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Stool Glial Fibrillary Acidic Protein Is Elevated in Progressive Multiple Sclerosis

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Abstract

Objectives

The gut microbiota and altered intestinal physiology have been implicated in multiple sclerosis (MS). Enteric glial cells regulate enteric nervous and immune function and express glial fibrillary acidic protein (GFAP) and S100β. Serum GFAP and neurofilament light chain can predict disease worsening; however, no clear markers differentiate relapsing from progressive disease.

Methods

To investigate enteric glial function in MS, we measured stool GFAP (st-GFAP) using an enzyme-linked immunosorbent assay in 31 healthy controls (HCs), 77 patients with relapsing remitting MS (RRMS), and 53 patients with progressive MS (ProgMS). Participants underwent clinical follow-up at 2 and 5 years after stool donation.

Results

We found higher st-GFAP levels in patients with ProgMS compared with those with RRMS and HCs. St-GFAP was positively correlated with baseline Expanded Disability Status Scale (EDSS) score, 25-foot walk time, and an increased EDSS score at 2 and 5 years. We found enteric glial hyperplasia in the colonic mucosa of a patient with primary progressive MS, as indicated by GFAP and S100β immunoreactivity, an effect not observed in duodenum tissue in patients with RRMS from our Milan cohort. St-GFAP in patients with ProgMS was negatively associated with *Eubacterium hallii*.

Discussion

These exploratory data indicate an altered enteric glial phenotype in patients with ProgMS and suggest that st-GFAP may be a prognostic biomarker.

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Supplementary Material

Introduction

The gut microbiota play an important role in multiple sclerosis (MS) pathogenesis[1-3] and are situated near large numbers of enteric glia that express glial fibrillary acid protein (GFAP) and S100β.[4] Serum markers of MS disease activity include neurofilament light chain (NfL), which is linked to neuronal damage, and GFAP, which is linked to disease progression.[5] Stool microbial metabolites are linked to disease progression in MS.[3] While stool inflammatory markers have been associated with Parkinson disease,[6] we have not observed elevated gut inflammation in MS.[7] In this study, we measured stool NfL, GFAP, and S100β, which, to our knowledge, have not been investigated in MS. We found elevated stool GFAP (st-GFAP) in patients with progressive MS (ProgMS), which is linked to disease worsening. These findings have implications for understanding gut physiology in MS and may provide a novel measure of disease progression.

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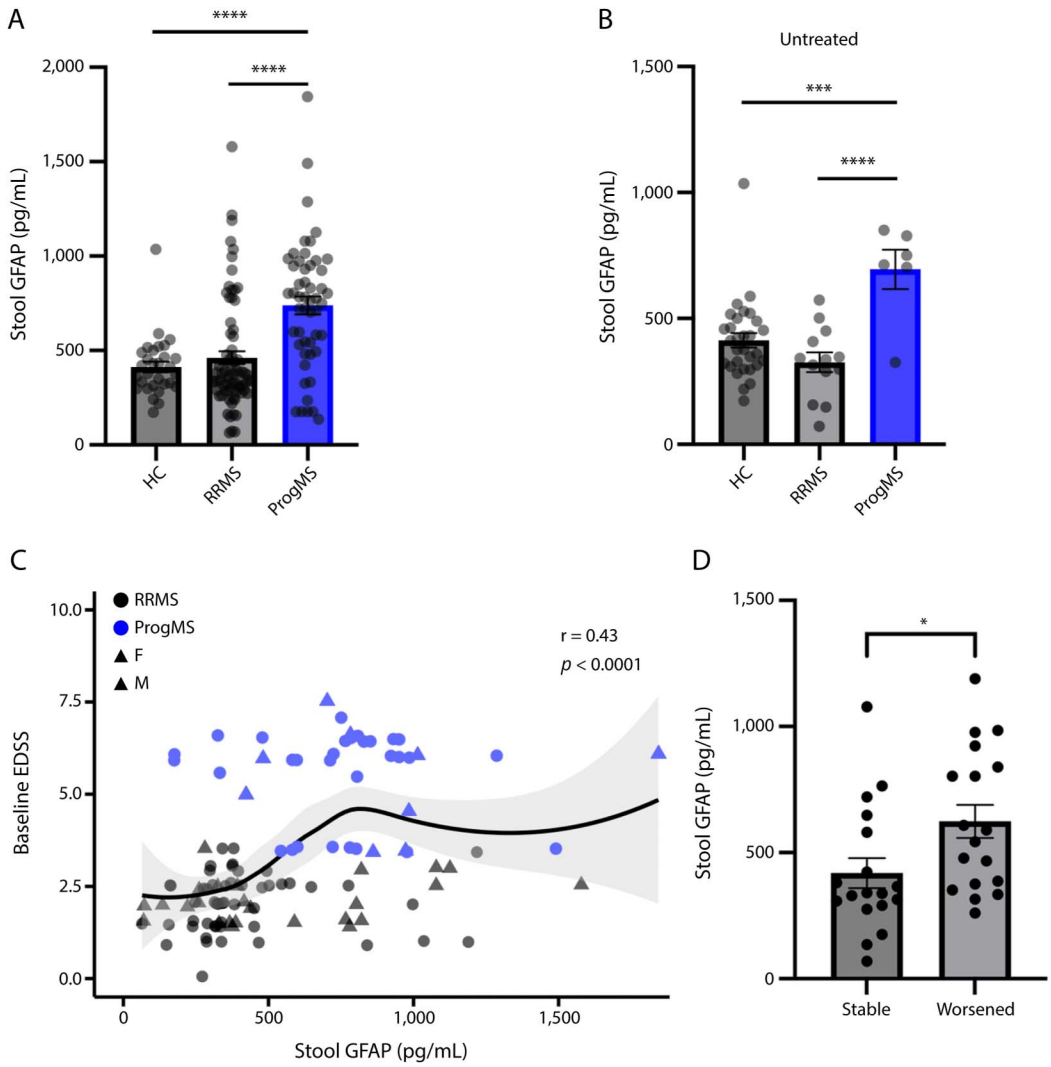
Figure 1 GFAP Is Elevated in Stool From Patients With ProgMS



Levels of st-GFAP in patients with MS (A). St-GFAP was lower in patients with ProgMS not receiving any disease-modifying therapy (B). St-GFAP levels are strongly correlated with baseline EDSS scores (C) and with a ≥1-point increase in EDSS at 2 years (worsened; D). Data in (A, B, D) are represented as mean ± SEM. Data in (C) are presented as a LOESS regression with 95% CIs plotted. Unadjusted *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. EDSS = Expanded Disability Status Scale; GFAP = glial fibrillary acidic protein; HC = healthy control; SEM = standard error.

Methods

Patients

Patients with MS were diagnosed according to the 2017 McDonald criteria. Exclusion criteria were pregnancy, a history of gastrointestinal surgery or disease, or antibiotic use within 3 months of sample collection. Additional exclusion criteria for Milan participants were corticosteroid use within 3 months of sample collection.[8] Experiments were approved by the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ethical Committee (#MS-GUT2019).[8] The Neuro-QoL survey assessed health-related quality of life.[9] Clinical measures were Expanded Disability Status Scale (EDSS) and timed 25-foot walk. Demographics are given in eTable 1.

Stool Protein Analysis

For enzyme-linked immunosorbent assays (ELISAs), stool was diluted 1:10 (w:v) in phosphate buffered saline (PBS) with 0.1% Tween-20 (ThermoFisher, Waltham, MA). Samples were homogenized on a Qiagen PowerLyzer-24 (Qiagen, Hilden, Germany) for 45s before being separated on a 4°C microcentrifuge at 6,000 rcf for 5 minutes and then 10,000 rcf for 10 minutes. Supernatants were used immediately for ELISAs to measure human GFAP, NfL (Abbexa, Cambridge, United Kingdom), or S100β (Abcam, Cambridge, MA). ELISAs were performed in triplicate, with values averaged per sample. Owing to healthy controls (HCs) being younger than patients with MS (eTable 1), we age corrected GFAP, NfL, and S100β quantities using a simple linear regression model.[7]

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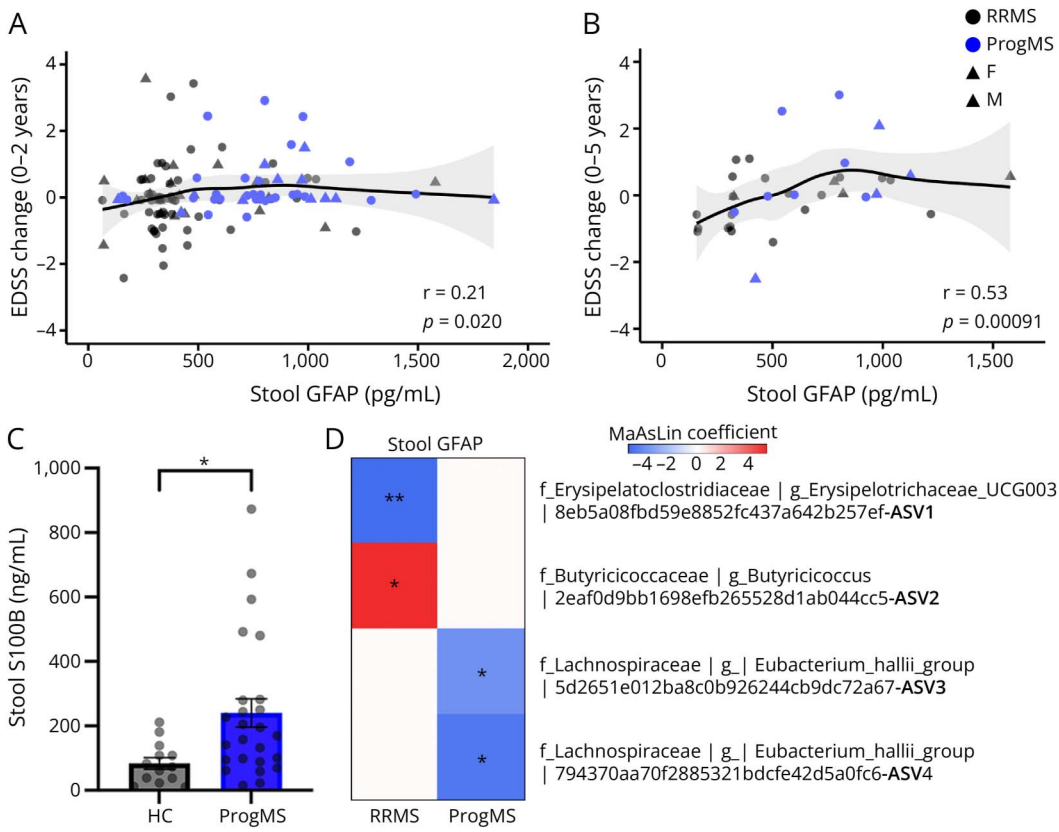
Figure 2 St-GFAP Correlates With Disease Worsening



Change in EDSS scores across 0–2 years (A) and 0–5 years (B) correlated with st-GFAP. Stool quantities of S100β in HCs vs patients with ProgMS (C). Heatmap showing MaAslin2 microbiota associations with GFAP, stratified by disease type and controlled for age and sex (D). Unadjusted *$p < 0.05$, **$p < 0.01$. EDSS = Expanded Disability Status Scale; GFAP = glial fibrillary acidic protein; HC = healthy control.

Immunohistochemistry

Colon biopsies were fixed and sectioned on a vibrating microtome (VT1000S; Leica, Wetzlar, Germany). Duodenums from Milan participants were paraffin embedded and sectioned before deparaffinization in xylenes and rehydration. Immunohistochemistry (IHC) was performed as previously described.[10] Tissue was incubated with polyclonal anti-GFAP (0.1 μg/mL; Immunostar, Hudson, WI) or monoclonal anti-S100β (2.4 μg/mL; Abcam). Images were taken on a BX61WI microscope (Olympus, Tokyo, Japan).

Statistics

ELISA data were analyzed by one-way analysis of variance (ANOVA) with the Tukey multiple comparisons test. Spearman correlations were performed in R, with a locally estimated scatterplot smoothing regression model and 95% confidence intervals. The Student *t* test without Welch correction was performed for 2-group comparisons. MaAslin2[11] was run in R to assess the association of st-GFAP quantities with microbiota, controlling for age, sex, and disease type. Data are represented as mean ± standard error (SEM).

Standard Protocol Approvals, Registrations, and Patient Consents

Participants in Boston were recruited under the Brigham and Women's Hospital CLIMB Study (IRB #2017P001169). Tissue collection was approved under Protocol #20204P001229. HC biopsy collection was approved by University of Colorado (UC) Health IRB#15–6051 and Colorado State University IRB#00010144. Informed consent was obtained from all study participants.

Data Availability

Anonymized data not published within this article will be made available by request from any qualified investigator.

Results

GFAP Is Elevated in Stool Samples of Patients With ProgMS

Patients with ProgMS had higher st-GFAP vs HCs ($p < 0.0001$) and those with RRMS (Figure 1A; $p < 0.0001$). St-GFAP was also elevated in untreated patients with ProgMS (Figure 1B). We then investigated st-GFAP's relation with EDSS score and found a positive correlation independent of

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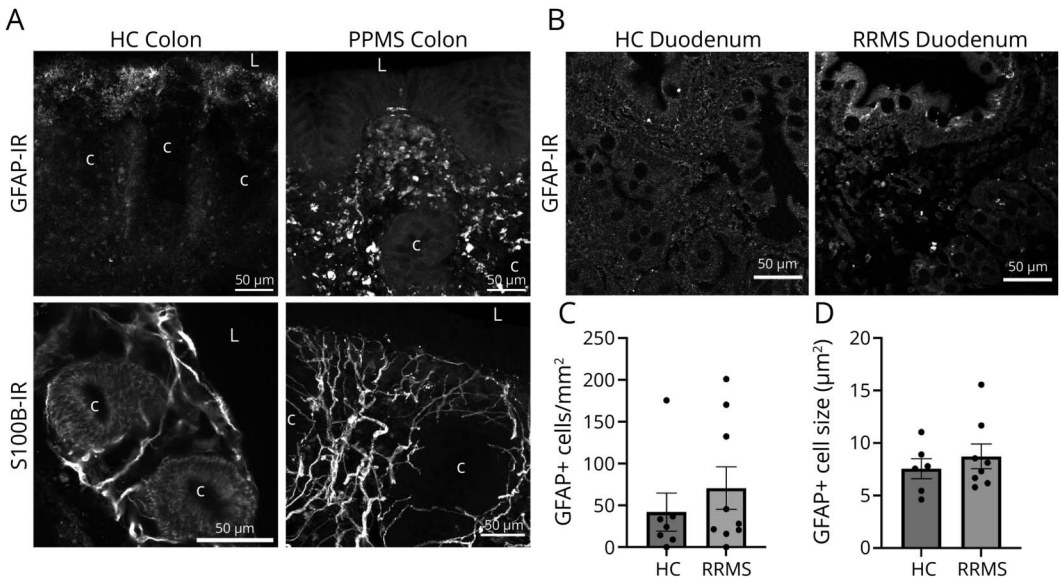
Figure 3 Enteric Gliosis in Patients With ProgMS



Immunoreactivity to GFAP and S100β demonstrates elevated quantities of enteric glial cells in a patient with primary progressive MS (PPMS) vs healthy controls (HCs) (A). Duodenum tissue processed for GFAP in HCs vs patients with RRMS (B). Quantification of GFAP + cells/mm^2 (C) and GFAP + mean cell size (D). Scale bars are given in 50 μm. Data in (C and D) are given as mean ± SEM. GFAP = glial fibrillary acidic protein; RRMS = relapsing remitting MS; SEM = standard error.

disease status (Figure 1C; Spearman $r = 0.43$, $p < 0.0001$). Furthermore, participants who worsened (ΔEDSS score ≥1)[12] had elevated st-GFAP vs stable participants matched for age, sex, treatment, and diagnosis (Figure 1D; $p < 0.05$). St-GFAP was positively correlated with EDSS change over 2 years (Figure 2A; $r = 0.21$, $p = 0.020$) and 5 years (Figure 2B; $r = 0.53$, $p = 0.00091$) and with timed 25-foot walk at baseline (eFigure 1A; $r = 0.36$, $p = 0.00031$). When we investigated the relationship between st-GFAP and Neuro-QoL scores, we found associations with baseline (0 years) and 2-year domains (eFigure 1B). Furthermore, st-GFAP was similar in HCs and patients with RRMS in the Milan cohort vs Boston cohort ($p = 0.15$). We then measured another enteric glial protein, S100β, in stool and found elevated levels in patients with ProgMS (Figure 1C).

To investigate the relationship between stool and serum, GFAP was measured in both biofluids from 20 HCs, 20 patients with RRMS, 20 patients with ProgMS; however, the levels were not correlated (Pearson $r = 0.147$, $p = 0.26$). We then investigated stool NfL and found similar levels between HCs, patients with RRMS, and patients with ProgMS ($p = 0.35$). Stool NfL was not associated with baseline (Spearman $r = 0.10$, $p = 0.32$) or 2-year ($r = 0.06$, $p = 0.56$) change in EDSS score.

Microbiota Linked to Elevated GFAP

Because the gut microbiome influences gut immune cells, including enteric glia, we characterized the gut microbiota by 16S rRNA sequencing and determined which microbial taxa were associated with st-GFAP levels in patients with RRMS and ProgMS. We found that 2 *Eubacterium hallii* amplicon sequence variants were negatively associated with st-GFAP in patients with ProgMS. In patients with RRMS, *Erysipelotrichaceae* was negatively and *Butyricicoccus* was positively associated with st-GFAP (Figure 2D). Relative abundance of GFAP-associated taxa was similar across disease groups (eFigure 2, A–D), suggesting a direct relationship between st-GFAP and these bacteria.

Enteric Glia in MS

To investigate whether the observed elevated st-GFAP was associated with changes in MS enteric glia, we obtained a colon biopsy from a patient with ProgMS. We found a marked increase in GFAP and S100β immunoreactivity (IR) in the ProgMS tissue (Figure 3A). While this is only a single subject, due to difficulty in obtaining colon biopsies from patients with ProgMS, it suggests that enteric glia are activated in patients with ProgMS and is consistent with our observed st-GFAP results (Figure 1). To further investigate enteric glia in MS, we obtained duodenal biopsies from n = 7 HCs and n = 9 patients with RRMS in Milan. We found similar GFAP-immunoreactivity (IR) in the duodena of patients with RRMS and HCs (Figure 3, B–D), consistent with st-GFAP findings. This suggests that GFAP is uniquely elevated in ProgMS.

Discussion

Enteric neuronal and glial proteins have not been well studied in MS. Enteric glial cells provide neuronal support, regulate immune responses to microbiota,[4] and influence gut

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motility.[13] We investigated whether markers of enteric glia were altered in MS and found that st-GFAP and S100β were elevated in ProgMS and linked to disease worsening.

Important questions related to our findings include the source of st-GFAP and its relationship with serum markers of progression. Serum GFAP marks CNS astrocytes and is a biomarker of disease progression in MS,[5] Alzheimer disease, and Parkinson disease.[14] We found no correlation between serum and st-GFAP, with st-GFAP being 5-fold higher than serum levels, suggesting that enteric glia are the likely source. Histochemical analysis of enteric glia in the colon of patients with ProgMS colon and duodena of patients with RRMS further supports enteric glial alterations in progressive disease. Of note, a previous study found hyperplastic enteric glial cells in the myenteric plexus of patients with MS.[15]

It is not clear which factors are driving observed elevations in st-GFAP. Activated enteric glia express more GFAP and S100β,[4] and we found these proteins to be elevated in the stool and colonic tissue of patients with ProgMS, consistent with an activated glial phenotype. Microbiota may influence enteric glial alterations that we found in ProgMS. We found an association of st-GFAP with 2 *Eubacterium hallii*, a taxa negatively associated with disease worsening, suggesting a beneficial effect.[3] Our study is limited by the availability of tissue from only 1 patient with progressive MS and by heterogeneity of disease-modifying therapies among patients. Nonetheless, our study identifies an altered enteric glial phenotype in patients with ProgMS and demonstrates that st-GFAP may serve as a biomarker with prognostic value.

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Author Contributions

L.A. Schwerdtfeger: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. F. Montini: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. M. Antonini Cencicchio: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. J.R. Christenson: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. B.I. Glanz: major role in the acquisition of data. M. Falcone: drafting/revision of the manuscript for content, including medical writing for content. M. Filippi: drafting/revision of the manuscript for content, including medical writing for content. L.M. Cox: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. T. Chitnis: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. H.L. Weiner: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data.

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Disclosure

The authors report no relevant disclosures. Go to Neurology.org/NN for full disclosures.

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