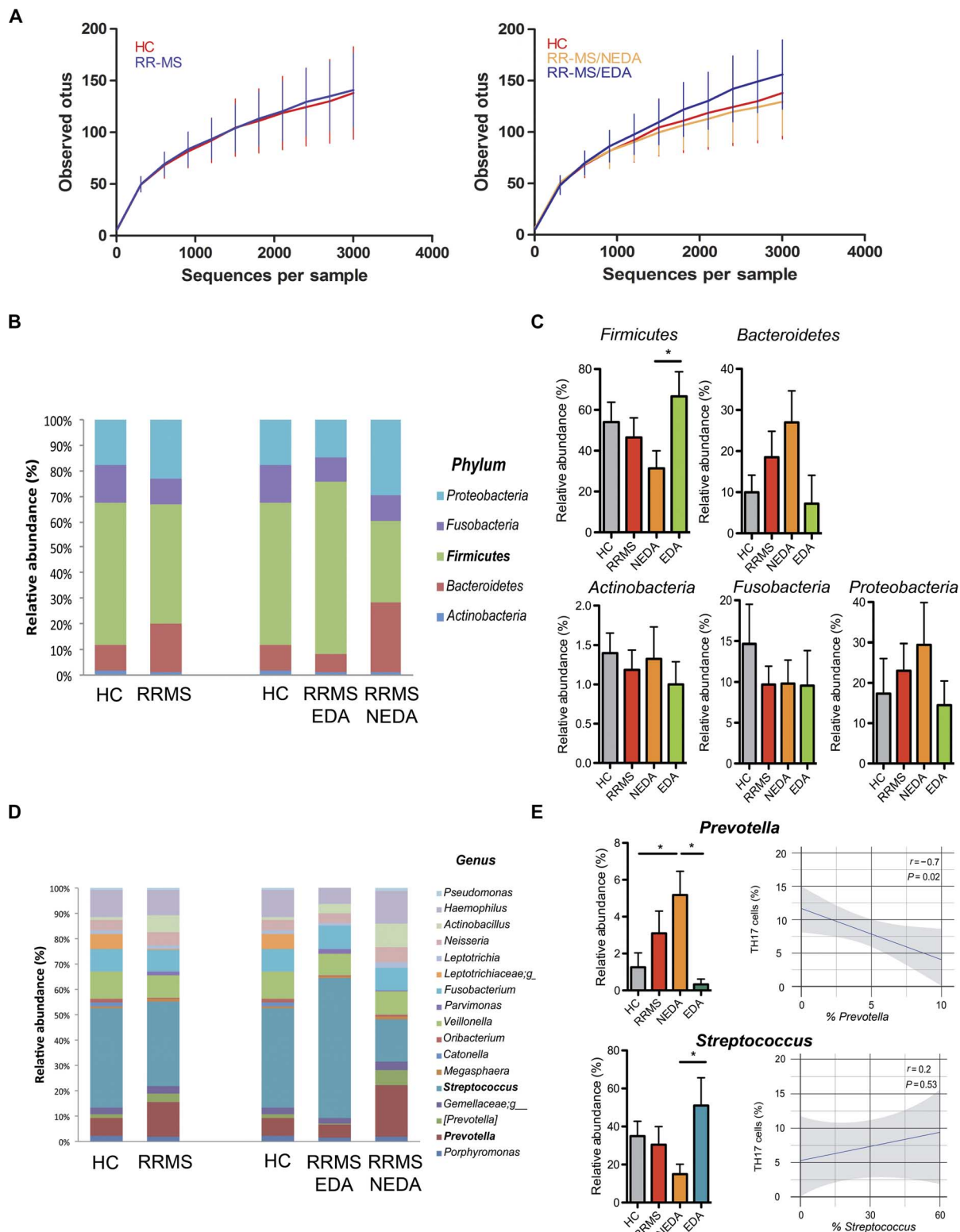
## DISCUSSION

Modifications of the intestinal environment induced by either diet or microbiota composition have been linked to the pathogenesis of MS (12–16), but the mechanisms underlying this association are still largely

unknown. In mice, a proinflammatory intestinal environment promotes brain autoimmunity by enhancing T<sub>H</sub>17 cell expansion and self-reactive T cell pathogenicity (4, 10). In these models, myelin-reactive T cells are driven toward a pathogenic T<sub>H</sub>17 phenotype in the small intestinal mucosa (11) and then migrate to the CNS where they mediate autoimmune damage of the myelin sheath. Our data confirm that brain autoimmunity is also associated in humans with intestinal expansion of T<sub>H</sub>17 cells. Although further investigation is needed to formally demonstrate that the T<sub>H</sub>17 cells residing in the small intestine of RRMS patients are myelin-specific, the selective T<sub>H</sub>17 cell expansion in RRMS/EDA patients with disease activity suggests that, similar to what is observed in mice with EAE, the gut environment enhances pathogenicity of self-reactive T cells by driving them toward a T<sub>H</sub>17 cell phenotype. In anti-MOG (myelin oligodendrocyte glycoprotein) T cell receptor transgenic mice that develop spontaneous EAE, the expansion of myelin reactive T<sub>H</sub>17 cells was detected in T cells residing in the intestinal wall (lamina propria T cells and in Peyer's patches) but not in peripheral organs, such as spleen and lymph nodes (4). Our observation that increased T<sub>H</sub>17 cell frequency is present in the small intestinal mucosa but not in peripheral blood of RRMS patients further underlies the similarities between pathogenic T<sub>H</sub>17 cell differentiation in humans and in animal models of MS.

The acquisition of an IL-22<sup>+</sup> phenotype by T<sub>H</sub>17 cells increased their effector function and proinflammatory properties (26, 27), and a recent study demonstrated that the presence of IL-22<sup>+</sup> T<sub>H</sub>17 cells correlates with high disease activity in rheumatoid arthritis patients (28). Our data show that RRMS/EDA patients with active disease also have an increased percentage of double-positive IL-22<sup>+</sup> T<sub>H</sub>17 cells. These results further suggest that increased differentiation and acquisition of a strong effector phenotype by T<sub>H</sub>17 cells in the small intestine correlate with disease activity in RRMS patients.

T<sub>H</sub>17 cell differentiation in the small intestine is regulated by the whole microbial community composition and also by some specific bacteria (29, 30). In rodents, a single bacterial type, the SFB, is sufficient to drive the accumulation of T<sub>H</sub>17 cells in the small intestinal lamina propria (8, 9). Studies in transgenic models of autoimmune arthritis (31) and EAE (10) demonstrated that SFB play a key role in the activation and expansion of self-reactive T<sub>H</sub>17 cells in the small intestine. Similarly, the T<sub>H</sub>17 cell expansion that we found in RRMS patients could be ascribed to overrepresentation of T<sub>H</sub>17-inducing commensal microbes that are present in the human small intestine. SFB have been found in multiple vertebrate species (32) and also in the human intestine (33). In a recent study, a functional analog of SFB, *Bifidobacterium adolescentis*, was isolated from the human intestine (21). In our human small intestinal samples, we did not detect SFB, and we found a limited amount of *Bifidobacterium* only in one intestinal tissue sample isolated from an RRMS patient. Hence, we exclude the possibility that increased T<sub>H</sub>17 cell frequency in RRMS was linked to excessive SFB or *Bifidobacterium* strain representation. However, T<sub>H</sub>17-promoting symbionts can be different in human populations residing in different geographical areas because of the effect of the environment on microbiota composition. In our human cohort, increased T<sub>H</sub>17 cell frequency in RRMS/EDA patients was associated with higher relative abundance of *Streptococcus* strains, in particular *S. mitis* and *S. oralis*. Previous studies showed that *Streptococcus* strains belonging to the *S. mitis* group (such as *Streptococcus pneumoniae*, *S. mitis*, and *S. oralis*) are capable of inducing T<sub>H</sub>17 cell differentiation in humans (24). *S. mitis* and *S. oralis*, the two *Streptococcus* strains that we found selectively increased in the small intestine of RRMS/EDA patients, are normally resident in the oral cavity, but both can cause local or invasive opportunistic infections (34). On the basis of



**Fig. 3. Differences of gut mucosa-associated microbiota composition between RRMS patients and HCs.** (A)  $\alpha$ -Diversity of gut microbiota profiles assessed by 16S amplicon sequencing of DNA purified from human small intestinal tissue of HCs and RRMS patients with active (RRMS/EDA) and nonactive (RRMS/NEDA) disease. (B) Average phylum-level composition of microbiota isolated from small intestinal samples of HC and total RRMS, RRMS/NEDA, and RRMS/EDA patients. (C) Means  $\pm$  SEM of the relative abundance of different phyla. \* $P < 0.05$  by unpaired  $t$  test. (D) Average genus-level composition in four cohorts of individuals. (E) Left: Means  $\pm$  SEM of the relative abundance of *Prevotella* and *Streptococcus* strains. \* $P < 0.05$  by unpaired  $t$  test. Right: Regression linear plot in all individuals (RRMS + HC) showing inverse relation between percentages of intestinal TH17 cells and relative abundance of *Prevotella* strains.

our findings, we speculate that, under certain conditions, or because of still unknown virulence factors, these *Streptococcus* strains can colonize the small intestine and favor T<sub>H</sub>17 cell differentiation in the human gut mucosa. Notably, both *Streptococcus* strains can also grow under low-pH conditions, which are a prerequisite for colonizing the duodenum (35).

Enhanced T<sub>H</sub>17 cell differentiation in the intestine can be induced not only by overrepresentation of T<sub>H</sub>17-inducing bacteria but also by decreased abundance of beneficial microbial strains that reduce T<sub>H</sub>17 cell polarization. For example, *Prevotella* is capable of producing the anti-inflammatory metabolite propionate (36) that limits intestinal T<sub>H</sub>17 cell expansion in mice (37). So far, three of five studies that analyzed microbiome composition in RRMS patients from different geographical areas (Japan and United States) found a reduction in the relative abundance of *Prevotella* strains in MS patients compared to HC subjects (13, 14, 16). We confirmed these findings in our Italian cohort of RRMS patients and also found that the *Prevotella* decrease is selectively present in RRMS patients with active disease. In our human cohort, which includes not only RRMS patients but also HC subjects, we demonstrated a statistically significant negative correlation between the relative abundance of *Prevotella* and the T<sub>H</sub>17 cell frequency in the human small intestine. These data indicate that reduced *Prevotella* representation in RRMS patients is linked to T<sub>H</sub>17 cell expansion and disease activity. In RRMS/NEDA patients with inactive disease, the *Prevotella* strains were more abundant in comparison not only with RRMS/EDA patients with active disease but also with the HC group, as if overrepresentation of this beneficial anti-inflammatory strain represents a protective mechanism to counterbalance the effect of T<sub>H</sub>17 cell-inducing microbes and MS pathogenesis in these patients.

The current standard of practice in neurology is to treat RRMS patients with immunomodulatory drugs (IFN- $\beta$ , GA, or fingolimod). Previous studies indicate that these immunomodulatory drugs can modulate gut mucosal immunity and microbiota composition. For example, IFN- $\beta$  was reported to play a beneficial anti-inflammatory role in the murine intestine (18, 19), whereas GA can influence the microbiota profile in treated MS patients (12). Here, we demonstrate that the percentage of intestinal T<sub>H</sub>17 cells was particularly high in the GA-treated group that contained a high number of RRMS/EDA patients with active disease (5 out of 9). This finding further supports the notion that the percentage of intestinal T<sub>H</sub>17 cells correlates with disease activity in RRMS patients. We also analyzed the microbiota composition in RRMS patients stratified according to immunomodulatory therapy and found a higher increase in the relative abundance of *Prevotella* strains in the IFN- $\beta$ -treated RRMS patients compared to the GA-treated cohort. Although our sample size was relatively small and we cannot make definitive conclusions on the capacity of IFN- $\beta$  treatment to augment *Prevotella* strains in the human intestine, our preliminary data do suggest that the beneficial effect of IFN- $\beta$  in RRMS may be related to modification of microbiota composition. Although a previous report showed several alterations in the microbiota composition in stool samples of GA-treated patients compared to untreated RRMS patients (12), our analysis on mucosa-associated microbiota did not reveal other significant differences in this subgroup. However, it must be noted that in our study, we compared GA-treated individuals only with RRMS patients treated with other immunomodulatory drugs (IFN- $\beta$ ) and not with untreated RRMS patients.

Our results point to a role of gut microbiota in regulating T<sub>H</sub>17 cell differentiation in RRMS patients; however, we cannot exclude the possibility that other environmental factors, such as diet composition, are also involved either directly or indirectly through microbiota modulation. High concentration of sodium chloride drives human and murine T cells

toward a biased T<sub>H</sub>17 cell phenotype (38, 39), thus leading to the hypothesis that T<sub>H</sub>17 cell differentiation in the human intestine is regulated by dietary consumption of salt. We administered a dietary questionnaire to our RRMS patients and HC group; although we did not detect relevant differences in the salt intake between patients and controls, we cannot rule out the possibility that the quantity of dietary salt influences T<sub>H</sub>17 cell differentiation in RRMS patients.

Although modifications of the dietary habits have been held responsible for the marked spike in incidence of autoimmune diseases, the so-called “autoimmune epidemic” that has occurred in the last half-century (40, 41), it is yet to be clarified how diet modulates autoimmune diseases such as MS (42). Dietary factors can affect brain autoimmunity by favoring T<sub>H</sub>17 cell differentiation in the intestine acting directly on myelin-reactive T cells (38, 39) or through microbiota modifications (4). Our results show that increased T<sub>H</sub>17 cell frequency and microbiota modifications are not present in all RRMS patients but only in those patients with active disease. This strongly suggests that T<sub>H</sub>17 cell expansion and high-*Streptococcus*/low-*Prevotella* strain representation in the small intestine are associated with recurrence of myelin-reactive T cell activation and RRMS pathogenesis. Beneficial *Prevotella* strains are enriched in the intestine of children fed with a high-fiber/low-fat diet (25), thus indicating that modifications of the dietary habits in RRMS patients could promote a beneficial *Prevotella*-enriched gut microbiota that reduces T<sub>H</sub>17 cell differentiation and disease activity. Our data provide the first evidence that alterations of gut mucosal immunity induced by specific microbiota composition are associated with brain autoimmune disease in humans and could pave the way to new therapeutic strategies in RRMS aimed at regulating intestinal T<sub>H</sub>17 cell expansion through dietary approaches and/or probiotic administration.

## MATERIALS AND METHODS

### Study population

RRMS patients were recruited from the Center for Diagnosis and Treatment of Multiple Sclerosis of the San Raffaele Hospital (IRCCS San Raffaele Scientific Institute, Milan, Italy). RRMS patients and HC subjects (sex- and age-matched with RRMS patients) included in this study were adult male and female individuals who performed EGD for diagnostic purposes (dyspepsia, gastroesophageal reflux, altered bowel habits, etc.). Exclusion criteria for both RRMS patients and HCs were as follows: no antibiotic and corticosteroid treatment in the 3 months before EGD; no history of gastroenteritis, gastric ulcer, irritable bowel disease, celiac disease, inflammatory bowel disease, and gastric and colorectal cancers. None of the RRMS patients had an active clinical relapse at the time of tissue sampling and were all treated with an immunomodulatory drug (IFN- $\beta$ , GA, or fingolimod) but not with immunosuppressive medications, such as axathioprine, mitoxantrone, and methotrexate. All individuals signed a written informed consent, complied with the study procedure, and were aware that they donated intestinal tissue fragments for research purpose. The study was approved by the Institutional Review Board of the IRCCS San Raffaele Scientific Institute (Ethical Committee Board), and all participants signed an informed consent before any data collection or study procedure.

### Intestinal tissue sample collection

All EGDs were performed at the Gastroenterology Unit of the San Raffaele Hospital. Tissue fragments of duodenal mucosa (one to two from each individual) obtained during EGD were collected in 10% RPMI [RPMI 1640 containing 10% fetal bovine serum, penicillin/streptomycin

(100 µg/ml), 2 mM glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 0.05 mM β-mercaptoethanol) and rapidly processed. Mucus and epithelial cells were removed from the tissue by incubation with 1 mM dithiothreitol and 5 mM EDTA in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) for 15 min at 37°C. Tissue samples were washed with HBSS; digested with collagenase A (1 mg/ml) (Roche Diagnostics Ltd.) in HBSS supplemented with calcium, magnesium, and deoxyribonuclease I (5 U/ml) (Roche Diagnostics Ltd.) for 1 hour at 37°C; and mechanically disrupted by gentle pipetting until dissociation was complete. One intestinal tissue sample was collected in RNeasy lysis buffer and immediately frozen at -80°C until DNA extraction for microbiota analysis.

### Multiparametric FACS analysis

Single-cell suspensions isolated from intestinal tissue samples were passed through a 70-µm cell strainer and washed with HBSS. The number of total cells isolated from intestinal tissue samples ranged from  $0.5 \times 10^6$  to  $3.7 \times 10^6$ , with a number of T cells (from  $1 \times 10^5$  to  $11 \times 10^5$ ) that were comparable between RRMS patients ( $5.7 \times 10^5 \pm 0.8 \times 10^5$ ) and HC ( $5.2 \times 10^5 \pm 0.7 \times 10^5$ ). PBMCs were isolated by Ficoll gradient and washed in complete RPMI 10. Intestinal cells and PBMCs were assessed for viability by Fixable Viability Dye eFluor 506 (eBioscience) and stained with APC-Cy7 (allophycocyanin-Cy7)-conjugated anti-human CD3 (clone SP34-2, BD Biosciences), PE (phycoerythrin)/Texas Red-conjugated anti-human CD4 (clone S3.5, Thermo Fisher), and Alexa Fluor 700-conjugated anti-human CD8 (clone OKT8, eBioscience) monoclonal antibodies. To stain for intracellular cytokines, cells were first stained with the monoclonal antibodies against surface molecules, fixed and permeabilized (BD Cytofix/Cytoperm solution kit, BD Biosciences), and then stained with eFluor 450-conjugated anti-human IFN-γ (clone 4S.B3, eBioscience), APC-conjugated anti-human IL-17A (clone eBio64-DEC17, eBioscience), and PE-conjugated anti-human IL-22 (clone 142928, R&D Systems). Data were acquired using a four-laser cell analyzer, LSRFortessa (BD Biosciences), and analyzed by using FACSDiva software (BD Biosciences).

### Microbiome analysis

The human microbiome analysis was performed by 16S amplicon sequencing on the 454-GS Junior platform (Roche). Total DNA was purified from human intestinal tissue biopsies of RRMS patients and HCs using QIAamp DNA Micro Kit (Qiagen), following the manufacturer's instructions. The V3-V5 region of the 16S rRNA gene was amplified starting from 500 ng of extracted DNA using the FastStart High Fidelity PCR System (Roche), barcoded sample-specific primers [16S-F331: ACTCCTACGGGAGGCAGC and 16S-R920: CCGTCAATTCMTT-GAGTTT] under cycling conditions (95°C for 3 min; 45 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and 72°C for 8 min), and then stored at 4°C until usage. Amplicons were loaded on 1% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen). Extracted amplicons were purified with AMPure XP beads (Beckman Coulter) and used for emulsion polymerase chain reaction and ultradeep pyrosequencing, following the 454-GS Junior manufacturer's instruction. Sequences with a high-quality score and a length of >250 bp were used for the taxonomic analysis with QIIME (Quantitative Insights Into Microbial Ecology version 1.9.0) software. The number of observed operational taxonomic units (OTUs) was calculated to determine the α-diversity. For all samples, at least 3000 sequences and a coverage rate of >99% were obtained.

The OTUs were identified using the UCLUST clustering method (43). Taxonomy to each sequence was assigned using the RDP Classifier

Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy, which assigns taxonomies using Naïve Bayes classification (44). The chimeric sequences were identified with ChimeraSlayer (<http://microbiomeutil.sourceforge.net/>) and filtered from the sequences of an OTU file. The identification of *Streptococcus* species was performed, as previously described with minor modifications, by amplification and sequencing of the bacterial *tuf* gene from small intestinal samples (45, 46).

### Statistical analyses

Statistical differences were calculated with PRISM 5.0 software (GraphPad) using unpaired, two-tailed *t* test. Logistic regression models adjusted for age, sex, ongoing MS therapy at the EGD, and previous immunosuppressant use were used to assess the association between gut T<sub>H</sub>17 cell percentages and subsequent disease activity at 2 years. Data were considered statistically significant when *P* values were ≤0.05.

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/7/e1700492/DC1>

fig. S1. Percentages of double-positive IL-17<sup>+</sup>IFN-γ<sup>+</sup> and IL-17<sup>+</sup>IL-22<sup>+</sup> T cells in the intestinal mucosa of HCs and RRMS patients with active disease (RRMS/EDA) or inactive disease (RRMS/NEDA).

fig. S2. Percentages of intestinal T<sub>H</sub>17 cell subsets in RRMS patients stratified according to immunomodulatory treatment.

fig. S3. Correlative statistical analysis between percentages of intestinal T<sub>H</sub>17 cells and age, EDSS score, and disease duration.

fig. S4. Characterization of *Streptococcus* species by amplification and sequencing.

fig. S5. Correlative analysis of relative abundance of *Prevotella* and *Streptococcus* strains in mucosa-associated microbiota and age, EDSS score, and disease duration.

fig. S6. Relative abundance of *Prevotella* and *Streptococcus* strains in HCs and RRMS patients stratified according to immunomodulatory treatment.

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## High frequency of intestinal T<sub>H</sub>17 cells correlates with microbiota alterations and disease activity in multiple sclerosis

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