Faecal mucoprotein MUC2 is decreased in multiple sclerosis and is associated with mucin degrading bacteria

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Summary

Background The gut microbiome is altered in MS and may contribute to disease by disrupting the intestinal barrier. The colonic mucus barrier, which is primarily composed of mucin protein 2 (MUC2), plays a crucial role in providing a barrier between colonic epithelial cells and the microbiome. Disruption of intestinal epithelial and mucus barriers has been reported in inflammatory bowel disease (IBD) and Parkinson's disease (PD) but has not been studied in the context of the microbiome in multiple sclerosis (MS).

Methods We investigated the epithelial tight junction protein zonulin occludins 1 (ZO-1), mucus protein MUC2, inflammatory stool markers (calprotectin), and gut microbiota composition in a cohort of subjects with relapsing and progressive MS.

Findings MUC2 was decreased in stool of subjects with both relapsing and progressive MS. ZO-1 was elevated in the serum of subjects with progressive MS but was not altered in the stool. Inflammatory markers typically elevated in IBD and PD, including calprotectin, were not altered in MS stool, suggesting disease specificity of altered gut physiology in MS. Microbiota with known mucus degrading capacity were elevated in the stool of subjects with MS and negatively correlated with mucus protein levels.

Interpretation Taken together, these findings suggest reduced gut barrier function in MS which is linked to increased mucin degrading bacteria.

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Keywords: Microbiome; Gut barrier; Mucus; Tight junction; MUC2; ZO-1

Introduction

It is now recognised that the gut microbiome plays a role in MS by modulating immunity,[1–5] but little is known about whether the microbiota is associated with altered gut physiology. One aspect of gut function that may be altered in MS involves regulation of gut barrier function. The intestinal mucus layer and epithelium provide an important barrier from the gut microbiota and disruption may contribute to MS. Studies using a direct measure of epithelial barrier integrity, the lactulose:mannitol assay, have reported increased barrier permeability in a portion (20–60%) of subjects with MS.[6,7] Serum analysis of tight junction proteins such as zonulin occludins-1[8,9] (ZO-1) and claudins[10] have demonstrated dysfunctional barrier integrity in MS. These studies suggest that a breakdown of tight junctions leading to a compromised epithelial barrier or a 'leaky' gut may exist in MS. Serum tight junction proteins may originate from any barrier tissue, including skin, endothelium, and gut, making faecal measures of tight junction related proteins more targeted readouts of epithelial integrity in the gut wall. While serum ZO-1 was found to be elevated in MS, it was not elevated in the stool.[11]

The mucus barrier overlays the intestinal epithelium and is an important regulator of gut homoeostasis. The mucus barrier constitutes a single layer in the small intestine and a bi-layer in the colon; mucus is produced by specialised gut wall epithelial cells known as goblet cells.[12] Neural and immune factors contribute to the generation of new goblet cells[13] and to triggering the secretion of mucus by goblet cells.[14] Gut mucus is

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Research in context

Evidence before this study

The gut microbiome is altered in multiple sclerosis (MS) and may contribute to disease by disrupting gut wall homoeostasis. Previous investigations surrounding epithelial barrier integrity have reported elevated tight junction proteins in serum of MS subjects, and in small cohorts, the lactulose:mannitol assay has demonstrated increased barrier permeability in ~20–60% of MS subjects. These studies suggest that a compromised epithelial gut barrier may exist in MS, however, serum tight junction proteins can originate from myriad non-gut barrier tissues, making interpretation difficult. One study has investigated tight junction proteins in stool of MS subjects and did not find altered levels. While microbes with mucus degrading capacity are routinely reported to be elevated in MS, no investigations have been done on mucoprotein levels in MS stool. Together these studies point towards the need for a large-scale investigation into the gut barrier and mucus layer in MS.

Added value of this study

We investigated epithelial and mucus barrier integrity in a cohort of relapsing and progressive MS. By quantification of tight junction, mucus, and inflammatory proteins in the serum and stool, we found evidence of gut epithelial barrier maintenance but a compromised mucus layer in both relapsing and progressive MS. Furthermore, we found that this was correlated with mucin-degrading bacteria, suggesting contribution of the gut microbiota to reduced barrier function.

Taken together, these findings suggest reduced gut barrier function in MS which is linked to increased mucin degrading bacteria.

Implications of all the available evidence

Our study provides a detailed report of epithelial and mucus barriers in MS, a critical area for investigation due to the gut microbiome's strong influence on disease. We provide a basis for future investigations to harness microbes that regulate gut mucus in clinical and translational studies, while also opening an area of investigation into the involvement of gut epithelial dysregulation in MS disease pathogenesis.

critically important to prevent microbial entry into the gut wall and serves as a source of nutrients for numerous species of bacteria, including *Bifidobacterium*, *Bacteroides* and *Akkermansia muciniphila*.[15,16] Given that several studies report elevated mucus degrading bacteria in MS,[5] the microbiota may be linked to reduced barrier function in MS.[15] Faecal levels of mucus structural proteins including MUC2[17] provide a measure of the gut mucus barrier and when coupled with microbiota sequencing technologies can determine the mucus degrading potential of the MS gut microbiome.[18]

We investigated epithelial and mucus barrier integrity in a cohort of relapsing and progressive MS. By quantification of tight junctions, mucus, and inflammatory proteins in the serum and stool we found evidence of gut epithelial barrier maintenance but a compromised mucus layer in both relapsing and progressive MS. Furthermore, we found that this was correlated with mucin-degrading bacteria, suggesting contribution of the gut microbiota to reduced barrier function.

Methods

Subjects and clinical metadata

Subjects for stool protein analysis were recruited from the Comprehensive Longitudinal Investigation of Multiple Sclerosis (CLIMB) study at Brigham and Women's Hospital (BWH). Inclusion criteria was a diagnosis of MS according to the latest McDonald criteria, with secondary and primary progressive MS being grouped together. Exclusion criteria were gastrointestinal surgical history, gastrointestinal disease, antibiotics within 3 months prior to sample, or current pregnancy. Healthy subjects from the PhenoGenetic Project at the BWH Genomics Center were used as controls and procedures for these samples were identical to those from subjects with MS. Upon enrolment, subjects were asked their sex (male or female) but not gender (e.g., man, woman, nonbinary). Of the n = 118 subjects with MS in our study, no subjects had a clinical relapse within ±1 month of sample collection. STROBE cross sectional reporting guidelines were used.[19] Complete subject demographics are in Table 1.

Ethics

All protocols were approved by the BWH institutional review board (IRB Protocol #: 2017P001169) and informed consent was obtained from every study subject.

Stool protein analysis

Enzyme linked immunosorbent assays (ELISA) were performed using 200 mg of stool diluted in 1 mL extraction buffer composed of phosphate buffered saline (PBS) with 0.1% v:v Tween-20 (Burlington, MA). Samples were homogenised on a Qiagen Powerlyzer 24 Homogeniser (Qiagen, Hilden, Germany) for 45 s. Samples were then separated on a RT centrifuge at 10,000×g for 10 min. Supernatants were used immediately for ELISAs to MUC2 (ab282871; Abcam, Waltham, MA), lactoferrin (ab200015; Abcam), neopterin

Characteristic	HC	RRMS	ProgMS	ANOVA or student's t-test result		
				HC vs. RRMS	HC vs. ProgMS	RRMS vs. ProgMS
Subjects, n	18	83	30			
Female subjects, n (%)	12 (66)	57 (69)	25 (83)			
Age, yr ± SD	37.1 ± 11.3	50.9 ± 11.0	56.0 ± 7.7	p < 0.0001	p < 0.0001	p = 0.058
BMI ± SD	25.5 ± 4.8	26.6 ± 5.8	27.8 ± 7.6	p = 0.79	p = 0.50	p = 0.74
Disease duration, yr ± SD	–	14.8 ± 8.3	17.2 ± 7.6	–	–	p = 0.16
EDSS, score ± SD	–	1.9 ± 0.9	5.4 ± 1.8	–	–	p < 0.0001
Treatment, n						
Anti-CD20	–	8	2			
Dimethyl fumarate	–	10	4			
Fingolimod	–	24	2			
Glatiramer acetate	–	8	2			
Interferon-B	–	7	4			
Natalizumab	–	7	2			
Mycophenolic acid	–	1	4			
Teriflunomide	–	2	2			
Untreated	18	16	8			

Table 1: **Study subject demographics.**

(abx252805; Abbexa, Sugar Land, TX), zonulin (KR5600; Immundiagnostik, Inc., Manchester, NH), calprotectin (EH62RB; Invitrogen, Waltham, MA), or S100A12 (RD191221200R; BioVendor R&D, Asheville, NC). All ELISAs were run on stool diluted 1:5 (w:v) for protein extraction and subsequently 1:2 (v:v) prior to ELISA assay procedures. Optical density was measured at 450 nm and analyte concentrations were calculated by interpolation of standard curve values based on kit manufactures guidelines. Final concentrations were adjusted for dilution factors prior to plotting. All ELISAs were performed following manufactures instructions.

Microbiome processing and analysis

Using 50–200 mg stool, DNA was extracted with DNAeasy PowerLyzer Microbiome DNA extraction kits (12255-50; Qiagen, Hilden, Germany). The V4-5 region of the 16S rRNA gene was amplified per the Earth Microbiome Project's updated barcoded primers.[20] Paired-end sequencing was performed at the Harvard Biopolymers facility on a MiSeq and all samples were sequenced on the same run. Sequencing analysis was performed in QIIME2 (v. 2023.9). Analysis pipeline was similar to previously performed,[1] briefly, data was denoised and filtered in DADA2 and paired samples were merged. The table was filtered at samples with less than 1000 reads, appearing in only 1 sample, or from known contaminates (e.g., Chloroplast, mitochondria, and bacteria not assigned at the phylum level). Taxonomic assignment was performed using a primer specific trained SILVA (November 2021 release). Prior to statistical analysis, species with less than 10% prevalence in any disease category were removed. Spearman's correlation was used to view differences in composition across samples, including Expanded Disability Status Score (EDSS), disease category, and MUC2 concentration.

Glycosyl hydrolase

Analysis of glycosyl hydrolases (GH) was performed using the carbohydrate-active enzyme database (CAZy[21]). Non-classified GH related enzymes were filtered leaving 853,046 total enzymes for comparative analysis. The GH data table was exported and further filtered for microbial taxa with exact species matches to taxa that were derived via BLAST (NIH) from our 16S dataset. GH enzyme identity, number, and taxa association were plotted in Prism 10. Full GH database that we used is available at https://www.cazy.org/Glycoside-Hydrolases.html.

Statistics

Statistical analyses were performed in Prism 10 (Graphpad, v10.4) or R (v4.3.3) with the RStudio suite (v2023.12.1+402). Data are presented as mean ± standard deviation (SD). ELISA comparisons were done with either a Student's t-test without a Welch's correction or via one-way ANOVA with a Tukey's multiple comparison's post-hoc test. Analyses using Student's t-tests and one-way ANOVA were assessed for normality via histogram plotting and for homogeneity via Levene's test or Bartlett's test, respectively. These assumptions were satisfied for all analyses except for the untreated subject MUC2 quantification in Fig. 1d, which had a significant result on Bartlett's test (Bartlett's statistic = 8.03, p = 0.018). This was corrected

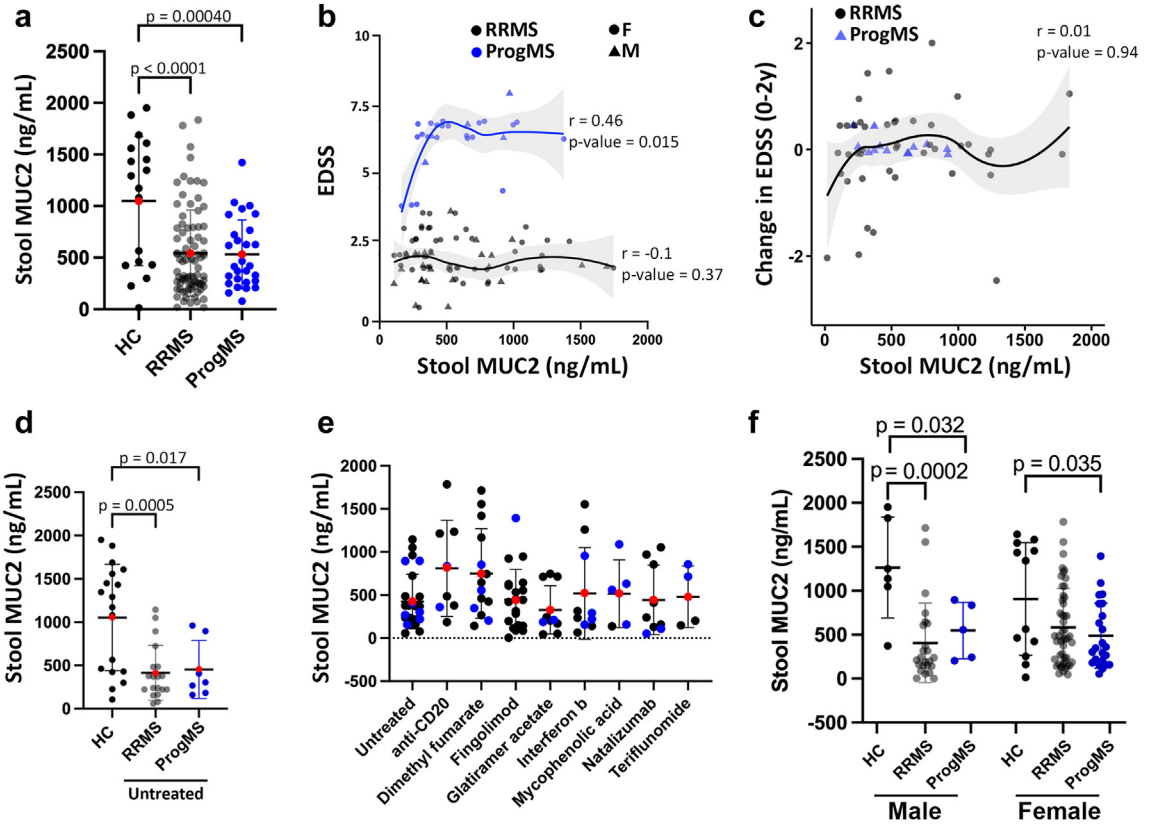

Fig. 1: **Intestinal mucus proteins are decreased in subjects with MS and correlated with EDSS**. MUC2 quantities were decreased in relapsing and progressive MS compared to HC (a). Stool MUC2 was not significantly correlated with EDSS in subjects with RRMS (b; r = −0.10, p = 0.37), but was correlated with EDSS in subjects with ProgMS (b; r = 0.46, p = 0.015). MUC2 was not correlated with a change in EDSS across 0–2 y (c; r = 0.010, p = 0.94). In untreated subjects, stool MUC2 was decreased in RRMS and ProgMS compared to HC (d). MUC2 was similar across DMT types (e), and sex (f). n = 18 HC, 85 RRMS, and 33 ProgMS for all plots except for (d) where n = 18 HC, 19 RRMS, and 7 ProgMS. Analyses were done via One-way ANOVA with a Tukey's post-hoc test (a, d, e), a 2-way ANOVA with Sidak's multiple comparisons post-hoc test (f), or a Spearman's correlation with LOESS smoothing (b–d). Panels b–d have linear regressions plotted with dotted lines showing 95% confidence intervals.

for by analysing via a Kruskal–Wallis non-parametric test. Sample size was chosen based on a pilot experiment in which stool MUC2 was quantified in n = 5 HC, 10 RRMS, and 10 subjects with ProgMS. Based on mean differences from this experiment and subsequent power analysis, we concluded that a minimum of n = 15 subjects per group was required. After a preliminary analysis with ~ n = 25 per group, we decided to increase the cohort of subjects with RRMS to account for variance in disease course of these subjects in our centre. Correlations were performed in R using default functions for Spearman's or Pearson's correlations. For data that considered EDSS in the model, Spearman's correlations were chosen due to the ordinal nature of EDSS. Spearman's correlations had a line fit via the default R function for the non-parametric, locally estimated scatterplot smoothing (LOESS) regression model, including 95% confidence intervals, with smoothing set at alpha = 0.7, and a window size = 1.2. Pearson's correlation was performed for serum vs. stool ZO-1 analysis, with an ordinary least square linear regression line and 95% confidence interval bands. Due to our HC subjects being younger than subjects with RRMS and ProgMS, we corrected MUC2, ZO-1, calprotectin, lactoferrin, neopterin, and S100A12 quantities for age using a simple linear regression model that was fit using the default R function lm() to generate residuals.

Bioinformatics

Microbiota analyses presented in Figs. 2 and 3 were done on a subset of subjects previously sequenced in our centre.[1,22] Briefly, the V4-5 region of the 16S rRNA gene was amplified per the updated Earth Microbiome Project's barcoded primers[20] and after library preparation and sequencing, microbiota were analysed with Qiime2 (version 2023.5) before running the PICRUSt2

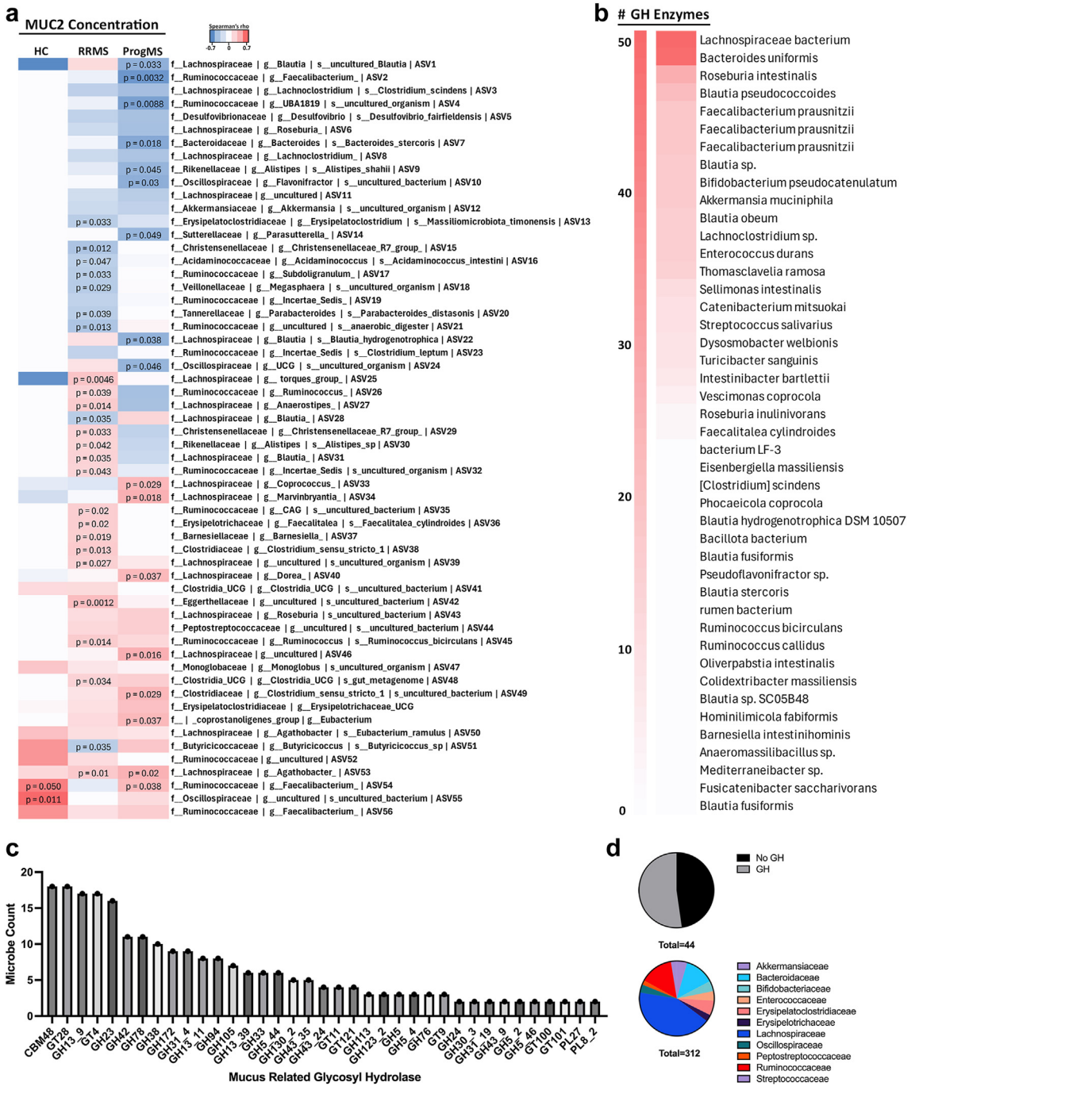

***Fig. 2:* Gut microbiota are correlated with MUC2 levels in MS**. Heatmap showing Spearman's rho values demonstrating microbial taxa correlated with MUC2 quantities in all subjects, RRMS, or ProgMS (a). Number of glycosyl hydrolases (GH) enzyme genes found in bacterial species from all subject's stool samples (b). Microbiota counts for each mucus related GH identified (c). Distribution of GH enzyme presence in n = 44 measured bacterial species that were negatively correlated with MUC2, and total number of GH enzymes in all microbes correlated with MUC2 (d). Data are individual microbial counts (c).

workflow (version 2.6.0).[23] After generating abundance files for KO and ECs in PICRUSt2, plotting and production of differential abundance tables were obtained through LinDA()[24] via the R package ggpicrust2().[25] Spearman's correlations were performed in R and Spearman's r and unadjusted p-values are reported.

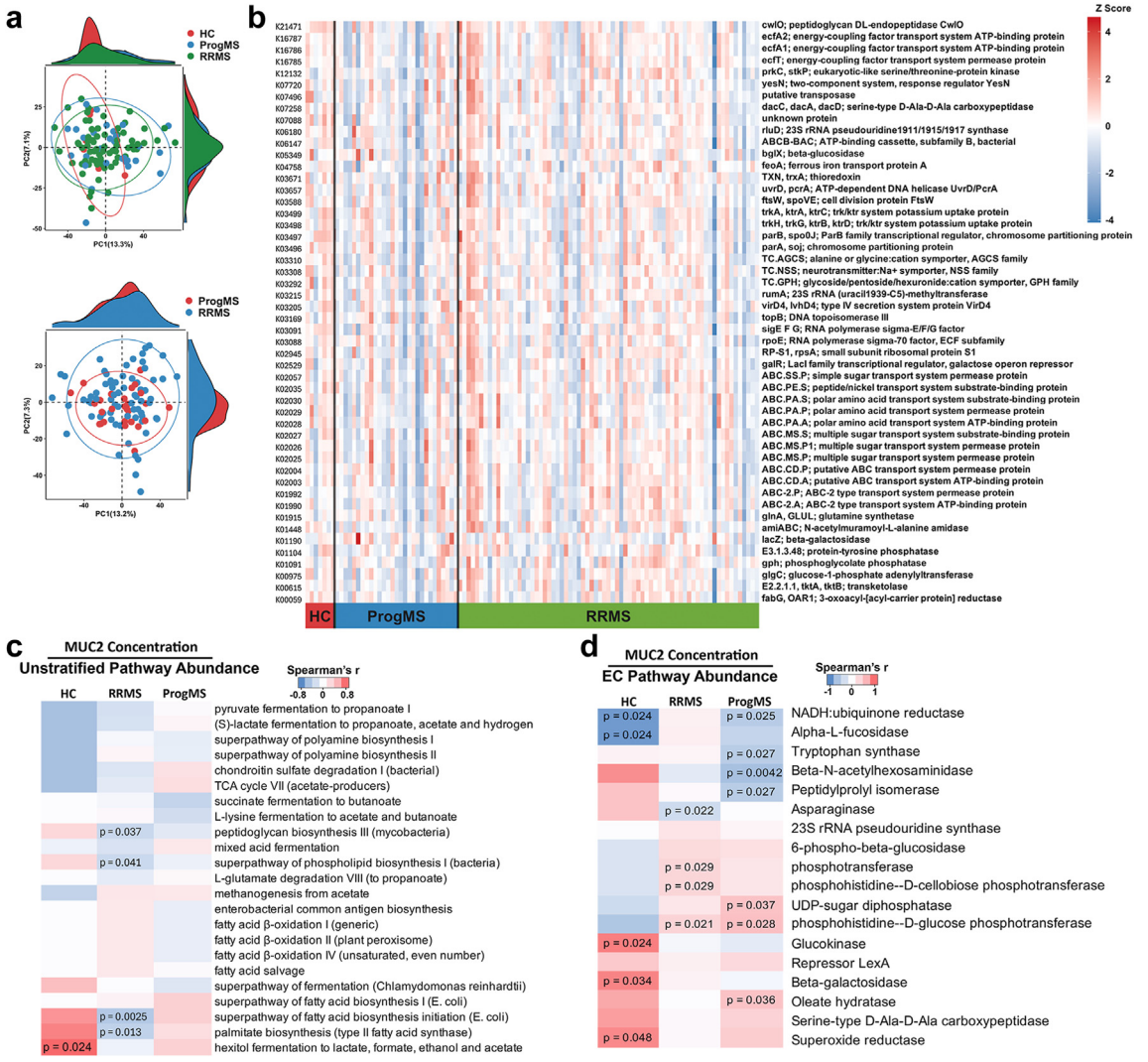

***Fig. 3*: Predicted metabolites altered in MS microbiome**. Microbial community genomes were used to predict microbial metabolite profiles with PiCrust2. PCA plots show distributions of HC, RRMS, and ProgMS samples (a). Heatmap showing the top 50 most altered LinDA results across disease status (b). Correlation and heatmap of MUC2 concentrations against unstratified Kegg ontology pathways by disease type (c). Correlation and heatmap of MUC2 concentrations against Kegg EC enzymatic pathways by disease type (d).

Role of funders

Funding sources were not involved in the study design, data acquisition, analysis or interpretation, writing, or submission of the manuscript.

Results

Mucus protein was decreased in stool from subjects with MS

To investigate colonic mucus levels in MS, we analysed the primary colonic muco-protein, MUC2. We found decreased levels of MUC2 in stool from relapsing ($p < 0.0001$, 95% CI = 237.1–774.7) and progressive ($p = 0.00040$, 95% CI = 203.7–828.4) subjects with MS compared to healthy controls (HC; Fig. 1a). In subjects with RRMS, stool MUC2 quantity correlation with EDSS showed a slightly negative correlation ($r = –0.1$, $p = 0.37$, 95% CI = –0.34 to 0.10), which did not reach significance on a Spearman's correlation, however, in subjects with ProgMS, MUC2 was positively correlated with EDSS ($r = 0.46$, $p = 0.015$, 95% CI = 0.087–0.72; Fig. 1b). In a subset of subjects with 2-year clinical follow-up, we observed a relatively flat Spearman's correlation between 2-year change in EDSS and stool MUC2 quantities, that did not reach significance ($r = 0.01$, $p = 0.94$, 95% CI = –0.26 to 0.28; Fig. 1c). Furthermore, when we investigated stool MUC2 levels in subjects not on any disease modifying therapies (DMTs) at the time of

sample, we found a decrease in MUC2 in RRMS ($p = 0.0005$, 95% CI = 187.9–925.7) and in ProgMS ($p = 0.017$, 95% CI = 21.3–1014) relative to HC subjects (Fig. 1d). Delineation of subjects by DMT type showed similar levels of MUC2 across all treatments ([$F(8,97) = 1.64$], $p = 0.12$; Fig. 1e). We next investigated whether MUC2 levels varied by sex. We found that MUC2 quantities were similar between both sexes ([$F(2,113) = 0.49$], $p = 0.48$), and there was not a statistical interaction between sex and disease status based on 2-way ANOVA ([$F(2,113) = 2.31$], $p = 0.10$; Fig. 1f).

Mucus degrading microbes elevated in MS

We next correlated microbiota levels with stool MUC2 and identified microbiota that are known to degrade mucus. We observed 8 bacterial taxa negatively correlated with MUC2 only in RRMS, pointing towards their potential to regulate mucus levels (Fig. 2a). Included in these taxa were multiple *Ruminococcaceae* family members, a *Tannerellaceae* and a *Christensenellaceae* (Fig. 2a). In subjects with ProgMS, 9 taxa were negatively correlated with stool MUC2 quantities, including multiple *Lachnospiraceae*, *Oscillospiraceae*, and a *Ruminococcus* (Fig. 2a). In HCs, 2 microbial taxa were significantly correlated, a *Ruminococcaceae* and an *Oscillospiraceae*. A majority of the microbes that were positively associated with MUC2 levels, pointing towards their not being mucus degraders, were from the *Lachnospiraceae* and *Ruminococcaceae* families. We then investigated which mucus related GH enzymes were associated with microbes using the CAZy[21] database and found microbial taxa from our subject's stool samples that contain GH enzyme genes (Fig. 2b). Furthermore, we found CBM48, GT28, GH13_9, GT4, and GH23 enzyme families associated with >15 microbial taxa in our subjects (Fig. 2c). Mucus related GH enzymes were present in n = 24 of the 44 microbial taxa (Fig. 2d) that we found to be negatively correlated with stool MUC2 levels (Fig. 2a). A majority of the n = 312 identified GH enzymes were from *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidaceae*, and *Akkermansiaceae* families (Fig. 2d).

Predicted metabolites altered in MS microbiome

The bioinformatics tool *Picrust2* was used to predict functional abundances based on 16S sequencing data from a subset of our cohort. PCA plots show similar distributions between HC, RRMS, and ProgMS, and in RR vs. Prog MS specifically (Fig. 3a). We then investigated the predicted relative abundance of Kegg ontologies in HC, RRMS, and ProgMS, and plotted a heatmap showing the top 50 most significantly altered pathways based on a LinDA correlation analysis (Fig. 3b). When correlating unstratified pathways with relevance to lipid, fatty acid, and polyamine synthesis, vs. stool MUC2 concentrations, we found 5 significantly altered pathways, all but 1 of which were in subjects with RRMS (Fig. 3c). While hexitol fermentation to short chain fatty acids was positively correlated with MUC2 in HC subjects, the subjects with RRMS and with lower MUC2 demonstrated decreased predicted palmitate biosynthesis (Fig. 3c). We then investigated enzymatic pathways altered across disease type in relation to MUC2 quantities, and 7 negatively and 9 positively associated with MUC2, including a negative association of tryptophan synthase only in subjects with ProgMS (Fig. 3d).

Tight junction proteins in MS

Investigating serum and faecal levels of the tight junction protein ZO-1 provided us with a measure of epithelial barrier integrity in MS. We found elevated levels of serum ZO-1 in ProgMS vs. subjects with RRMS (Fig. 4a; $t = 2.30$, $p = 0.027$), however, when we correlated serum ZO-1 protein quantities with EDSS, the association did not reach significance on Spearman's correlation for either RRMS ($r = 0.10$, $p = 0.62$, 95% CI = –0.35 to 0.51) or ProgMS ($r = –0.19$, $p = 0.48$, 95% CI = –0.63 to 0.38; Fig. 4b). Quantities of ZO-1 in serum were positively correlated with levels in stool, however, this did not reach significance on Pearson's correlation (Fig. 4c; $r = 0.20$, 95% CI = –0.11 to 0.48). When we investigated stool quantities of ZO-1, we found similar levels in relapsing and progressive MS vs. HC (Fig. 4d; [$F(2,66) = 0.37$], $p = 0.69$). When we correlated stool ZO-1 with EDSS, the associations were positive but did not reach significance on Spearman's correlation for RRMS ($r = 0.044$, $p = 0.80$, 95% CI = –0.31 to 0.39) or ProgMS ($r = 0.22$, $p = 0.34$, 95% CI = –0.26 to 0.61; Fig. 4e). In subjects not on any DMT, quantities of stool ZO-1 were similar between RRMS and ProgMS vs. HC ([$F(2,26) = 0.032$], $p = 0.97$; Fig. 4f).

Inflammatory markers in MS stool

To determine if the MS gut wall had an altered inflammatory state, we quantified stool inflammatory markers that have been reported altered in other intestinal and neurologic diseases. Levels of calprotectin ([$F(2,65) = 1.70$], $p = 0.19$; Fig. 5a), lactoferrin ([$F(2,62) = 0.28$], $p = 0.76$; Fig. 5b), neopterin ([$F(2,72) = 1.0$], $p = 0.36$; Fig. 5c), and S100A12 ([$F(2,80) = 1.19$], $p = 0.31$; Fig. 5d), were similar in relapsing or progressive MS compared to HC. When we performed Spearman's correlations on these four inflammatory markers with EDSS, associations did not reach significance for calprotectin (RRMS – $r = –0.16$, $p = 0.54$, 95% CI = –0.58 to 0.27; ProgMS – $r = 0.43$, $p = 0.10$, 95% CI = 0.006–0.78; Fig. 5e), lactoferrin (RRMS – $r = –0.20$, $p = 0.27$, 95% CI = –0.53 to 0.14; ProgMS – $r = –0.32$, $p = 0.38$, 95% CI = –0.81 to 0.27; Fig. 5f), neopterin (RRMS – $r = –0.23$, $p = 0.20$, 95% CI = –0.52 to 0.10; ProgMS – $r = 0.25$, $p = 0.37$, 95% CI = –0.23 to 0.64; Fig. 5g), or S100A12 (RRMS – $r = –0.004$, $p = 0.98$, 95% CI = –0.34 to 0.33; ProgMS – $r = –0.05$, $p = 0.85$, 95% CI = –0.52 to 0.4; Fig. 5h).

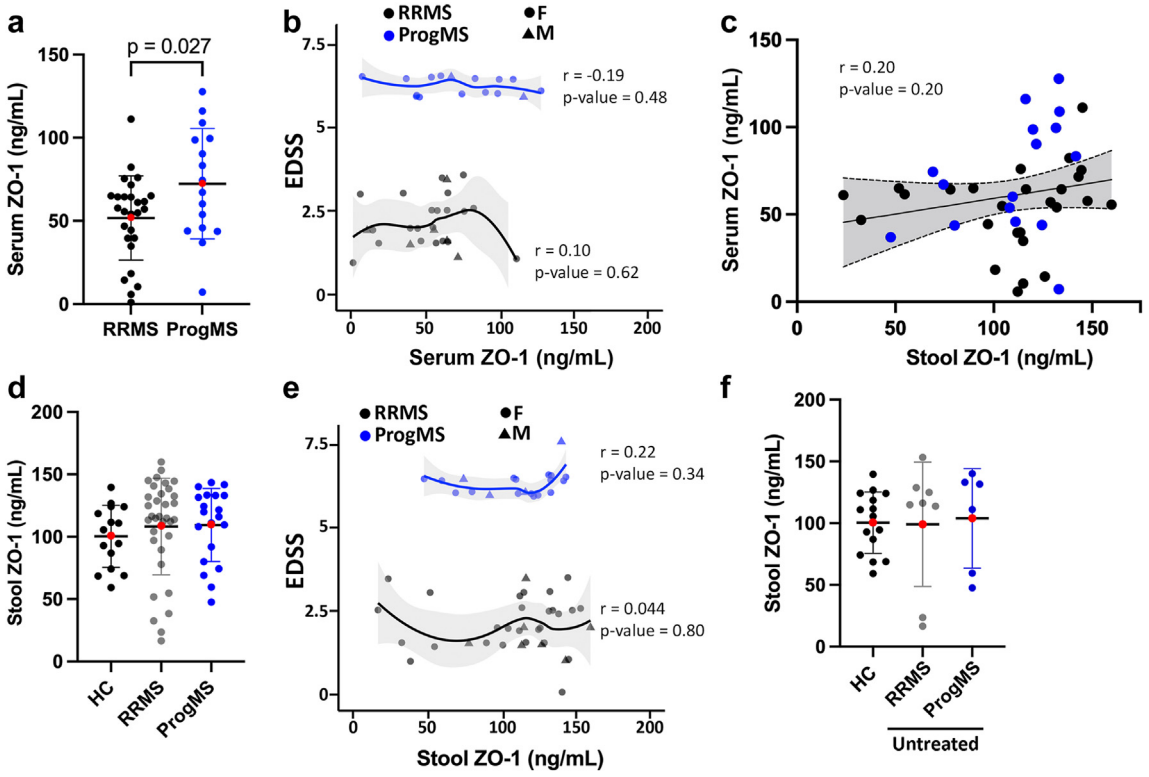

Fig. 4: **Tight junction protein ZO-1 was altered in ProgMS serum but not stool**. Serum ZO-1 was elevated in ProgMS vs. RRMS (a), but was not correlated with EDSS (b), or with stool ZO-1 quantities (c). Stool levels of ZO-1 were similar between RR- and Prog- MS compared to HC (d). EDSS was not correlated with stool ZO-1 levels (e). In untreated subjects, stool ZO-1 was similar across HC, RRMS, and ProgMS (f). Serum ZO-1 n = 27 RRMS, 16 ProgMS. Stool ZO-1 n = 15 HC, 35 RRMS, 20 ProgMS. Analyses were done via a student's t-test without a Welch's correction (a), one-way ANOVA with Tukey's post-hoc test (d), and Spearman's correlations with LOESS smoothing (b, e). Linear regression was performed in (c). Shaded/dotted lines on panels (b, c, e) are 95% confidence intervals. Data are mean ± SD with individual values on graphs.

Discussion

Intestinal barrier function at the epithelial and mucus levels tightly controls the entry of environmental stimuli and pathogens into the gut wall. Disruptions in these barriers has been implicated in driving a host of inflammatory diseases[8] including Parkinson's disease peripheral pathophysiology.[26] The gut barrier in MS has not been well investigated, with some studies showing 'leaky' barriers in a subset of subjects with MS,[6,7] but often in small cohorts and without analysis of progressive subjects with MS or duration of disease. Further, the intestinal mucus layer in the small intestine or colon has not been described in MS.

Gut wall mucus plays a vital role in filtering environmental compounds prior to contact with the gut epithelial layer while simultaneously creating a habitable environment for commensal microbiota.[27] Numerous diseases have dysregulated gut mucus layers, primarily peripherally-mediated inflammatory diseases like ulcerative colitis.[28] In this study, we show substantially decreased levels of mucus protein in the stool of subjects with MS. This decrease in mucus may be driven by altered goblet cell production/secretion or by elevated levels of mucus degrading microbiota.[15] Numerous microbial taxa with mucus degrading capacity[18] have been correlated with mucus protein levels in subjects with MS, including *A. muciniphila*,[15,16] *Bacteroides* genera members, *Ruminococcus torques*, *Bifidobacterium*, and *Blautia* genera members.[18] This points towards degrading of mucus by microbiota as the potential mechanism that decreases levels of mucoproteins in MS stool. However, the possibility of decreased goblet cell production or secretion cannot be discounted, and future investigations will focus on this possibility using human intestinal biopsies.

Dysregulation of the gut epithelial barrier can lead to infiltration of environmental products and pathogenic microbes, resulting in mucosal immune activation and inflammation.[29] Epithelial tight junction proteins have been shown elevated in stool from subjects with Parkinson's disease,[30] but have not been well elucidated in stool of those with MS. A general elevation in serum tight junction proteins has been shown in multiple neurodegenerative diseases, including Parkinson's,[30]

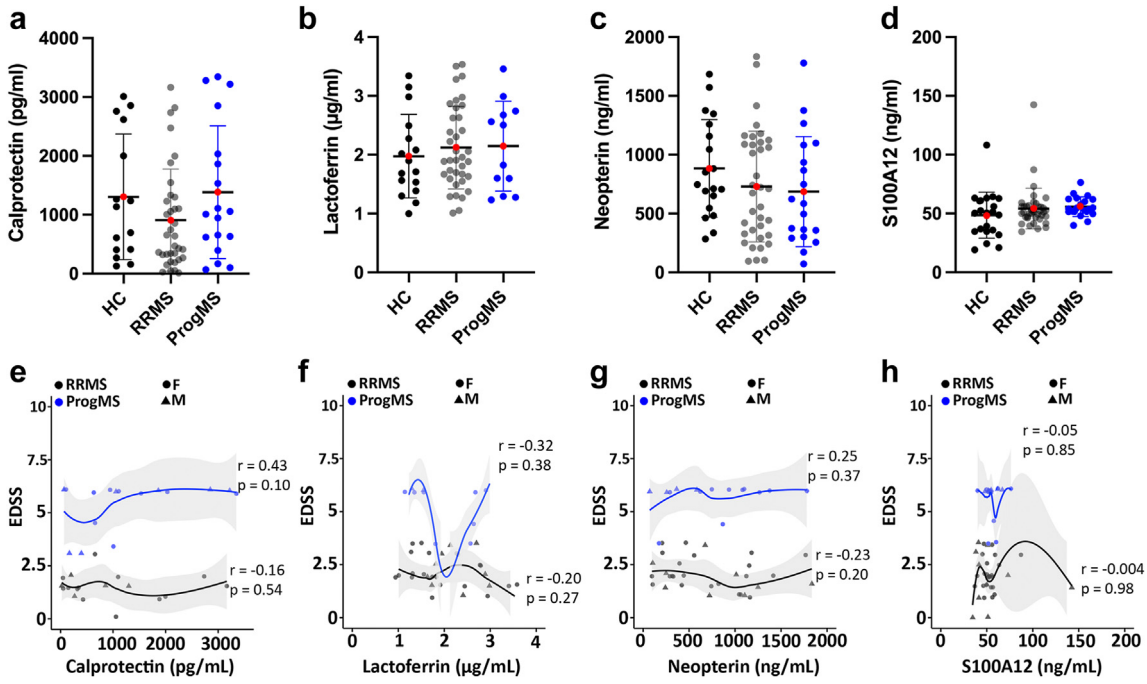

Fig. 5: **Faecal inflammatory markers are not altered in MS**. Calprotectin (a), lactoferrin (b), neopterin (c), and S100A12 (d) levels in stool by disease type. Correlation of stool levels of calprotectin (e), lactoferrin (f), neopterin (g), and S100A12 (h) with EDSS. n = 18 HC, 42 RRMS, 20 ProgMS. One-way ANOVA was performed on (a–d), and Spearman's correlations with LOESS smoothing (e–h). Shaded area in (e–h) represents 95% confidence intervals. Data are mean ± SD with individual values on graphs.

relapsing MS,[8–10] and clinically isolated syndrome.[9] We found elevated serum tight junction proteins in progressive MS relative to relapsing subjects, showing potential for disease status specific intestinal wall damages. We then screened faecal zonulin levels in relapsing and progressive MS compared to healthy controls and observed no alterations between groups. Our data suggest intact gut epithelial barrier function, an altered mucus layer in MS, and shows the potential for subjects with progressive disease to have increased serum tight junction proteins, originating somewhere outside of the gut wall. Additional confirmation of such mucosal disruption and the consequences for MS pathogenesis is warranted. Remarkably, we did not find differences in multiple inflammatory markers in subjects with MS stool as are routinely reported in Parkinson's disease[26,30–33] and IBD.[34–36] Together, these data support the notion that the intestinal tight-junction barrier is relatively in-tact, and there is not widespread mucosal inflammation in MS. This further indicates that gastrointestinal dysfunction may be disease-specific in neurologic disease, with a greater reduction in mucus barrier in MS vs. reduced tight-junctions and a GI inflammatory profile in PD.

The intestinal host and microbial environments are varied in people with MS, and differences in microbiome composition and bacterial metabolites are seen in relapsing compared to progressive disease.[1,5,37,38] Subjects with progressive disease have been shown to harbour increased *Akkermansia*,[39] *Clostridium*,[37] and *Methanobrevibacter*,[40] with lower quantities of *Gemmiger* and *Butyricicoccus*.[37] These findings were confirmed by the International Multiple Sclerosis Microbiome Study (iMSMS), in which we participate, using a multi-centre study of 576 subjects with multiple sclerosis and matched household healthy controls across 7 sites in 4 countries.[1,5] Furthermore, we have recently shown that microbes and their metabolites are differentially altered in subjects who worsened over time clinically and radiologically, and in those who transitioned to progressive disease.[22] Importantly, our study found that many of the metabolic alterations in these subjects were downregulated, indicating that subjects who became progressive were missing potentially beneficial microbes and their metabolites. Differing gut environments between relapsing and progressive MS are being illuminated, however, focus thus far has been predominantly on microbiome and metabolomes. In this manuscript, we have shown modifications to the epithelial barrier and mucus layer of the gut of subjects with MS which is associated with an altered microbial environment.

Compromised barrier integrity of the gut epithelial and mucus layers allows for increased entry of

environmental stimuli, while also influencing microbiota community structures. In this study, we show a dysregulated mucus barrier in subjects with MS which is associated with elevated levels of mucus degrading microbiota. We did not observe altered tight junction proteins in this cohort, nor did we see elevated general inflammatory markers. Taken together these data point towards microbiota dysbiosis linked to a decreased mucus layer in the gut of subjects with MS.

Limitations of the study

Our study provides important data on the gut environment in MS, demonstrating an altered mucus layer potentially influenced by muco-degrading bacteria, however, it is not without its limitations. We include a relatively small cohort of subjects when subdivided by disease type and sex, and HC subjects were significantly younger than our MS cohort (Table 1). While we accounted for this by correcting protein measurements for age, the differing biology between young and older individuals can't be discounted. Our cohort was also on various DMTs, with only a small subset being untreated, making it possible that inflammatory processes driving leaky gut are resolved in treated subjects. Dietary habits were not monitored during these investigations, creating an uncontrolled variable by which gut environment may have been altered. Taken together, our study provides data on gut wall alterations in a dysregulated microbial environment in MS, and highlights the need for further investigations into gut wall physiology of subjects with MS with particular attention paid to progressive disease.

Contributors

All authors meet all four criteria for authorship as defined in the ICMJE recommendations and all authors read and approved the final version of the manuscript. All authors confirm that they had full access to all the data in the study and accept responsibility to submit for publication. Drs. Schwerdtfeger and Weiner have directly accessed and verified the underlying data reported in the manuscript. LAS conceived the study, performed the experiments, analysed and interpreted the data, and wrote the manuscript. FM performed the experiments, interpreted the data, and edited the manuscript. TC provided biorepository samples and edited the manuscript. LMC and HLW conceived the study, edited the manuscript, and oversaw all aspects of the study.

Data sharing statement

Microbiota sequencing data used in this study is a subset of previously archived data collected in our centre.[22] All raw sequencing data is accessible at the NCBI Sequence Read Archive: SUB14686820 and BioProject PRJNA1152580. We used standard bioinformatic workflows throughout this study which are available on a GitHub repository to increase reproducibility of our analyses (https://github.com/LukeSchwerdtfeger/MS-barrier).

Declaration of interests

L.A.S. has received grant support from the NIH. F.M. has no interests to declare. T.C. has received grants or contracts from the BrightFocus Foundation, Bristol Myers Squibb, EMD Serono, Genentech, I-MAB Biopharma, the NIH, NMSS, Masachusetts Life Sciences Center, US Department of Defense, Novartis Pharmaceuticals, Octave Biosciences, Sanofi Genzyme, Tiziana Therapeutics, Wesley Clover International, and Celgene Corp. She has also received consulting fees from Bristol Myers Squibb, Cabaletta Bio, Cycle Pharmaceuticals, Genentech, Inc., Janssen, Merck KGaA, MJH Life Sciences, Novartis Pharmaceuticals AG, Novartis Pharmaceuticals KK, Octave Biosciences, F. Hoffman-La Roche Ltd, Sanofi, UCB Biopharma, and Siemens. She has received presentation honoraria from Intellisphere, LLC, and Prime Education, LLC, and participates in Advisory Board roles for Bristol Myers Squibb, Genentech, Inc., Novartis Pharmaceuticals Corporation, Octabe Bioscience, Inc., Roche, and Sanofi US Services, Inc. L.M.C. has received consulting fees from Anaerobe Systems. H.L.W. has received grants or contracts from the Cure Alzheimer's Fund, NIH, NMSS, stock or stock options from vTv Therapeutics, and Tiziana Life Sciences, and participates in Advisory Board roles for Sanofi and Tiziana Life Sciences.

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