Elevated F-EDN correlates with mucosal eosinophil degranulation in patients with IBS—A possible association with microbiota?

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Summary Sentence: We show, for the first time, a correlation between F-EDN levels and degranulation of eosinophils in the colon of IBS patients which might play a role in IBS pathophysiology.

Abstract
Eosinophils have been linked to functional dyspepsia; however, less is known about their role in irritable bowel syndrome (IBS). This study tested the hypothesis of alterations in levels of fecal eosinophil-derived neurotoxin (F-EDN) and eosinophil density and degranulation within the colonic mucosa of IBS patients compared with healthy controls (HC). Colonic biopsies were collected from 37 IBS patients and 20 HC and analyzed for eosinophil numbers and local degranulation of eosinophil cationic protein (ECP) by histologic procedures. Fecal samples were collected for F-EDN and microbiota analysis. Differentiated 15HL-60 cells were used in vitro to investigate the direct effect of live bacteria on eosinophil activation measured by a colorimetric assay with o-phenylenediamine (OPD) substrate. We observed a higher number of eosinophils and increased extracellular ECP in the mucosa of IBS patients compared with HC. Moreover, F-EDN levels in IBS samples were elevated compared with HC and positively correlated to extracellular ECP. Metagenomic analysis showed significant correlations between bacterial composition and eosinophil measurements in both HC and IBS patients. In vitro experiments revealed an increased degranulation of 15HL-60 after stimulation with Salmonella typhimurium, Salmonella enterica, and Yersinia enterocolitica. To conclude, we could demonstrate alterations related to eosinophils in IBS, and, for the first time, a positive correlation between F-EDN levels and degranulated eosinophils in the colonic mucosa of IBS patients. Together our results suggest that eosinophils play a role in the pathophysiology of IBS and the mechanisms might be linked to an altered microbiota.

KEYWORDS
bacteria, eosinophil cationic protein, irritable bowel syndrome, fecal eosinophil-derived neurotoxin

Abbreviations: Arbitrary units, AU; eosinophil cationic protein, ECP; eosinophil protein X, EPX; fecal eosinophil-derived neurotoxin, F-EDN; gastrointestinal, GI; HADS anxiety, HADS-A; HADS-depression, HADS-D; healthy controls, HC; hospital anxiety and depression scale, HADS; IBS-constipation, IBS-C; IBS-diarrhea, IBS-D; IBS-mixed, IBS-M; IBS-severity scoring system, IBS-SSS; inflammatory bowel disease, IBD; irritable bowel syndrome, IBS; minutes, (min); o-phenylenediamine, OPD; ulcerative colitis, UC
1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a very common brain–gut disorder, with a largely unknown pathophysiology. However, it appears that IBS symptoms are driven by factors such as motility abnormalities, visceral sensation, altered brain–gut interactions, psychosocial distress, altered immune responses, imbalance in gut microbiome, and abnormal intestinal permeability. In both colon and ileum of patients with IBS, low-grade mucosal inflammation is frequently observed. There is increasing evidence showing elevated number of mast cells in IBS and increased mast cell degranulation with elevated secretion of mediators such as tryptase and histamine. In addition, studies have shown an increased number of T cells and increased secretion of proinflammatory cytokines. While studies have confirmed the role for eosinophils in functional dyspepsia, another functional disorder of the brain–gut axis, which is considerably overlapping with IBS, studies investigating the role of eosinophils in IBS are inconsistent, showing both equal and increased eosinophil density in IBS patients compared with healthy controls (HC). We recently showed an increased count of eosinophils in the colonic mucosa of IBS-mixed (IBS-M) patients compared with HC, which was positively correlated to increased intestinal paracellular permeability. In healthy mucosa, there is only moderate amounts of active eosinophils. We have previously demonstrated increased numbers of activated eosinophils in the colonic mucosa of both active and inactive ulcerative colitis (UC), indicating both a proinflammatory and a tissue-repairing role of eosinophils in UC. Eosinophil-derived neutrotoxin (EDN), also known as eosinophil protein X (EPX), is a protein secreted by activated eosinophils. Fecal levels of EDN (F-EDN) have shown to reflect disease activity in patients with inflammatory bowel disease (IBD). We previously showed increased F-EDN levels in patients with IBD that were further related to an increased risk of relapse. However, EDN measured in stool samples from IBS patients were equal to the EDN levels of HC, even though 11% of patients had EDN levels above the upper normal limit, previously defined.

To our knowledge, there is no study present investigating F-EDN in IBS patients, and for correlating F-EDN levels with the presence or activation of mucosal eosinophils. In the present study, biopsies and fecal samples from patients with moderate to severe IBS were collected and compared with HC. Eosinophil density and distribution of intracellular and extracellular eosinophil cationic protein (ECP) were quantified in colonic biopsies by histologic techniques followed by microscopy. In addition, EDN and metagenomic analyses of the intestinal microflora were performed on fecal samples. To investigate the direct effect of live bacteria on eosinophil activation, in vitro experiments were performed using differentiated human eosinophil cell line 15HL-60. Degranulation was assessed by a colorimetric assay using o-phenylenediamine (OPD) substrate.

2 | MATERIALS AND METHODS

2.1 | Patients

The total material constituted of 37 women with IBS (median age 31 years, range 19–55 years) with moderate to severe symptoms and 20 age-matched female HC (27 years, 21–47), recruited at the Gastroenterology Department, University Hospital, Linköping. IBS patients were classified into IBS-M (n = 21), IBS-diarrhea (IBS-D) (n = 8), or IBS-constipation (IBS-C) (n = 8) subgroup based on the predominant stool consistency according to the Rome III criteria. The HC were recruited by advertising. The inclusion criteria of HC included no medical history of chronic gastrointestinal (GI) symptoms or disorders, and no medication intake. Exclusion criteria for both groups included organic GI disease, metabolic, neurologic, or severe psychiatric disorders, and self-reported nicotine intake within 2 months before the entry to the study, allergy, and use of nonsteroidal anti-inflammatory drugs. None of the patients related the onset of their symptoms to infectious gastroenteritis. The regional ethical review board approved the study (2013/111-31), and a written, informed consent was obtained from each patient included in the study in accordance with the Helsinki declaration.

2.2 | Questionnaires

2.2.1 | IBS-severity scoring system

IBS-severity scoring system (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. Each item generates a score between 0 and 100 with a maximal sum score of 500. Sum score indicates mild (75–175), moderate (175–300), or severe (>300) disease.

2.2.2 | GI symptom diary

IBS patients recorded their GI symptoms on 14 consecutive days. Symptoms, bowel movements, and stool consistency defined by Bristol Stool Chart were reported.

2.2.3 | Hospital anxiety and depression scale

The hospital anxiety and depression scale (HADS) was used to measure symptoms of depression and anxiety. The scale consists of 7 items for depression (HADS-D) and anxiety subscales (HADS-A), with scores on each subscale ranging from 0 to 21. Cut-off values are indicated as ≥8 for subclinical (suspicious) anxiety or depression and ≥11 as definite cases on both the HADS-D and HADS-A, respectively.
2.3 | Collection of biologic samples

After 8 h of fasting, biopsies were taken, without sedation, with scope insertion approximately 30–40 cm orally from linea dentata during a flexible sigmoidoscopy. Biopsies were directly put in ice-cold oxygenated Krebs buffer (115 mM NaCl, 1.25 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 2 mM KH\(_2\)PO\(_4\), and 25 mM NaHCO\(_3\), pH 7.2) and transported to the laboratory.

Fecal samples were collected using feces sample containers (Sarstedt, Helsingborg, Sweden) within 2 weeks prior to the sigmoidoscopy. They were sent by mail on the day of collection to the University Hospital in Linköping and were stored in –70°C within 24 h from collection.

2.4 | Analysis of F-EDN

Fecal extracts from 33 IBS patients (median age 31 years, range 19–55 years) and 20 HC (27 years, 21–47) were prepared as described previously\(^{27}\) and analyzed for EDN in a blinded fashion. Levels of F-EDN were measured by ELISA (Diagnostics Development AB, Uppsala, Sweden). The concentration of F-EDN was adjusted for water content as previously described\(^{27}\) and expressed as µg/g semidy feces. Normal levels were set to ≤2.15 µg/g, as previously described.\(^{27}\) The intra- and interassay variations were less than 10% for the assay.

2.5 | Quantification of mucosal eosinophils

Colonic biopsies from 37 IBS patients and 20 HC were fixed in 4% paraformaldehyde in PBS for 24 hours at 4°C followed by embedding in paraffin and sectioning at 5 µm. Slides were hydrated according to standard procedures and incubated in Harris hematoxylin (Histolab, Gothenburg, Sweden) for 4 minutes (min). After rinsing in tap water, slides were immersed in 1% hydrochloric alcohol for 10 seconds, reimmersed in tap water followed by tap water with 2–3 ammonia drops for 1 min. Last, slides were incubated in eosin (Analytical Standards, Mölnlycke, Sweden) for 1 min and mounted with Pertex mounting media (Histolab). The total amount of eosinophils was quantified in a blinded fashion using a Nikon E800 microscope connected to software NIS elements (Nikon Instruments Inc., Tokyo, Japan). The number of eosin-positive cells were quantified manually at 40x objective. A minimum of 8 fields of view per biopsy section were quantified. Only fields of view that were completely covered by the tissue were included in the analysis. The mean value for each section was estimated for eosin-positive cells and a median value of the number of eosinophils from patients with IBS and HC, respectively.

2.6 | Quantification of ECP and study of intra- and extracellular distribution

A subsample of 23 IBS patients (median age 30 years, range 21–55 years) and 18 HC (28 years, 22–47) was randomly selected to study the granule distribution of ECP in colonic biopsies. Sections were hydrated, immersed in citrate buffer at 60°C for 30 min, and incubated for 5 min with background sniper (Histolab). Slides were rinsed and incubated over night at 4°C with mouse anti-human ECP 1:200 (Diagnostic Development). After rinsing, slides were incubated with 1:400 secondary antibody Alexa Fluor 594-conjugated rabbit-anti-mouse (Life Technologies, Thermo Fisher Scientific, Stockholm, Sweden) for 1 hour at room temperature. Slides were rinsed and mounted with Prolong® Gold-DAPI (Life Technologies). One transversal section was used for each staining and negative controls were included in all experiments. Between 8 and 14 images of single eosinophils (single when possible) were acquired with LSM800 Zeiss Inverted Confocal with a 60x oil immersion objective. Images were processed using ImageJ/ FIJI\(^{33}\) and analyzed using Cell Profiler 2.2.0 software,\(^{34}\) as previously described.\(^{15}\) To avoid artefacts, only eosinophils deeper into the mucosa were included in the analysis, and not those located close to the epithelial surface. In brief, all granules detected inside defined cellular limits were considered as intracellular and the granules localized outside these limits were considered as extracellular content and interpreted as degranulated ECP. After setting the threshold, the average of integrated intensity per cell and the extracellular content surrounding the cell was measured. The median value was calculated from all the averages and expressed in arbitrary units (AU).

2.7 | Fecal microbiota analysis

Frozen fecal samples were sent to Eurofins Genomics (Ebersberg, Germany) for whole genome sequencing. After thawing, samples went through cell wall lysis and elimination of compounds (e.g., digested food, bile acids, and bilirubin) followed by DNA purification and extraction. DNA sequencing was performed using an Illumina Technology HiSeq 4000 (read mode 2 × 150 bp). The sequence reads were inspected for base quality from the 3't and 5'tends for removal of low-quality calls using a sliding window approach. Bases with average read quality below 15 were considered of low quality. Filtered reads were then aligned to the GRCh37/hg19 genome using the BWA sequence aligner.\(^{35}\) After quality filtering and trimming steps, only mate pairs (forward and reverse) reads were used for the downstream taxonomy analysis.

Taxonomy profiling of the nonhost sequences was performed using Metaphlan2, which is a popular tool for clade-specific marker gene-based taxonomic profiling.\(^{36,37}\) This method rapidly measures the presence and abundance of microbial taxa at different taxonomic levels. Metaphlan2 profiles provide the composition of gut microbial species and both absolute and relative abundances based on the ~1 M unique clade-specific marker genes database. Bacterial genera that were not present in at least 10% of the samples were filtered away.

2.8 | Effects of live bacteria on eosinophil degranulation in vitro

To study the effects of live bacteria on the degranulation of eosinophils, the human eosinophil cell line 15HL-60 (ATCC, Manassas, Virginia,
USA) was used. Cells were grown in a 5% CO₂ incubator at 37°C in RPMI 1640 media (Gibco, Thermo Fisher Scientific, Stockholm, Sweden) supplemented with 10% FBS (Gibco), 10 mM/ml penicillin–streptomycin (Gibco), and 5 mM HEPES (Gibco), final pH 7.8. For differentiation into an eosinophil-like phenotype, 15HL-60 cells were treated with 0.5 mM sodium butyrate (Sigma–Aldrich, Stockholm, Sweden) for 6 days at a final density of 10⁵ cells/ml, based on a previously described differentiation protocol. Differentiation was previously set-up and confirmed using different methods, as previously described. For each experiment, differentiated 15HL-60 cells were washed and 10⁵ cells/well were seeded in HBSS (Gibco) on a 96-well plate. Following previous methodology, cells were primed with 10 ng/ml of GM-CSF (Sigma–Aldrich) for 1 hour at 37°C. Live bacteria, Yersinia (Y.) enterocolitica, S. enterica, S. typhimurium, and the adherent invasive Escherichia coli (AIEC) HM427, were prepared as previously described. Bacteria were grown to the middle of the exponential growth phase in liquid culture, that corresponds to optical density of 0.6 (OD₆₀₀).

Differentiated primed 15HL-60 cells were infected with bacteria with a multiplicity of infection (MOI) of 100, or HBSS as control, for 2 hours at 37°C. In parallel, nonprimed and undifferentiated cells acted as controls. Supernatants were collected and cells were lysed with 0.1% Triton X-100 (Sigma–Aldrich) in PBS. In order to evaluate the cell degranulation, eosinophil peroxidase release was measured both in lysed cells and supernatants by colorimetric assay using OPD as substrate. Results were pooled together to calculate the total amount of eosinophil peroxidase release per well. Experiments were repeated 5 times and performed in triplicates. Results are presented as eosinophil peroxidase release in 15HL-60 cells stimulated with bacteria normalized to control cells (vehicle).

2.9 Statistical analysis

Data were analyzed using the GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Normality test showed that the data did not follow the normal distribution and are presented as median (25th–75th percentile). Comparisons between 2 groups were performed using Mann–Whitney U test. Correlations between different parameters were performed using 2-tailed Spearman correlation test followed by simple linear regression analysis to test intercorrelations. Correlations between bacteria composition and number of eosinophils, extracellular ECP, intracellular ECP, and F-EDN were performed using the cov.test from the R package Psych, stratified for HC or IBS patients. Correlations with values *P < 0.05 and rho > 0.3 were kept for further analyses. Significance was set to *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. For processing of microbiota data, a principal component analysis of Bray–Curtis dissimilarities and correlation analysis were calculated using R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) and packages vegan and labdsv and visualized with ggplot2.

3 | RESULTS

3.1 Symptom scores

IBS patients had a median to severe symptom score of 341 (interquartile range 285–406), according to IBS-SSS. Increased number of mucosal eosinophils in IBS

Eosin staining showed an increased number of eosinophils per mm² in IBS patients compared with HC, *P < 0.0001 (Fig. 1A), which is in line with our previous findings in a smaller cohort of IBS-M patients. Representative photographs of cells identified as eosinophils are shown in Figure 1B. When comparing microscopy results patient by patient, it was shown that approximately 95% of the IBS patients possessed higher eosinophil numbers compared with the mean of HC, which indicates that a mucosal immune activation seems to be present in the colonic mucosa of almost all IBS patients. There was no significant difference in the number of eosinophils between stool consistency subgroups according to Rome III criteria. There were negative correlations between number of eosinophils and the proportion of hard stools, pain, bloating, and IBS-SSS (Figs. 1C–1F).

3.3 Equal amounts of total ECP in IBS patients and HC

Immunofluorescent staining and image analysis showed equal amounts of total ECP (the sum of intracellular and extracellular ECP) in biopsies of IBS patients compared with HC (Fig. 2A). This indicates an equal total production of ECP per eosinophils in both HC and IBS patients. There was no significant difference in total ECP between IBS subgroups (data not shown).

3.4 Decreased intracellular ECP in IBS

Further image analysis showed decreased levels of intracellular ECP in eosinophils from IBS biopsies compared with HC, *P < 0.001 (Fig. 2B). Representative images are shown in Figure 2D, where intracellular ECP is indicated in cyan. There was no significant difference in intracellular ECP between IBS subgroups (data not shown). Noticeably, there was a significant negative correlation between intracellular ECP and proportion of defeactions with urgency (r = −0.54, p = 0.02), but no significant correlation to other symptoms.

3.5 Increased extracellular ECP in IBS

The extracellular ECP levels were increased in biopsies from IBS patients compared with HC, *P < 0.05 (Fig. 2C). Representative images
FIGURE 1  Elevated eosinophil numbers in the colonic mucosa of irritable bowel syndrome (IBS) compared with healthy controls (HC) and correlation with symptoms. (A) Quantification of eosinophils in IBS and HC stained by hematoxylin and eosin. (B) Representative images of cells identified as eosinophils (arrows) in IBS and HC, 60× objective. (C) Linear regression analysis between number of eosinophils and the proportion of stools with hard consistency in patients with IBS. (D) Linear regression analysis between the number of eosinophils and IBS-severity scoring system (IBS-SSS). (E) Linear regression analysis between the number of eosinophils and total abdominal pain. (F) Linear regression analysis between the number of eosinophils and abdominal bloating. Bars represent median (25th–75th percentile) and comparisons were done with Mann–Whitney U test. ****P < 0.0001. Analysis in C–F was performed with simple linear regression are shown in Figure 2D, where extracellular ECP is shown in magenta. There was no significant difference in extracellular ECP between IBS subgroups (data not shown). Extracellular ECP was not significantly correlated to any symptoms in IBS patients.

### 3.6  Increased F-EDN in IBS

The levels of F-EDN were higher in IBS feces compared with HC, P < 0.05 (Fig. 3). There was no significant difference in F-EDN between IBS subgroups (data not shown). In IBS patients, F-EDN significantly correlated positively with HAD scores for anxiety (r = 0.37, P = 0.03) but not with depression (r = 0.3, P = 0.08). There was no significant correlation between F-EDN and other symptoms in IBS.

### 3.7  Extracellular ECP correlates with increased levels of F-EDN

Spearman correlation analysis revealed significant associations between extracellular ECP and F-EDN in IBS patients (r = 0.6, P < 0.01), while no association was found in HC (r = 0.2, P = 0.4). Linear regression analysis revealed a weak significant association between extracellular ECP and F-EDN in HC (r = 0.28, P < 0.05) (Fig. 4A), while a stronger association was shown in IBS patients (r = 0.53, P < 0.001) (Fig. 4B).

### 3.8  Microbiota

After removal of bacterial genera absent in 90% of the samples, 62 bacteria on genus taxonomic level remained. On a beta diversity level, there were no significant differences in microbiota composition between IBS patients and HC (Fig. 5). Correlation analysis between the abundance of certain bacterial genera and eosinophil parameters (F-EDN, intra- and extracellular ECP, number of eosinophils) yielded 10 significant correlations for IBS patients (Table 1) and 5 for HC (Table 2) after statistical correction.

### 3.9  Increased degranulation of eosinophils in vitro by Salmonella and Yersinia

The in vitro degranulation challenge using the 15HL-60 cells during bacterial infection and the quantification through the colorimetric assay using OPD substrate showed a higher degranulation of the 15HL-60 cells when infected with S. enterica (P < 0.01), S. typhimurium (P < 0.01), and Y. enterocolitica (P < 0.01) as compared with vehicles.
The localization of eosinophil cationic protein (ECP) is mostly intracellular in eosinophils of healthy controls (HC) while mostly extracellular in patients with irritable bowel syndrome (IBS). Quantification of (A) total, (B) intracellular, and (C) extracellular ECP in colonic biopsies of HC and patients with IBS by immunofluorescence procedures followed by image analysis. (D) Representative images of eosinophils with identified plasma cell membrane (white line), intracellular ECP (cyan), and extracellular ECP (magenta) in HC and IBS. Quantification was done in 60x objective confocal images and expressed in arbitrary units (AU). Bars represent median (25th–75th percentile). Comparisons between groups were done with Mann–Whitney U test. *P < 0.05 and ***P < 0.001

TABLE 1 Correlations between bacterial genera and eosinophil parameters in patients with irritable bowel syndrome

| Parameter               | F-EDN | rho     | P value |
|-------------------------|-------|---------|---------|
| Anaerotruncus           | F-EDN | 0.396   | 0.037   |
| Coprobacillus           | F-EDN | 0.440   | 0.019   |
| Eggerthella             | F-EDN | 0.412   | 0.030   |
| Pseudoflavonifractor    | F-EDN | 0.423   | 0.025   |
| Solobacterium           | F-EDN | −0.451  | 0.016   |
| Clostridiaceae          | Eosinophil density | −0.436 | 0.020   |
| Gordonibacter           | Eosinophil density | 0.516 | 0.005   |
| Odoribacter             | Eosinophil density | −0.398 | 0.036   |
| Adlercreutzia           | Extracellular ECP | −0.532 | 0.028   |
| Corynecreatia           | Intracellular ECP | −0.445 | 0.043   |

F-EDN = Fecal eosinophil-derived neurotoxin; ECP = eosinophil cationic protein. For processing of data, a principal component analysis of similarities and correlation analysis were calculated using R version 4.0.2 and packages vegan.
TABLE 2  Correlations between bacterial genera and eosinophil parameters in healthy controls

| Parameter       | rho  | P value |
|-----------------|------|---------|
| Eggerthella     | −0.708 | 0.005 |
| Methanobrevibacter | 0.538 | 0.047 |
| Akkermansia     | −0.732 | 0.007 |
| Holdemania      | −0.69  | 0.013 |
| Prevotella      | −0.741 | 0.006 |

ECP = eosinophil cationic protein. For processing of data, a principal component analysis of similarities and correlation analysis were calculated using R version 4.0.2 and packages vegan.

FIGURE 3  Levels of fecal eosinophil-derived neurotoxin (F-EDN) are significantly higher in patients with irritable bowel syndrome (IBS) compared with healthy controls (HC). F-EDN was measured by enzyme-linked immunosorbent assay. Data are presented as median (25th–75th percentile) and comparisons between groups were done with Mann–Whitney U test. *P < 0.05

(Fig. 6). The only pathogen that resulted in a nonsignificant increase of the 15HL-60 cells degranulation was AIEC HM427.

4 | DISCUSSION

In the present study, we confirmed our previous findings of an increased number of eosinophils in the colonic mucosa of IBS-M patients compared with HC and showed increased eosinophil density in all IBS subtypes. We detected increased degranulation of ECP in IBS colonic biopsies and demonstrated, for the first time, a positive correlation between extracellular ECP and F-EDN. In vitro experiments using differentiated 15HL-60 cells infected with pathogens often associated with IBS onset, such as *S. typhimurium* and *Y. enterocolitica*, resulted in eosinophil degranulation. Together, these results suggest that eosinophil activation might be associated with the presence of bacteria, such as *Coprobacillus* and/or *Clostridiaceae*, and this might play a role in IBS pathophysiology.

Previous findings of eosinophil density in IBS mucosa are inconsistent. Our group found an increased number of eosinophils in the colon of patients with IBS-M. Similarly, Singh and colleagues recently showed a higher density of eosinophils in colon of youth with IBS as compared with HC. In line with this, Dyadyk et al. showed an increased severity of eosinophil infiltration in the colonic mucosa of IBS patients in comparison with individuals without any GI disease. De Silva et al. found equal eosinophil counts in the colon of IBS patients and HC with significantly more eosinophils in the cecum of IBS patients; however, in this study, only patients with postinfectious IBS were investigated, a patient group that might have a different pathophysiology. Walker et al. showed increased eosinophil levels only in the colonic mucosa of IBS patients who also suffered from colonic spirochetosis, defined by histologic observation of spirochetal strains of *Brachyspira* within the biopsies. In our study, the microbiome data did not indicate the presence of spirochetosis in any fecal sample; however, we did not investigate the presence of mucosal-bound *Brachyspira*. Jabbar et al. recently showed that mucosal *Brachyspira* colonization was observed in 31% of the IBS patients but in none of the HC investigated. The presence of mucosal *Brachyspira* was also associated with mild mucosal inflammation with expansion of plasma cells, mast cells, and eosinophils. Considering this, it might be that mucosal-bound *Brachyspira* is present in some individuals.

When looking at the total levels of ECP in IBS mucosa compared with HC, we observed no differences, but interestingly further investigation of the ECP distribution revealed that intracellular ECP levels in IBS patients were significantly lower when compared with HC. In line with this, the levels of extracellular ECP were significantly higher in IBS when compared with HC. Magnusson et al. showed increased levels of eosinophil markers, in particular EPX/EDN, in feces from patients with food-related GI symptoms. Of the 13 patients investigated, 8 reached the criteria for IBS according to ROME II. Similar observations have been reported by Fritscher-Ravens et al. who challenged IBS patients with atypical food allergens during endoscopy. Authors observed eosinophil degranulation in the duodenum, when patients were challenged with wheat, for example. We, on the other hand, observed increased eosinophil degranulation in the colon. Although, we have carefully excluded patients with known allergies, we did not...
Extracellular eosinophil cationic protein (ECP) and fecal eosinophil-derived neurotoxin (F-EDN) show a positive correlation. (A) Linear regression analysis between ECP and F-EDN in healthy controls (HC). (B) Linear regression analysis of ECP in biopsies and F-EDN in patients with irritable bowel syndrome (IBS). Analysis was performed with simple linear regression.

Principal coordinate analysis plot based on the relative abundance of 62 bacteria on genus taxonomic level. HC = healthy controls, IBS = irritable bowel syndrome, IBS-M = IBS-mixed, IBS-D = IBS-diarrhea, IBS-C = IBS-constipation.

Infection with pathogens induces eosinophil degranulation in vitro. Degranulation of the human eosinophil cell line 15HL-60 was measured by a colorimetric assay using o-phenylenediamine substrate. Differentiated 15HL-60 cells were exposed to vehicle or live Salmonella enterica (S. ent), Salmonella typhimurium (S. typh), Yersinia enteroocolitica (Y. ent), or the adherent invasive Escherichia coli HM427 (AIEC) for 2 hours. Experiments were repeated 5 times and performed in triplicates. Data are presented as eosinophil peroxidase release in 15HL-60 cells stimulated with live bacteria normalized to vehicle (%). Data are presented as median (25th–75th percentile) and comparisons between groups were done with Mann–Whitney U test. **P < 0.01.

exclude IBS patients with subjective food intolerance and we did not exclude patients with functional dyspepsia, a condition that often overlaps with IBS and is known to be related to duodenal eosinophilia. The question that rises from the observations above is if the underlying mechanism of eosinophil degranulation is similar in these studies. The simplest answer can be that the increased eosinophil degranulation may be related to dietary factors, which were not measured in our study. This is supported by previous published evidence showing increased ECP levels in feces that can be used as a marker for food hypersensitivity in IBS. Furthermore, the same study reports that 25% of IBS patients suffer from food hypersensitivity. In our study, we cannot completely exclude unknown food allergies, atypical allergies, or food hypersensitivity since all these conditions may be undiagnosed or difficult to diagnose with the methods currently available.

How eosinophils are activated in IBS is not clear, but there may be an association with bacterial presence. Our in vitro studies using differentiated 15HL-60 cells showed that S. typhimurium, for example, induced the eosinophils to degranulate, and it might be that the increase in extracellular ECP and F-EDN that we observed in IBS is due to an altered fecal microbiota composition. Previous research has demonstrated that IBS patients’ bacterial composition is often compromised and possibly linked with the disease severity. Further than an altered microbiome, we have previously shown an increased bacterial passage in IBS patients related with
mucosal eosinophils and the secretion of eosinophilic proteins into the feces.

We found that in IBS patients, F-EDN significantly correlated positively with HADS-A, but not with other symptoms. Anxiety is a very common comorbidity in IBS and eosinophilia has recently been shown to be independently linked to anxiety in patients with functional dyspepsia, another common functional brain–gut disorder that is considerably overlapping with IBS. Singh et al. recently showed in IBS patients that rectosigmoid eosinophilia was associated with higher anxiety scores. Our data show that eosinophilic activity, but not eosinophilic density was significantly positively correlated with anxiety. Our findings combined with previously published data indicate that F-EDN is positively correlated with reported anxiety in IBS and eosinophil degranulation in the colon. Further work is necessary to assess if F-EDN and/or other molecules secreted by eosinophils during degranulation, like ECP, can become useful as clinical biomarkers in IBS subgrouping. Eosinophilic density was inversely related to proportion of hard stools but also to IBS severity, pain, and bloating. The IBS patients in this study all had moderate to severe symptoms, and these findings are therefore indicating that eosinophils are not primarily responsible for the severe abdominal pain and bloating reported by these IBS patients. Chronic visceral pain has a complex pathophysiology involving several mechanisms in the central nervous system and therefore our findings are not unexpected.

IBS is a heterogenic patient group regarding pathophysiology, and as one could expect, some of the IBS patients possessed levels of ECP and/or F-EDN equal to HC. Therefore, our findings indicate that the measurement of ECP and F-EDN could be of importance for IBS subgrouping in relation to mild inflammation and this could be relevant for the prediction of treatments.

In conclusion, we showed an increased number of mucosal eosinophils and increased eosinophil degranulation assessed through extracellular ECP and F-EDN in patients with IBS compared with HC, which may be related to bacteria presence. Also, we showed for the first time, a positive correlation between F-EDN levels and degranulated mucosal eosinophils in patients with IBS. Together our results suggest that eosinophils play a potential role in the pathophysiology for at least a subgroup of IBS patients, and the mechanisms might be related to an altered microbiota.

**AUTHORSHIP**

M.C.-B. designed the study, performed the experiments, analyzed the data, and participated in drafting the manuscript. F.M.F. performed the experiments, analyzed the data, and cowrote the manuscript. O.B. analyzed and interpreted the data and participated in drafting the manuscript. C.M.L. performed the microbiota analysis, visualization, and interpretations. P.D.R. contributed with the microbiota analysis, visualization, and interpretations. O.B. helped with the acquisition of patients and clinical data, interpreted the data, and cowrote the manuscript. C.P. performed the experiments and analyzed the data. S.A.W. worked on the conception and design of the study, acquisition
of patients and clinical data, interpreted the data, provided financial support, and participated in drafting the manuscript. M.C. worked on the conception and design of the study, interpreted the data, provided financial support, and cowrote the manuscript. Å.V.K. initiated, conceptualized, and designed the study, interpreted the data, provided financial support, and had main responsibility for drafting the article and for the final version before approval.

ACKNOWLEDGMENTS
We would like to thank all patients and HC for their participance. We also thank Mrs Lena Svensson, Linköping University, for preparing the fecal samples for EDN-analysis; Mr Martin E Winberg, Linköping University, for sectioning and assisting in the ECP degranulation analysis, and Mr Vesa Loitto at the Microscopy Core Facility, Linköping University, for assisting with imaging gear and expertise.

This work was supported by grants from the Apotekare Hedberg Foundation (Å.V.K.), Magnus Bergvall Foundation (2018-02604, Å.V.K.), Bengt Ihre Foundation (SLS-788111, Å.V.K.; SLS-882561, Å.V.K.), Ruth and Richard Jolin Foundation (2017-00350, 2019-00347, Å.V.K.), County Council of Östergötland (Lio-934618, Å.V.K.), Mucosa Infection and Inflammation Center-MIIC (Å.V.K./S.A.W.), Medical Faculty, Uppsala University, Uppsala Sweden (M.C.).

DISCLOSURES
The authors declare no conflicts of interest.

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How to cite this article: Casado-Bedmar M, de-Faria FM, Biskou O, et al. Elevated F-EDN correlates with mucosal eosinophil degranulation in patients with IBS – a possible association with microbiota?. J Leukoc Biol. 2022;111:655–665. https://doi.org/10.1002/JLB.4A0521-228R