Altered interaction between enteric glial cells and mast cells in the colon of women with irritable bowel syndrome

Felipe Meira de-Faria1 | Maite Casado-Bedmar1 | Carl Mårten Lindqvist2 | Michael P. Jones3 | Susanna A. Walter1,4 | Åsa V. Keita1

Abstract
Background: Enteric glial cells (EGC) and mast cells (MC) are intimately associated with gastrointestinal physiological functions. We aimed to investigate EGC-MC interaction in irritable bowel syndrome (IBS), a gut-brain disorder linked to increased intestinal permeability, and MC.

Methods: Parallel approaches were used to quantify EGC markers in colonic biopsies from healthy controls (HC) and patients with IBS. Data were correlated with MC, vasoactive intestinal polypeptide (VIP) and VIP receptors (VPAC1/VPAC2) expressions, and bacterial translocation through biopsies mounted in Ussing chambers. In addition, we investigated the effects of EGC mediators on colonic permeability and the pharmacological-induced responses of EGC and MC cell lines.

Key Results: Immunofluorescence of IBS colonic mucosa, as well as Western blotting and ELISA of IBS biopsy lysates, revealed increased glial fibrillary intermediate filament (GFAP) expression, indicating EGC activation. Mucosal GFAP correlated with increased MC and VPAC1+MC numbers and decreased VIP+MC, which seemed to control bacterial translocation in HC. In the contrary, EGC activation in IBS correlated with less MC and VPAC1+ MC numbers, and more VIP+ MC. In vitro, MC and EGC cell lines showed intracellular calcium responses to each other's mediators. Furthermore, EGC mediators prevented VIP-induced MC degranulation, while MC mediators induced a reactive EGC phenotype. In Ussing chambers, EGC mediators decreased paracellular passage through healthy colonic biopsies.

Conclusions & Inferences: Findings suggest the involvement of EGC and MC in the control of barrier function in the human colon and indicate a potential EGC-MC interaction that seems altered in IBS, with detrimental consequences to colonic permeability. Altogether, results suggest that imbalanced EGC-MC communication contributes to the pathophysiology of IBS.

Keywords
bacterial translocation, enteric glial cells, enteric nervous system, mast cell, mucosal immunology
1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a gut-brain disorder characterized by recurrent abdominal pain linked to disturbed bowel habits with a global prevalence of about 11% of the population. The pathogenesis of IBS is driven by a tangle of factors including abnormalities in motility, visceral sensation, brain-gut interaction, psychosocial distress, changes in immune responses, imbalance in gut microbiome, and abnormal intestinal permeability.

Gastrointestinal (GI) functions are predominantly controlled by the enteric nervous system (ENS), an intrinsic network of enteric neurons and enteric glial cells (EGC) that communicates bidirectionally with the central nervous system (CNS), mainly through the vagus nerve, to maintain homeostasis. EGC, distributed throughout all colonic layers, are a diverse population of cells distinguished by their morphology and location within the gut wall. EGC vary express glial cell markers, such as glial fibrillary intermediate filament (GFAP), S100 calcium-binding protein β (S100β) and SRY-box transcriptional factor 10 (Sox-10). This fact suggests functional diversity and plasticity among EGC types that is observed, for example, in response to environmental triggers, such as lipopolysaccharide. EGC are strategically positioned close to enteric neurons, smooth muscle, and intestinal epithelium that together influence neural circuits, taking part in the regulation of GI functions. In the GI tract, EGC are known to participate in epithelial barrier function, fluid secretion, and motility. Therefore, EGC may play an important role in the pathophysiology of IBS, but there are still few studies present. Recent experimental and clinical findings have shown changes in EGC in IBS animal models, presenting hyperplasia in myenteric plexus and colonic-mucosal alterations in GFAP expression and S100β-positive area. In IBS patients, these correlated with abdominal pain and bloating.

Mast cells (MC) are active players in the gut and are well known for communicating with neurons within the ENS. MC contribute to low-grade inflammation in the intestinal mucosa and to visceral hypersensitivity in IBS patients. Our group previously showed that MC and vasoactive intestinal polypeptide (VIP), a neuroimmune-endocrine mediator majorly produced by enteric neurons and MC in the gut, contribute to control barrier function in the ileum of healthy humans and in rats submitted to chronic stress. More recently, we reported higher levels of tryptase, an increased density of MC, and percentage of VIP+ and VIP receptor 1 (VPAC1) MC, but not VIP receptor 2 (VPAC2) MC in IBS compared to healthy controls (HC). Further, we found that translocation of live bacteria through colonic mucosa is partially controlled by MC and VIP in both IBS and healthy subjects.

Existing evidence supporting a direct glial-MC communication is limited to the CNS, where, when perturbed, it appears to contribute to the etiology of neurodegenerative diseases such as Parkinson’s disease, multiple sclerosis, traumatic brain injury depression, and Autism spectrum disorder. Neuron-MC communication has been studied in human submucosal plexus preparations, showing clear bidirectional signaling. Another study showed that functional dyspepsia patients presenting gliosis displayed impaired neuronal function, which was correlated with MC infiltration in duodenal submucosa.

Despite all scientific effort, mechanisms by which the MC-EGC communication influences the IBS pathophysiology remain unclear. Based on previous findings, we hypothesized that EGC are a functional part of the neuroimmune hub formed by enteric neurons and MC. Since human EGC express toll-like receptors and expression levels are modulated by both pathogenic and probiotic bacteria, we further hypothesized that the EGC might be influenced by the patient’s own microbiota. Thus, this study aimed to (1) explore phenotypical changes of EGC in IBS and evaluate the relationship between EGC and MC in the colon of healthy and IBS subjects; (2) evaluate associations between EGC and fecal microbiota; (3) investigate EGC and MC activation in relation to barrier function and by using in vitro model systems. We hypothesized that EGC are contributing to the neuroimmune hub formed by enteric neurons and MC, and that this hub may be altered in IBS.

2 | MATERIALS AND METHODS

2.1 | Subjects

Thirty women with moderate-to-severe IBS (mean 31.2 years, range 19–55) meeting Rome III criteria were recruited from the Gastroenterology Department, University Hospital, Linköping.
IBS subjects were classified based on predominant bowel habit into IBS-diarrhea (IBS-D, n = 7), IBS-constipation (IBS-C, n = 7) and IBS-mixed (IBS-M, n = 16). All patients were experiencing symptoms during the study. Twenty-two age-matched females (mean 31.6 years, range 21–56) without a medical history of GI symptoms or complaints were recruited as HC. Exclusion criteria were organic GI disease, metabolic, neurological, or severe psychiatric disorders, self-reported nicotine intake within 2 months before the study, allergy and use of non-steroidal anti-inflammatory drugs or central acting pain medications. The regional ethical review board approved the study, and all participants gave their written informed consent in accordance with the Declaration of Helsinki.

2.2 | Questionnaires and GI symptom diaries

Questionnaire data from all participants were collected at inclusion for sample characterization and for the assessment of IBS-related variables. Symptom diaries were obtained from IBS patients, but not HC. Patients recorded their GI symptoms for 14 consecutive days using validated diary cards.27 The symptoms (abdominal pain, nausea, bloating) and every single bowel movement and stool consistency (defined by the Bristol Chart, BSC) were reported along a 24-h time axis. The values were manually scored, and the mean frequency of symptom episodes/week and symptom duration/day was extracted from the diary data.

2.3 | IBS severity scoring system (IBS-SSS)

IBS-SSS is a five-item questionnaire evaluating overall IBS symptom severity by assessing the frequency and the intensity of abdominal pain and distension, the satisfaction with bowel habits, and interference with daily life.28 Each item generates a score between 0 and 100 with a maximal sum score of 500. Sum scores indicate mild (75–175), moderate (175–300), or severe (>300) disease.

2.4 | Hospital Anxiety and Depression Score (HADS)

The HADS was used to measure symptoms of anxiety and depression.29 The scale consists of seven items of anxiety (HADS-A) and depression subscales (HADS-D), with scores on each subscale ranging from 0 to 21.

2.5 | Sigmoidoscopies

Flexible sigmoidoscopies were performed according to a standardized protocol regarding stretching and thickness of the biopsies. Participants were fasting for 8 h and completed a colon preparation with an enema (klyx®) early the same morning. The procedure was performed without sedation with scope insertion approximately 30–40 cm ascending from linea dentata. Colonic biopsies were taken with biopsy forceps without a central lance and placed directly in ice-cold-oxygenated Krebs buffer.30

2.6 | Fecal samples

Fecal samples from 33 patients with IBS and 20 HC were collected in feces containers (Sarstedt, Helsingborg, Sweden) within 2 weeks prior to the sigmoidoscopy and within 24 h sent to the laboratory in room temperature (RT). Immediately after arrival, samples were frozen at −80°C.

2.7 | Microbiota analysis

Fecal microbiota was analyzed by the Genetic Analysis GA-map® Dysbiosis Test (Genetic Analysis AS, Oslo, Norway), which is a gut microbiota DNA analysis tool to identify dysbiosis. Briefly, the test involves sample homogenization, mechanical bacterial cell disruption, and total bacterial gDNA extraction as previously described.31 The test utilizes 54 predetermined bacterial DNA markers targeting the 16S rRNA sequence in seven variable regions (V3–V9) that measure the abundance of bacteria according to the intensity of the fluorescent signal detection. The 54 DNA probes on the GA-map targeted ≥300 bacteria on different taxonomic levels and were selected based on the ability to distinguish between HC, IBS, and IBD patients.32 Analyses of bacterial abundances were performed using the fluorescent signal.

2.8 | Ussing chambers experiments

Colonic biopsies from IBS patients and HC were mounted in Ussing chambers as previously described.30 After 40 min of equilibration, 10⁸ CFU/ml live green fluorescent protein (GFP)-labeled Escherichia coli HS or GFP-Salmonella (S.) typhimurium were added to the mucosal side of each chamber. Serosal samples were collected over time, and bacterial passage was measured.

To investigate the influence of glial cell mediators on permeability, biopsies from 3 HC were mounted in Ussing chambers and added 100 µM S-nitrosothiolane (GSNO) (Sigma-Aldrich), 3 nM GDNF (Thermo Fisher), or 100 nM S100β (Sigma-Aldrich), or Krebs buffer as control, on the mucosal side. Concentrations were based on previous publications.10,32,33 The paracellular marker fluorescein isothiocyanate (FITC)-dextran 4000 (Sigma-Aldrich) was added on the mucosal sides at 2.5 nM, and serosal samples were collected over time and analyzed for FITC-dextran passage at 488 nm in a VICTOR™ X3 multileader plate reader.
2.9 | Immunofluorescence

Biopsies from IBS and HC were fixed, embedded in paraffin, sectioned at 5 µm, and individually stained for 1:500 rabbit anti-GFAP (DakoCytomation) and 1:250 mouse anti-S100β (Invitrogen) overnight at 4°C. Antibody specificity was verified by Western blotting on human intestinal tissues and cell lines. Slides were rinsed and incubated for 1 h at RT with 1:2000 Alexa Fluor 594-conjugated secondary antibodies (Invitrogen). Slides were mounted with Prolong® Gold DAPI (Life Technologies, Thermo Fisher); negative controls were obtained by omitting primary antibodies. Images (400x) were acquired using a Nikon E800 widefield fluorescence microscope and NIS elements software (Nikon Instruments Inc). Only the mucosal layer was evaluated to prevent acquisition of uneven fluorescent information due to the presence of submucous ganglionic structures. Ten images/section were acquired and analyzed using the opensource platform Fiji package for Image J software.24 Automatic thresholding Triangle25 was employed before measuring positive signals; data expressed as fluorescence intensity (arbitrary units). Staining for MC-tryptase, VIP, VPAC1, and VPAC2 is described elsewhere.21

2.10 | Western blotting

Frozen biopsies from IBS and HC were subjected to protein extraction followed by Western blotting as previously described.26 Membranes were incubated overnight at 4°C with 1:5000 rabbit polyclonal antibody anti-GFAP (DakoCytomation), 1:1000 mouse monoclonal anti-S100β antibody (Thermo Fisher), and 1:10,000 mouse monoclonal anti-β-actin antibody (Cell Signaling, BioNordika, Solna, Sweden), in PBS pH 7.6 + 3% BSA + 0.01% Tween 20. After washing, membranes were incubated with 1:20,000 Alexa Fluor 790-conjugated goat polyclonal anti-mouse (Invitrogen) and Alexa Fluor 680-conjugated goat polyclonal anti-rabbit secondary antibodies (Invitrogen) for 1 h at RT in PBS pH 7.6 + 5% non-fat milk +0.01% Tween 20. Membranes were washed and fluorescent bands were detected and quantified by Odyssey CLx and Image Studio software (LI-COR Biosciences). GFAP and β-actin protein levels were corrected to their brightest signal within each individual membrane and normalized to β-actin loading control corrected values. Values were given as fold change relative to HC.

2.11 | Determination of GFAP, GDNF and S100β levels in biopsy lysates by ELISA

To measure GFAP, GDNF, and S100β levels, we employed sandwich ELISA kits. Biopsy lysates diluted 1:20, cell culture medium, and standard samples were added to plates pre-coated with primary antibody. Plates were incubated at 37°C, and after 90 min, a secondary-biotinylated antibody was added. After 60 min at 37°C, plates were further treated following manufacturer’s instructions (Nordic Biosite). Absorbance was measured at 450 nm in VersaMax Tunable Microplate Reader (Molecular Devices) using SoftMax pro 5 (Molecular Devices). The software generated a standard curve from which the concentrations of the samples were calculated. Biopsy lysates values were corrected to dilution and normalized to protein concentration.

2.12 | In vitro intracellular calcium [Ca2+]i responses live cell imaging

To study the ability of MC in evoking responses in EGC and vice-versa, we employed live cell recordings using [Ca2+]i mobilization assay with Fluo-4 NW on EGC cell line EGC/PK0600399egfr (ATCC, CRL-2690) and rat basophil/MC-like cell line RBL-2H3 (ATCC, CRL-2256). CRL-2690 are myenteric-derived glial cell line from the jejunal portion of the small intestine of rats37 and are widely used in EGC studies.26,38 RBL-2H3 cells were used in this setting due to their ability to attach to culture dishes, which is required during recording in our experimental settings. For live cell imaging experiments, cells were seeded at 5.0 × 104 cells/ml in 35 mm confocal dishes in Dulbecco’s Modified Eagle Medium (DMEM) or Minimal Essential Medium (MEM); after 24 h, cells were washed with HEPES-buffered-Hank’s Balanced Salt Solution (HBSS) and loaded with Fluo-4 NW for 30 min at 37°C in a 5% CO2 incubator. An insert (RC-37F, Warner instruments) connecting the culture dish to a perfusion system kept HBSS perfusing constantly throughout the experiment at 1 ml/min. Cells were treated for 20 s with 10 µM adenosine triphosphate (ATP), 3 µM VIP, 10 µM histamine, 1 nM tryptase, 10 nM GDNF, or 30 nM S100β. Images were acquired for 2 (EGC) and 4 min (MC) every 1 s under 100 ms exposure on a Nikon Ti Eclipse Spinning Disk inverted confocal microscope. Data were presented as ΔF/FO (difference between rise in signal corrected to background and expressed as % relative to baseline).

2.13 | GSNO, GDNF, and S100β effects on VIP-induced degranulation in HMC-1.1

To investigate the effect of the EGC products GDNF and S100β on MC, a MC-degranulation assay was set up29 using the human MC line HMC-1.1 (Merck Chemicals and Life Science AB). Cells were cultured in Isove Medium (EMD, Millipore) supplemented with 1.2 mM α-thioglycerol (Sigma-Aldrich), 10% fetal bovine serum, and 1% peni-cillin/streptomycin at 37°C in a 5% CO2 incubator. Upon in vitro experiments, cells were washed 3 times and seeded in 96 well plates (3.0 × 104 cells/well in DPBS). After 30 min acclimation at 37°C, cells were pre-treated with 1, 10, or 30 nM GDNF (Thermo Fisher) or 10, 30, or 100 nM S100β (Thermo Fisher) for 20 min followed by 30 min stimulation with 3 µM VIP (Sigma-Aldrich) or vehicle. Concentrations were based on previous publications.10,32,33 Cells were collected and lysed to quantify the degranulation rate of HMC-1.1 with a β-hexosaminidase assay, as previously described.39 Results were nor-malized to maximum degranulation and expressed as the percentage of β-hexosaminidase release relative to control samples.
2.14 | EGC cell line activation by tryptase, serotonin (5-HT), histamine, VIP, and live bacteria

To evaluate EGC activation in vitro, EGC cell line CRL-2690 were grown until confluence (~10 × 10^6 cells) in DMEM supplemented with 10% fetal bovine serum +1% penicillin/streptomycin. Cells were washed in DPBS and kept in non-supplemented DMEM without phenol red. Cells were then treated with 1 nM tryptase, 100 µM 5-HT, 10 µM histamine, or 3 µM VIP. In addition, EGC were exposed to S. typhimurium, E. coli HM427, or Yersinia enterocolitica (cell:bacteria, 1:100) After 24 h, medium was collected to determine the levels of S100β and GDNF by ELISA, and cell protein lysates were subjected to Western blotting, as described above, to measure the increase in GFAP expression as an glial activation signature (gliosis).

2.15 | Statistics

Correlational data were calculated in Mplus, formal statistical inference employed a nonparametric bootstrap. Data were processed using GraphPad Prism 8 (GraphPad Software Inc); all correlational data were treated as nonparametric. For correlations, Spearman’s correlation settings were employed; for comparison between group’s correlations, linear regression was performed to test differences between slopes (intercorrelation). Comparison between 2 groups was done with Mann-Whitney test, while comparison between >2 groups was done by one-way analysis of variance (ANOVA), followed by Tuckey’s multiple comparison test. Statistical significance was set as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. For microbiota, a principal component analysis of similarities and correlation analysis were calculated using R version 4.0.2 and packages vegan and labdsv and visualized with ggplot2.

3 | RESULTS

3.1 | Characteristics of IBS subjects

Subjects (n = 30) were diagnosed with moderate-to-severe IBS (IBS-SSS 341 [303–418]) and divided into IBS-D (n = 7), IBS-C (n = 7) and IBS-M (n = 16). IBS patients presented significantly higher HADS compared to HC (Table 1). Prospectively recorded GI symptom diary confirmed that IBS patients had a moderate-to-severe symptom burden (Table 2).

3.2 | Reactive EGC in the colon of IBS patients

Immunofluorescence showed increased levels of mucosal GFAP in biopsies of IBS compared to HC, p < 0.001 (Figure 1A) while there was no difference in S100β expression (Figure 1B). Immunofluorescence finding of GFAP was confirmed by Western blotting (Figure 1C) and ELISA (Figure 1D) of biopsy lysates showing significantly increased levels in IBS. Further, ELISA confirmed the immunofluorescence

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**TABLE 1** Characteristics of the subjects involved in the study

|                | HC (n = 22) | IBS total (n = 30) | IBS-M (n = 16) | IBS-D (n = 7) | IBS-C (n = 7) | p-value |
|----------------|------------|--------------------|----------------|--------------|--------------|---------|
| Age            | 31.6 (21–56) | 31.2 (19–55)       | 30.6 (19–52)   | 33.5 (22–42) | 32.8 (19–55) | 0.75    |
| IBS-SSS        | 3 (0–24)   | 341 (303–418)      | 369 (325–411)  | 341 (292–446) | 323 (255–402) | <0.0001 |
| Anxiety HADS   | 4 (2–5)    | 10.5 (8–14.0)      | 12 (8–14.7)    | 11 (8–14)    | 10 (6–13)    | <0.0001 |
| Depression HADS| 1 (0–3)   | 5.5 (4–9.3)        | 6 (4–13.5)     | 6 (3–10)     | 4 (4–6)      | <0.0001 |

Note: Data presented as median (25th–75th percentile), age is presented as mean (range). Mann-Whitney test was performed between healthy controls (HC) and irritable bowel syndrome (IBS) group. Abbreviations: HADS, Hospital Anxiety and Depression Scale; IBS-SSS, IBS severity scoring system.

**TABLE 2** Gastrointestinal symptoms diary from the irritable bowel syndrome (IBS) patients

|                                | IBS total (n = 30) | IBS-M (n = 16) | IBS-D (n = 7) | IBS-C (n = 7) |
|--------------------------------|--------------------|----------------|--------------|--------------|
| Abdominal pain (episodes/week) | 8.4 (4.6–14.3)     | 8.4 (2.6–14.1) | 13.5 (5.0–17.5) | 7.5 (5.0–12.0) |
| Abdominal pain (h/day)         | 5.5 (1.2–10.3)     | 4.7 (0.6–10.6) | 6.3 (1.2–9.6)  | 5.3 (2.8–11.9) |
| Bloating (episodes/week)       | 5.8 (3.0–8.9)      | 5.5 (2.7–10.4) | 5.5 (3.0–9.5)  | 7.0 (3.5–8.5)  |
| Bloating (h/day)               | 6.1 (2.4–11.2)     | 8.4 (1.2–13.1) | 4.5 (0.9–10.4) | 5.8 (4.0–10.0) |
| % Bristol 1-2                  | 6.1 (0.0–29.4)     | 7.9 (0.0–49.1) | 0.0 (0.0–1.8)  | 23.0 (8.8–55.6) |
| % Bristol 3–5                  | 42.9 (29.3–66.7)   | 38.7 (14.5–66.7) | 50.0 (30.8–90.9) | 39.3 (31.0–73.2) |
| % Bristol 6–7                  | 41.18 (7.27–56.00) | 33.33 (9.86–55.83) | 50 (7.27–69.23) | 20.6 (0.0–48.6) |

Note: Data presented as median (25th–75th percentile). Abbreviations: IBS-C, IBS-constipation; IBS-D, IBS-diarrhea; IBS-M, IBS-mixed.
FIGURE 1  Quantification of glial fibrillary acidic protein (GFAP) (A) and S100 calcium-binding protein β (S100β) (B) by measurements of fluorescence intensity (FI) following immunofluorescence in the mucosa of colonic biopsies of irritable bowel syndrome (IBS) patients and healthy controls (HC). (C) GFAP protein expression measured by Western blotting in lysates from colonic biopsies of IBS patients and HC. Expressions of GFAP were normalized according to the β-actin loading control (D) GFAP protein expression measured in biopsy lysates by ELISA (E) S100β protein expression in biopsy lysates by ELISA (F) Expressions of glial-derived neurotrophic factor (GDNF) quantified by ELISA in biopsy lysates. Data are expressed by median (IQR) and compared with Mann-Whitney test; *p < 0.05, ***p < 0.001
findings of S100β, showing equal levels in biopsy lysates of IBS and HC (Figure 1E). Measurements of GDNF, measured by ELISA in biopsy lysates, revealed decreased levels in IBS compared to HC, \( p < 0.05 \) (Figure 1F). Spearman analysis revealed no significant correlations between mucosal parameters and symptoms (data not shown).

### 3.3 Decreased levels of Actinomycetales in IBS but no relation between microbiota and EGC

Out of the 54 probes investigated, all identifying a phylogenetic group of bacteria, only the group Actinomycetales showed out to differ between HC and IBS. There were significantly lower levels in IBS patients, \( p < 0.01 \) (Figure 2A), with a majority of the lower data points belonging to the IBS-D subgroup (red dots in Figure 2A). A visualization of the data from the 54 probes with principal coordinate analysis (PCoA) showed that the HC has a similar spread of the microbiota as IBS-D and IBS-M, whereas IBS-C is slightly separated from the other groups (Figure 2B). Test of the difference between the groups with Analysis of similarities (ANOSIM) was not significant, \( p = 0.26 \). The orientation of the microbiota did not seem to be related to expression/abundance of GFAP, analyzed both by immunofluorescence (Figure 2C) and Western blotting (Figure 2D). The abundance of Actinomycetales did not correlate with GFAP, neither for immunofluorescence (Pearson correlation, \( p = 0.39 \)) (Figure 2E) nor Western blotting (Pearson correlation, \( p = 0.20 \)) (Figure 2F). Neither did the abundance correlate significantly with the immunofluorescence expression of S100β (Supplementary Figure S1).

### 3.4 EGC and MC are phenotypically changed in IBS

GFAP expression positively correlated with MC density, percentage of VPAC1* and VPAC2* MC in HC, while the same set of correlations were negative in IBS. VIP* MC and GFAP showed negative correlation in HC but positive correlation in IBS. To compare groups, the difference between the slopes (intercorrelations) from the correlations of HC and IBS was analyzed. Significant differences were found in GFAP versus MC density (Figure 3A), GFAP versus VIP* MC (Figure 3B), GFAP versus VPAC1* MC (Figure 3C), but not between GFAP and VPAC2* MC (Figure 3D).

### 3.5 EGC phenotype correlates differently to live bacterial passage through colonic biopsies of patients with IBS and HC

To evaluate the involvement of EGC in barrier function, we first analyzed intercorrelations from IBS and HC on the passage of live commensal or pathogenic bacteria (an event partly controlled by MC and VIP signaling) and GFAP expression. In HC, higher expression of GFAP correlated with less E. coli and Salmonella passing through colonic mucosa. In IBS, however, there was a slightly positive correlation between GFAP and the number of live bacteria translocating through the mucosa. Intercorrelation analysis between IBS and HC showed that GFAP levels had a significant interaction with the passage of Salmonella (Figure 3E), but not to E. coli, \( p = 0.12 \) (Supplementary Figure S2).

### 3.6 EGC mediators control paracellular permeability

To test whether GSNO, GDNF, and S100β control barrier function, colonic biopsies were mounted in Ussing chambers. All treatments significantly decreased the passage of FITC-Dextran 4000 (Figure 3F).

### 3.7 In vitro [Ca\(^{2+}\)] responses in EGC and MC suggest their ability to communicate

The [Ca\(^{2+}\)], mobilization assay with EGC CRL-2690 and MC RBL-2H3 cell lines revealed that both cell types responded with [Ca\(^{2+}\)], transients, providing evidence for signaling by the means of MC mediators on EGC (Figure 4A) and EGC mediators on MC (Figure 4B). Although marginally, experiments showed that EGC responded to all the mediators tested. Tryptase (1 nM) induced [Ca\(^{2+}\)], rise in 16% of the cells with long-standing effect but low peak rise (about \( \Delta F/F_0 = 4\% \)). Histamine triggered more cells (34%) showing slow and flipping [Ca\(^{2+}\)], transients (maximum mean peak \( \Delta F/F_0 = 6.8\% \)), finally VIP-induced responses in 24% of the cells (\( \Delta F/F_0 = 7.3\% \)). As expected, ATP (smaller embedded graph within Figure 4A) induced a clear [Ca\(^{2+}\)], rise (\( \Delta F/F_0 = 150\% \)) in all EGC (100%). ATP, GDNF, and S100β elicited [Ca\(^{2+}\)], transients in MC. ATP evoked responses in 62% of MC (peak \( \Delta F/F_0 = 48\% \)), GDNF induced [Ca\(^{2+}\)], rise (\( \Delta F/F_0 = 14\% \)) in 21% of the MC. Finally, S100β-induced [Ca\(^{2+}\)], rise in MC (26% of cells) reached a similar magnitude (\( \Delta F/F_0 = 11\% \)).

### 3.8 GDNF and S100β prevent VIP-induced degranulation in HMC-1.1

We further tested if GDNF or S100β could activate the HMC-1.1 or influence VIP-induced HMC-1.1 degranulation. Neither GDNF nor S100β-induced degranulation. In contrast, GDNF prevented MC degranulation induced by VIP (3 μM), both at 1 and 10 nM, \( p < 0.05 \) (Figure 4C). A similar pattern was seen for S100β, both at 10 and 30 nM, \( p < 0.05 \) (Figure 4D). No difference was observed in cells exposed to GNDF (1/10/30 nM) or S100β (10/30/100 nM) without addition of VIP.
3.9 Live bacteria, MC and neuronal products induce GFAP expression in EGC cell line, but with different profiling

EGC incubated with trypatse (1 nM), 5-HT (100 µM), histamine (10 µM), VIP (3 µM), or with live S. typhimurium, E. coli HM427, and Y. enterocolitica (cell:bacteria, 1:100) for 24 h displayed gliosis evidenced by increased GFAP expression (Figure 5A–B). Interestingly, the release of S100β was markedly inhibited, p < 0.0001, by all mediators (Figure 5C) while all bacteria strains led to increased S100β release, however only significantly by S. typhimurium (Figure 5D). GDNF was significantly increased by trypatse and Y. enterocolitica (Figure 5E–F).

4 DISCUSSION

In this study, we explored EGC phenotypical changes in IBS and how the activation of EGC is associated with MC-VIP increased translocation of live bacteria through the colonic mucosa.22 We found an increased expression of GFAP in the colonic mucosa of IBS, in which EGC acquire a reactive phenotype.45 We did not count the number of GFAP+ EGC nor S100β+ EGC, instead, we measured the intensity of GFAP expression, which in the literature is considered as a sign of EGC activation (gliosis).40 Data showed evidence of physiological interaction between EGC and MC in the human colon that could play a role in the pathophysiology of IBS, if unbalanced. However, the present study does not allow any conclusions about causality, nor disease specificity. Moreover, the data are restricted to females and there were no significant correlations between phenotypical changes in EGC and IBS symptoms. Nevertheless, the present study shows novel results that emphasize the important role of EGC in regulating the gut barrier function and, consequently, their overall relevance in GI functions.

Previous studies associating EGC and IBS symptoms have shown conflicting results. Wang and colleagues15 showed that increased expression of colonic GFAP in IBS patients correlated positively with severity and frequency of abdominal pain. Lilli et al.16 found decreased S100β-stained area in the colon of IBS patients that was negatively correlated with both frequency and intensity of pain and bloating. However, no differences in S100β and GFAP protein expression were found. S100β is constitutively expressed by a subset of EGC,8 presenting neuroprotective properties at low concentrations and pro-inflammatory properties when upregulated. In the present study, no changes in S100β could be identified, and the EGC phenotype in IBS did not correlate with abdominal pain nor bloating.

GFAP is expressed in CNS astrocytes, non-myelinating Schwann cells in the peripheral nervous systems, and in EGC.41 GFAP expression is regulated by several compounds such as lipopolysaccharide.42 Increased S100β and GFAP expression were observed in human small bowel-derived EGC exposed to lipopolysaccharide and IFN-γ.43 In our study, colonic GFAP expression in HC correlated positively with numbers of MC, VPAC1+ MC, and VPAC2+ MC, but negatively with VIP+ MC, contrasting to the correlations found in IBS. We previously showed that VIP and MC influence the translocation of live bacteria in IBS.21 Here, GFAP levels were negatively correlated with the translocation of S. typhimurium through the colonic mucosa in HC, while this interaction was positive in IBS patients. Hence, increasing EGC activation (as paralleled by GFAP levels) corresponded to limited passage of bacteria under healthy conditions. But reactive EGC, as found in IBS, seems to contribute to bacteria translocation. Our findings point to a physiological interaction between MC and EGC in the human colon. Such interaction could be disrupted in IBS, suggesting that both EGC and MC contribute to the downregulation of barrier function in IBS.

Bassotti44 reported increased MC numbers in patients with obstructed defecation. Even though not statistically significant, the authors highlighted that degranulated MC were always in close proximity to EGC in both patients and controls (also seen by our group, unpublished data). More recently, Cirillo25 showed gliosis and impaired neuronal function in duodenal submucous ganglia, which was associated with eosinophil and MC infiltration in patients with functional dyspepsia. Another study45 suggested the involvement of MC in inducing gliosis in an irinotecan-induced small intestinal mucositis model. In this study, Nogueira46 showed that compound 48/80-induced MC degranulation in mice prevented irinotecan-induced neuronal loss and reactive gliosis. Irinotecan itself induced MC degranulation followed by gliosis, but no changes in neurons and EGC were found after treatment with compound 48/80 alone. Another study,46 however, reported enteric neurons and visceral afferents activation by compound 48/80 independently of MC, suggesting that actors other than MC influence the ENS changes seen by Nogueira.45 All these findings together with ours suggest that both MC and EGC participate in the regulation of GI functions and likely contribute to pathological scenarios. In the CNS, glia-MC crosstalk is long known and has important implications to conditions such as Parkinson’s disease, multiple sclerosis, depression and autism spectrum disorder.28 CNS glia-MC crosstalk accelerates disease progression and neuroinflammation by feedback loops involving mediators like brain-derived neurotrophic factor, ATP, and a variety of cytokines.45 Regardless of the similarities between CNS-glial...
FIGURE 3  Intercorrelations of (A) glial fibrillary acidic protein (GFAP) expression (measured as fluorescence intensity (FI) in arbitrary units (a.u.) and mast cell (MC) density, (B) GFAP and percentage of vasoactive intestinal polypeptide (VIP)* MC, (C) GFAP and VIP receptor 1 (VPAC1)* MC, (D) GFAP and VPAC2* MC, and (E) GFAP and passage of Salmonella (S.) typhimurium in the colonic mucosa of irritable bowel syndrome (IBS) patients and healthy controls (HC). (F) Effects of S-nitrosoglutathione (GSNO), glial-derived neurotrophic factor (GDNF) and S100 calcium-binding protein β (S100β) on permeability to fluorescein isothiocyanate (FITC) dextran 4000 across colonic biopsies mounted on Ussing chambers for 120 min. Data are expressed as median (IQR), two-way ANOVA, followed by Bonferroni's test, *p < 0.05, **p < 0.01 and ***p < 0.001; each symbol represents the pool of three independent experiments. Data in A–E were processed with Spearman correlation followed by linear regression to test differences between slopes.
cells (oligodendrocytes, non-myelinating Schwann cells and astrocytes) and EGC, the latter represents a distinct glial lineage. Understanding the role EGC in the gut is imperative to comprehend the impact that EGC-MC interaction may have in health and during intestinal disease such as IBS.

MC are often found in close proximity to nerves. Barbara et al. demonstrated that MC and afferent nerves proximity may contribute to the abdominal pain in IBS. Nevertheless, interaction between MC and enteric nerves has also been described. EGC envelop and nourish enteric neurons throughout the gut; therefore, communication between MC and enteric neurons most likely evokes indirect or direct responses in EGC and may even rely on EGC for fine-tuning. The existence of a neuroimmune hub claims for EGC involvement. Our data showed EGC expression of GFAP increases along with MC density in the colonic mucosa. This correlation switched to negative in IBS patients, suggesting altered EGC-MC interactions in IBS. Data further showed that the relationship between GFAP expression and the percentage of VIP+ MC in HC and IBS was significantly different. In HC, there was a negative correlation with VIP+ MC, being more frequent when GFAP expression was lower. In IBS, however, there was a positive correlation. VPAC1+ and VPAC2+ MC showed a positive correlation to GFAP in HC, but negative in IBS.

VIP triggers responses through different receptors, VPAC1 and VPAC2, both inducing cellular adenyl cyclase activity and eventually [Ca2+]i responses. Both VPAC1 and VPAC2 produce either pro- or anti-inflammatory signals when bound to VIP. VIP can promote neuroprotection by inducing the glial release of neurotrophic factors in CNS-glial cells. Recently, Fung showed a VIP-regulated neural-EGC circuit in the mouse submucosal plexus. They showed that VIP activates VPAC1 in cholinergic neurons which, by the release of purines, led to [Ca2+]i transients in EGC. Interestingly EGC were inhibited by the activation of VPAC2 in their experimental settings; together, these findings indicate EGC involvement on VIP-signaling innervating the intestinal mucosa. Beyond cholinergic circuits, VIP

**FIGURE 4** [Ca2+]i responses in (A) enteric glial cell (EGC) cell line exposed to vasoactive intestinal polypeptide (VIP), histamine, tryptase, or adenosine triphosphate (ATP), as positive control (small-embedded graph) (B) mast cell (MC) cell line HMC-1.1 exposed to glial-derived neurotrophic factor (GDNF), S100 calcium-binding protein β (S100β) or ATP. Images were acquired for 2 min in assays with EGC cell line and for 4 min in those with HMC-1.1, every 1 s with exposure time of 100 ms. Arrows indicate addition of treatments and black bars represent duration of perfusion of treatments (20 s), and dotted light-gray line at (1.0) indicates baseline. ΔF/F0 is the difference between rise in signal corrected with background and expressed as % relative to baseline. VIP-induced degranulation of HMC-1.1 was measured by β-hexosaminidase release after pre-treatment with (C) GDNF or (D) S100β. White bars represent non-VIP-treated cells and light-gray bars represent VIP-treated cells. Data are expressed as median (IQR), one-way ANOVA, followed by Dunns’ test, ‘p < 0.05, compared to C treated with VIP. #p < 0.01, compared to C untreated; each symbol represents independent experiments in triplicates.
Figure 5 Enteric glial cell (EGC) activation induced by vehicle, tryptase (1 nM), serotonin (5-HT) (100 µM), histamine (10 µM), vasoactive intestinal polypeptide (VIP) (3 µM) or with live bacteria *Salmonella typhimurium* (*S. typh*), *Escherichia coli* HM427 (*E. coli*), and *Yersinia enterocolitica* (*Y. enter*), cell:bacteria, 1:100, for 24 h. C in graphs represents vehicle as control. Cell protein lysates were prepared for Western blotting, and the expression of GFAP and housekeeping protein β-actin were quantified. (A) GFAP expression induced by neuronal and mast cell (MC) mediators, (B) glial fibrillary acidic protein (GFAP) expression induced by live bacteria. Medium was collected and used for determination with ELISA of (C) S100 calcium-binding protein β (S100β) release induced by neuronal and MC mediators, (D) S100β release induced by live bacteria, (E) glial-derived neurotrophic factor (GDNF) release induced by neuronal and MC mediators, (F) GDNF release induced by live bacteria. Data are expressed as median (IQR), one-way ANOVA, followed by Dunns’ test or Kruskal-Wallis nonparametric t test, *p < 0.05, **p < 0.01 and ****p < 0.0001; each symbol represents independent experiments.
signaling in the gut implies the activation of adrenergic and serotonergic receptors whose expression and function on VIP-positive neurons has been demonstrated.9

The VIP-dependent translocation of bacteria21 correlated differently to EGC phenotype in IBS and HC. GFAP expression in the colonic mucosa of HC negatively correlated with the bacterial passage, suggesting protective involvement of EGC. Whereas in IBS, higher GFAP levels were linked to the loss of beneficial EGC activity. GFAP physiological expression may reflect protective mechanisms promoted by EGC, whilst IBS-reactive EGC might influence its microenvironment negatively, due to altered release of glial mediators. In fact, we showed that EGC activation induced by tryptase, 5-HT, histamine and VIP, displayed a variable pattern of S100 β expression with protein levels.60

Another recent study from Lee et al.59 reported higher GDNF mRNA expression in biopsies from patients with IBS-D. In contrast to our finding, Lin and colleagues58 showed increased GDNF protein levels in colon biopsy samples from patients with IBS-D. Another recent study from Lee et al.59 reported higher GDNF mRNA expression in biopsies from patients with IBS-C, but no difference between IBS-D and HC. These apparent contradictions may rely on several reasons such as number of study population and methods employed. Lin et al.58 studied a specific subgroup of patients with IBS-D, including both males and females. Lee and colleagues59 also included both male and female patients in their study; however, they assessed mRNA levels only, which often show poor correlation with protein levels.60 GDNF, as GSNO, is a potent regulator of barrier function10,61 and its decreased values in IBS reinforces that altered barrier function in IBS may result from EGC activity as well. As referred above, tryptase induced gliosis followed by increased release of GDNF by EGC in our in vitro assay. On the other hand, IBS biopsies presented lower levels of GDNF and more activated MC (thus releasing more tryptase) and EGC, which would point to a higher release of GDNF by EGC. However, other mediators, such as histamine and VIP, also induced gliosis, yet kept GDNF levels to that of untreated EGC, suggesting that other mediators can block GDNF release by activated EGC. In addition to that, GDNF is highly expressed by epithelial cells,33 hence biopsy GDNF levels more likely reflect overall mucosal levels rather than EGC-GDNF only. We used GSNO and GDNF to confirm their ability to tighten the colonic epithelia of HC biopsies in Ussing chambers. In addition, we tested whether S100β could also influence passage through the biopsies. All treatments resulted in diminished passage of FITC-dextran, indicating that all these EGC mediators (at physiological concentrations) influence the paracellular route through the colonic mucosa.

Both MC and EGC respond to environmental triggers, that is, bacteria and/or their products in the gut.64 Physiological loads of pathogens and/or antigens breaking into the epithelial layer are consequentially sampled by resident immune cells in the intestines, which could explain the positive association between GFAP and MC numbers in HC. This load of pathogens and antigens within the mucosa may be slightly higher, or altered in its composition, in IBS patients,21 which could potentially be a disturbing factor to EGC-MC interaction. Fettucciaro and colleagues recently suggested a role of EGC in the pathogenesis of Clostridium (C.) difficile infection.62 Further, authors hypothesized that EGC surviving C. difficile toxins acquire a senescence state which could be associated with an increased risk of IBS.64

In the present study, we could not find any correlation between microbiota composition and GDNF or S100β expressions, although we did find decreased levels of Actinomycetales. Actinomycetales is an order of bacteria where one member is the Actinomycetes, known to have antibacterial activity in the GI tract65 and confirms a previous study showing decreased levels of Actinomycetales in patients with IBS-D,66 which is coherent with our study. However, we could not find any significant correlation to EGC parameters and more detailed analyses are needed to further investigate this matter.

EGC activation results in an altered release of mediators such as GSNO, S100β and GDNF,48 which may also affect MC. On the other hand, MC respond with a wide range of packed mediators that are known to influence EGC.16 Here, we confirmed this and provided new evidence proving MC-EGC communication. Both EGC and MC are activated in IBS and seem to present altered interactions, possibly due to the presence of other disturbing factors. It is important to highlight that we focused on EGC and MC in our study, which per se constitutes a limitation, as there are several other cells participating in the regulation of the gut mucosa.

[Ca2+]i responses in vitro revealed that EGC responded to the MC mediators VIP, histamine and tryptase; whereas MC responded to EGC mediators S100β and GDNF, indicating their ability in communicating with each other. Furthermore, in vitro MC-degranulation assay showed that HMC-1.1 exposed to either S100β or GDNF kept normal response. Both GDNF and S100β prevented VIP-induced degranulation of HMC-1.1. Even though, in vitro models are limited by the nature of the cells, our findings provide substantial evidence of regulatory roles between EGC and MC.

Our data showed that VIP+ MC correlated positively with GFAP expression in IBS patients. Even though MC are not the main source of VIP,67 MC contain58 and release69 VIP and may thereby contribute to EGC activation. VIPergic enteric neurons most likely influence both MC and EGC activities in the GI. Altogether this indicates that this neuroimmune hub in the gut appears to ground significant events that ultimately contribute to developing IBS.

In summary, the study suggests that EGC-MC interaction in the human colon presents distinct profiles in health and in IBS, as overviewed in Figure 6. Despite limitations, we provided compelling evidence suggesting that, under homeostasis, EGC-increasing GFAP level is followed by increased MC numbers. The MC are VIP-tuned to regulate bacterial translocation in the colon, contributing to the barrier function. In IBS, however, dysregulated VIP circuitry together with reactive EGC destabilize MC. This might contribute to weakening the barrier and, in turn, may result in increased passage of antigens, creating a feedback loop. Altogether, these findings point to a VIP-tuned EGC-MC activity in the human colon with a potential relevance to IBS.
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DISCLOSURES

The authors have no conflicting interests to declare.
AUTHOR CONTRIBUTIONS
FMF designed the study, performed experiments, analyzed data, and wrote the manuscript; MCB performed experiments, analyzed data, co-wrote the manuscript, and made the graphical artwork; CML contributed to microbiota analysis, visualization, and interpretations; MPJ was involved in statistical analysis and interpretation; SAW designed the study, provided financial support, interpreted data and co-wrote the manuscript; ÁVK designed the study, provided financial support, interpreted data, co-wrote the manuscript, and was responsible for the final version. All authors read and approved the final manuscript.

ORCID
Felipe Meira de Faria https://orcid.org/0000-0002-7875-0691
Michael P. Jones https://orcid.org/0000-0003-0565-4938
Ása V. Keita https://orcid.org/0000-0002-6820-0215

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.