

Spain suggest colonic mast cells are increased in IBS, whereas studies from Northern Europe and the United States have not confirmed the findings (8–12). Increased intraepithelial lymphocytes (IELs) in colonic biopsies were observed in IBS in a New Zealand study (13), but the small intestine was not examined, and little other data are available. However, none of the past studies were population-based, and selection bias is difficult to exclude in the reports to date.

As there may be intestinal dysbiosis and increased intestinal permeability in IBS (14), we speculated Paneth cells may be altered in IBS which has not been previously explored, although they are reduced in Crohn disease (15). Paneth cells are located throughout the small intestine and importantly play a role in host-microbe interactions through secretion of antimicrobial peptides, as well as helping to maintain intestinal integrity (16). Increased metaplastic Paneth cells are seen in the colon in damage and infection (17).

We aimed to determine what is normal in terms of immune cell counts in the ileocolonic region in subjects identified from a population-based colonoscopy study and determine whether there is histological evidence of increased IELs, mast cells, eosinophils, or Paneth cell changes in the terminal ileum and colon in subjects with IBS. On finding alterations in ileocolonic IELs in IBS, we then investigated whether the mucosa-associated microbiota (MaM) and fecal microbiota (FM) differed between subjects with high and low IEL counts where samples were available.

METHODS

Participants

In this study, ileocolonic biopsies from subjects from the population-based colonoscopic (PopCol) study with a normal colonoscopy, i.e., no inflammatory bowel disorder or spirochetosis or other detected intestinal pathogens that were not taking proton pump inhibitors (PPIs) or nonsteroidal anti-inflammatory drugs (NSAIDs) at the time of investigation, were evaluated histologically.

The PopCol study has previously been described in detail (18). In short, a validated questionnaire was sent out to a sample of 3,556 randomly selected persons of the general population in 2 parishes in Stockholm, Sweden. All 2,293 responders were contacted by phone with an invitation to a hospital visit comprising an interview by a gastroenterologist for medical history and further questionnaires, plus a subsequent research ileocolonoscopy and biopsies. Of the 1,643 responders reached by the invitation, 1,244 accepted a hospital visit, and of these, 745 had the ileocolonoscopy.

The mean age was 44.5 years, and 51% were women in the original study population (the 3,556 randomly selected from the inhabitants in the 2 parishes). It was more difficult to reach younger participants as they may have their registered address with their parents while living elsewhere and often had unregistered prepaid cell phone cards. Also, they were less inclined to take time off work to do the investigation. In addition, IBS is more common in women. Of those who had the colonoscopy, the mean age was 51.7 years and 57.2% were women (18). Otherwise responders were very similar to nonresponders in terms of clinical characteristics, as previously reported in detail (18).

Of the 745 subjects who had the colonoscopy, 13 had inflammatory bowel disease including microscopic colitis, 17 had spirochetosis, 12 had other intestinal pathogens such as

Clostridium difficile, *Salmonella*, *Candida*, or spring worms, and PPIs and NSAIDs were taken by 32 and 52 subjects, respectively. Only subjects who had a completely normal colonoscopy and no evidence of organic or infectious gastrointestinal (GI) disease and who were not taking PPIs or NSAIDs are included in this study. All the subjects in this study were born in Sweden; the Swedish population is genetically similar to other populations of Northern Europe (19) and the diet in Sweden, and especially Stockholm, is also similar to Europe including the intake of fermentable carbohydrates (20).

IBS cases

IBS was defined by applying criteria congruent to Rome criteria to the validated Abdominal Symptom Questionnaire (ASQ) (2,18), as previously described. IBS cases were defined as having abdominal pain or discomfort at least weekly in the last 3 months paired with at least 2 of 3 of the following: (i) pain (or discomfort) associated with defecation (pain at defecation or pain improved or relieved by defecation), (ii) onset of pain or discomfort associated with a change in usual number of bowel movements, or (iii) onset of pain or discomfort associated with change in stool consistency, plus a normal colonoscopy. Subtypes were based on stool form reports: IBS—diarrhea was defined as having loose but not hard stools, IBS—constipation was defined as having hard but not loose stools. IBS—mixed was defined as having both, and IBS—unspecified as having neither.

Controls

Controls were similarly identified from the PopCol cohort and were selected if they had reported no abdominal pain or discomfort, or reported pain or discomfort not more than monthly in the past 3 months, plus a normal colonoscopy.

Assessment of possible confounders

Age, sex, and smoking are potential risk factors for IBS and were included in the analyses to control for confounding (21). Participants reported their current smoking status (current smoker or not currently smoking) in a questionnaire at the hospital visit.

The 36-Item Short-Form Survey (SF-36) is a validated measure of health-related quality of life including mood. We included in a post hoc secondary analysis the emotional well-being scale as a potential confounder (22).

Histopathology

Biopsies were taken at ileocolonoscopy from the terminal ileum, cecum, transverse colon, sigmoid colon, and rectum. Blinded assessment of histology was undertaken, and slides were dual read and any disagreement resolved by consensus. The counts were all performed in well-orientated areas of the sections, with epithelial nuclei in parallel by an expert GI pathologist (M.M.W.) (23).

A biopsy from each site was fixed in 10% formalin and routinely processed and stained with hematoxylin and eosin. IELs, eosinophils, and mast cells were counted with an Olympus BX51 light microscope using $\times 40$ objective. IELs were counted per 100 enterocytes/colonocytes, excluding crypts and enterocytes/colonocytes overlying lymphoid aggregates. Immunohistochemistry for mast cells was performed using rabbit anti-human polyclonal antibodies for mast cells, CD117 (c-kit, Dakocytomation) and counterstained with Harris's hematoxylin. Eosinophils and mast cells were counted in 5 nonoverlapping high-power fields and summed to calculate the number of cells per 5 high-power fields. When counting CD117

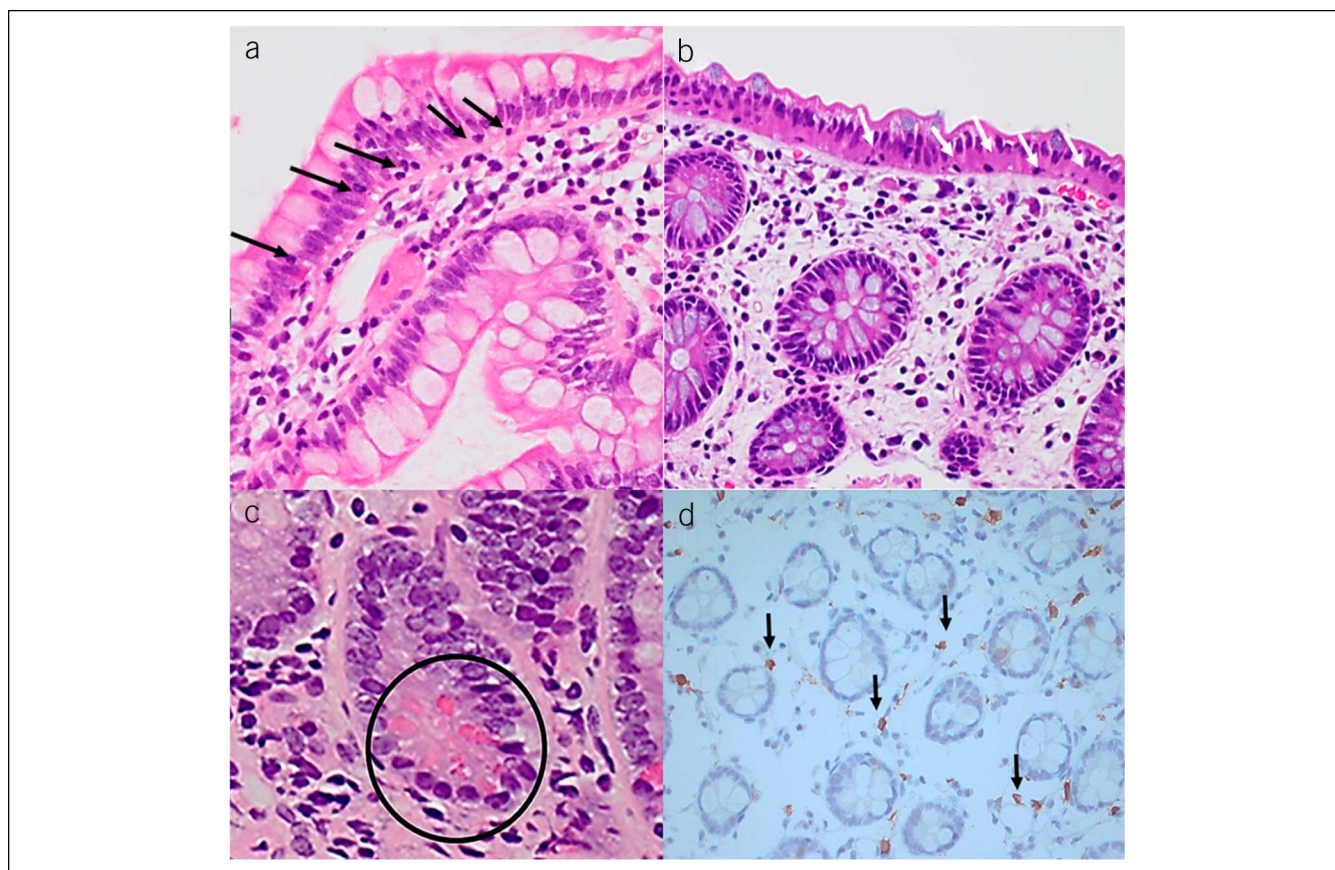


Figure 1. Ileocolonic histopathological alterations in the irritable bowel syndrome. (a) Terminal ileum biopsy hematoxylin and eosin stain, intraepithelial lymphocytes, arrowed. (b) Cecal biopsy hematoxylin and eosin stain, scant eosinophils in lamina propria, intraepithelial lymphocytes, arrowed. (c) Terminal ileum biopsy hematoxylin and eosin stain, paneth cells, circled. (d) Cecal biopsy immunohistochemistry CD117 stain, mast cells, arrowed.

immunostained mast cells, only those cells with unquestionable cytoplasmic staining or a visible blue nucleus were recorded positive. Paneth cells were identified by their secretory granules on hematoxylin and eosin and location at the base of the crypts and number of Paneth cells/5 crypts counted (Figure 1a–d).

For the purposes of the microbiome analysis, terminal ileal (TI) IEL counts were dichotomized using >15 per 100 enterocytes to define a high IEL count (based on control data).

Microbiota analyses

16S rRNA gene sequencing data from sigmoid MaM and FM were taken from the PopCol study, with sequencing and bioinformatics processing performed as described previously (24). The data were further subsampled at 10,000 reads, such that 57 samples were removed, and singleton and rare sequences were discarded. Chao and Shannon indices were generated, and Bray-Curtis distance measures were calculated as a measure of Beta diversity. Nonmetric dimensional scaling plots and permutational multivariate analysis of variance tests were performed using the Vegan library within R. Taxonomic profiles were compared at genus level in STAMP (25).

Statistics

Differences in background variables between cases and controls were calculated with the χ^2 test for dichotomous variables and the Mann-Whitney rank-sum test for ordinal variables.

Contrasts of cell counts proceeded in 2 stages. First, IBS and controls were compared using binary unconditional logistic regression with cell counts as the independent variables and probability of IBS as the outcome. Second, the probability of being in any IBS subtype (with controls as the reference) was modeled using multinomial logistic regression with cell counts as the outcome. In both sets of models, an odds ratio >1.0 indicates higher cell counts that are associated with increased probability of having IBS (or a specific subtype) as opposed to controls. Conversely, an odds ratio <1.0 indicates higher cell counts that are associated with decreased probability of IBS. Both univariate associations and multivariable associations adjusted for variables selected a priori (age, sex, and smoking) were calculated. A sensitivity analysis was performed also adjusting for mood applying the SF-36 in addition to age, sex, and smoking.

All cell counts were standardized in the regression analyses, so that relative risk ratios (RRRs) are comparable between cell types and localities as a measure of strength of association. Missing data for histology due to missing biopsies or unreadable slides differ between cell types and locations and numbers included in the analyses are provided.

Five subjects had missing data on IBS status, and 6 subjects without IBS but with daily or monthly abdominal pain were excluded leaving 154 subjects.

All statistical analyses were performed in Stata 15.1 (Stata-Corp, College Station, TX). All *P* values calculated were 2-tailed,

Table 1. Characteristics of the study population

	Total	Control	IBS	IBS—mixed	IBS—diarrhea	IBS—constipation	IBS—unspecified
N	154	106	48	20	10	9	9
Sex (males), n (%)	77 (46.7)	51 (48.1)	22 (45.8)	6 (30.0)	8 (80.0)	2 (22.2)	6 (66.7)
Age, median (IQR)	53 (40–59)	53 (40–60)	48.5 (35.5–58)	49.5 (35.5–59)	43.5 (33–55)	48 (38–56)	51 (43–63)
Current smoker, n (%)	28 (17.0)	18 (17.0)	9 (18.8)	4 (20.0)	2 (20.0)	1 (11.1)	2 (22.2)
Mood (SF-36 emotional well-being), median (IQR)	84 (68–92)	84 (72–92)	72 (40–88)	64 (38–88)	72 (36–88)	72 (52–84)	84 (68–92)

IBS, irritable bowel syndrome; IQR, interquartile range; SF-36, 36-Item Short-Form Survey.

and an alpha level of 0.05 was used for statistical significance in the reporting of the results.

The sample size available was constrained by that available in the original study; however, statistical power was evaluated as adequate (power = 0.87 at $\alpha = 0.05$) for a minimally important odds ratio of 1.5 or greater when contrasting IBS overall with controls. However, statistical power is low (power = 0.35 at $\alpha = 0.05$) when contrasting the smallest IBS subtypes with controls under the same conditions.

RESULTS

Demographic data in IBS cases and controls

Study group characteristics are given in Table 1. About half of the participants were men (47%), and the mean age was 53 years. IBS subjects ($n = 48$) and controls ($n = 106$) were similar in terms of age, sex, and smoking. IBS cases had lower (worse) mood scores than controls on the SF-36 ($P = 0.002$).

Histopathology in IBS cases and controls

Median and interquartile range of cells counts in total and divided on IBS status are given in Table 2. In total, 39 of the 106 controls reported monthly abdominal pain; there were no significant differences in histological cell counts between controls reporting monthly pain and controls reporting no pain (data not shown).

Univariately, there was a statistically significant increase of IELs (Figure 1a) in the terminal ileum in IBS ($P = 0.016$) and IBS-mixed ($P = 0.017$). These associations remained after adjustment of age, sex, and smoking (Table 3). There were also increased IELs in IBS—diarrhea in the cecum univariately ($P = 0.005$) (Figure 1b), and this also held true after adjustment for age, sex, and smoking (Table 3). In a post hoc sensitivity analysis also adjusting for mood, a major component of the mental health scale of the SF-36, in the multivariable models as well as age, sex, and smoking, the association of increased cecal IELs in IBS—diarrhea was retained (RRR = 2.43, 95% confidence interval [CI] 1.26–4.71, $P = 0.008$), as was the association of TI IELs with IBS overall (RRR = 1.60, 95% CI 1.00–2.56, $P = 0.048$) and IBS—mixed (RRR = 1.89, 95% CI 1.02–3.49, $P = 0.042$).

Paneth cells (Figure 1c) were not reduced in the terminal ileum in IBS or increased in the colon (Table 3). Eosinophils (Figure 1b) and mast cells (Figure 1d) were not increased in the terminal ileum or colon in IBS (Table 3).

Mucosa-associated and FM in IBS cases and controls

There were 55 participants in whom paired IEL count and microbiota data were available. All 55 participants had MaM data, and 20 of these participants also had FM data; 23 (42%) had IBS, and 31 (56%) had a high IEL count in the terminal ileum. There was no statistical difference in sex ($P = 0.83$, χ^2 test) or age ($P = 0.30$, Mann-Whitney rank-sum test) between subjects with available microbiota data and subjects without data.

No difference was observed in Chao richness and Shannon diversity of MaM or FM between participants with low and high TI IEL counts (Figure 2a–d). Neither Chao richness nor Shannon diversity differentiated participants based on IBS diagnosis (data not shown).

Difference in beta diversity of the MaM between low and high terminal ileum IEL count groups did not reach statistical significance (Figure 3a $P = 0.08$), and no difference in FM beta diversity was observed (Figure 3b $P = 0.85$). There was no difference in beta diversity of either the MaM or FM between IBS and non-IBS participants (Figure 3c,d; $P = 0.20$ and 0.54 respectively).

When taxonomic profiles were compared in the MaM, the genus *Blautia* and unclassified *Clostridiales* were associated with high TI IEL count (Figure 4a,b; $P = 0.024$ and 0.036 , respectively). No taxonomic associations were seen with IEL count in the FM. IBS status did not associate with any taxa in the MaM or FM.

MaM and FM alterations according to cecal IEL count were also evaluated, and none were significant after P value correction (data not shown).

DISCUSSION

In the large PopCol study, we carefully assessed community subjects by quantitative histology and investigated whether there was an increase in inflammatory cells in subjects with IBS, defined by the Rome criteria, vs. IBS—free controls. All subjects were identified and selected from the same sampling frame minimizing any selection bias, a major strength of this nested case-control design. Blinded assessment of histology was undertaken to avoid measurement bias, and slides were dual read. Potential confounders were adjusted for in the multivariable analyses. We did observe a modest but significant increase in IELs in the terminal ileum in IBS vs. controls, and when IBS subgroups were analyzed, IBS—mixed was associated with increased IELs. In the cecum, similarly increased IELs in IBS—diarrhea were observed. Mast cells and eosinophils were not increased in IBS. Paneth cell numbers were not altered, but this is likely due to Paneth cells being largely absent in

Table 2. Cell counts, crude values, per site presented as median (IQR)

	Total	Control	IBS	IBS—mixed	IBS—diarrhea	IBS—constipation	IBS—unspecified
Mast cells (n per 5 HPF)							
Ileum	337.5 (256–429)	326.5 (247–415)	402 (327–474)	393.5 (348–448)	283 (206–360)	294 (186–402)	473.5 (407–507)
Cecum	154.5 (123.5–203)	149.5 (122.5–197.5)	172 (129–214)	207.5 (124–235)	151 (129–191)	152 (132–170)	184.5 (136.5–205.5)
Transverse colon	167 (134–216)	165 (134–207)	197 (137–221)	221 (199–261)	142 (90–208)	152 (131–204)	153.5 (111–199)
Sigmoid colon	176 (136–221)	166 (136–203)	195 (156–233)	210 (182–235)	198.5 (114–233)	193 (180–219)	172 (153–220.5)
Rectum	140 (113–173.5)	138 (113–163)	159 (114–191)	165 (140–193)	143 (108–198)	135 (100–193)	168 (75–177)
IEL (n per 100 epithelial cells)							
Ileum	16 (11–20)	15 (10–19)	17 (14–24)	20 (15.5–24)	23 (17–23)	12 (11–24)	14 (11–25)
Cecum	9 (5–12)	9 (5–11)	9 (6–13)	8 (5–12)	12 (10–16)	8 (5.5–13.5)	8 (6–14)
Transverse colon	9 (5–12)	9 (5–12)	9 (5–13)	8 (5–14)	11 (7–14)	11 (6.5–12)	6 (4.5–11)
Sigmoid colon	8 (6–12)	8.5 (6–11.5)	8.5 (6–12)	8 (6–10)	9 (7–12)	10 (10–17)	7 (5.5–11)
Rectum	8 (5–11)	8 (4–11)	8 (6–12)	8.5 (5–13)	7 (4–8)	9 (7–13.5)	8.5 (5.5–12)
Eosinophils (n per 5 HPF)							
Ileum	9 (6–18)	9 (6–17)	15 (6–19)	15 (10–21)	10.5 (4.5–16)	19 (17–37)	6 (3–24)
Cecum	30 (18.5–43)	28 (18–41)	32 (25–44)	29.5 (25.5–42)	34 (19–41)	32.5 (22–51)	41.5 (22–51)
Transverse colon	15 (10–23)	14 (9–22)	20 (11–27)	20.5 (11–29)	20 (16–26)	16.5 (8.5–23.5)	19 (12–27)
Sigmoid colon	9 (4–15)	8 (3–14)	9 (4–14)	9 (2–15)	8 (6–16)	5 (1–9)	10 (5.5–11)
Rectum	2 (1–5)	2 (1–6)	2 (1–5)	2 (1–5)	1 (1–3)	1.5 (0–3)	6 (2–8)
Paneth cells (n per 5 crypts)							
Ileum	82.5 (65.5–103.5)	78.5 (62–103.5)	87 (68–100)	96 (65–112)	87 (73–88)	89 (81–128)	79.5 (65.5–85.5)
Cecum	0 (0–1)	0 (0–1)	0 (0–0)	0 (0–0)	1 (0–2)	0 (0–0)	0 (0–0)
Transverse colon	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Sigmoid colon	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
Rectum	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)

All significant associations ($P < 0.001$) are shown in bold.

HPF, high-power fields; IBS, irritable bowel syndrome; IEL, intraepithelial lymphocyte; IQR, interquartile range.

the large intestine. Subjects taking aspirin and NSAIDs were excluded, so these drugs do not explain the histological findings.

Age and female gender are risk factors for IBS, and smoking is a risk factor for postinfectious and IBS—diarrhea (21,26). It is notable that IELs were increased in the terminal ileum in IBS adjusting for age, sex, and smoking. This association remained significant when adjusted for mood in a sensitivity analysis, although this secondary analysis needs to be interpreted with caution as it was post hoc. Normal values for IELs in the terminal

ileum have been uncertain. In a study of 61 asymptomatic patients undergoing colonoscopy and biopsy, counts of IELs per 100 enterocytes were low, with a median of 3 (range 0–9) (27), but this was not based on a random population sample in contrast to the PopCol study. Increased IELs in the small intestine are not specific, but may occur in celiac disease or with small intestinal bacterial overgrowth (SIBO) (28,29). All subjects in this study had a normal colonoscopy, and we excluded cases of celiac disease; however, measures of SIBO were not available, although most

Table 3. Multivariable associations between ileocolonic cell counts on histology and IBS status adjusted for age, sex, and smoking

	IBS			IBS—mixed			IBS—diarrhea			IBS—constipation			IBS—unspecified		
	RRR ^a	95% CI	P	RRR	95% CI	P	RRR	95% CI	P	RRR	95% CI	P	RRR	95% CI	P
Mast cells (n per 5 HPF)															
Ileum	1.3689	0.81010–2.3131	0.241	1.5218	0.77612–2.9839	0.222	—	—	0.996	0.70360	0.07722–6.4102	0.755	1.6778	0.78786–3.5731	0.180
Cecum	1.1027	0.75044–1.6203	0.619	1.2982	0.78322–2.1520	0.311	0.83596	0.29181–2.3947	0.739	0.55615	0.16739–1.8478	0.338	1.2389	0.71408–2.149	0.446
Transverse colon	1.0069	0.67940–1.4924	0.972	1.5623	0.9461–2.5800	0.081	0.53171	0.17507–1.6148	0.265	0.71277	0.27059–1.8775	0.493	0.60324	0.21618–1.6832	0.334
Sigmoid colon	1.1069	0.76087–1.6104	0.595	1.2321	0.76248–1.9911	0.394	0.96311	0.41302–2.2458	0.931	1.0498	0.41494–2.656	0.918	1.0529	0.53958–2.0547	0.880
Rectum	1.2035	0.8266–1.7524	0.334	1.4324	0.91380–2.245	0.117	1.0100	0.46194–2.208	0.980	0.75712	0.24387–2.350	0.630	1.2657	0.60854–2.6327	0.528
IEL (n per 100 epithelial cells)															
Ileum	1.6976	1.0789–2.6710	0.022	2.048	1.1488–3.6521	0.015	1.6336	0.42804–6.2347	0.473	1.3513	0.54999–3.3204	0.511	1.2684	0.52059–3.0906	0.601
Cecum	1.2996	0.90454–1.8674	0.156	0.8843	0.47358–1.651	0.700	2.0281	1.1323–3.6327	0.017	1.0589	0.45024–2.490	0.896	1.2804	0.58857–2.7857	0.533
Transverse colon	1.0593	0.7172–1.5644	0.772	1.1262	0.64974–1.9522	0.672	1.4284	0.68853–2.9633	0.338	0.98363	0.43739–2.2120	0.968	0.66438	0.2713–1.6268	0.371
Sigmoid colon	1.0678	0.7315–1.5587	0.734	0.86461	0.47556–1.5719	0.633	1.0786	0.51708–2.2501	0.840	1.7580	0.90251–3.4246	0.097	0.8321	0.34973–1.9799	0.678
Rectum	1.4179	0.97412–2.0638	0.068	1.3649	0.80787–2.3062	0.245	1.1478	0.58713–2.2442	0.687	1.9572	0.96416–3.9730	0.063	1.4216	0.71956–2.8087	0.311
Eosinophils (n per 5 HPF)															
Ileum	1.1347	0.74997–1.7170	0.550	1.1573	0.70505–1.8997	0.563	1.2274	0.14722–10.234	0.850	1.2915	0.66429–2.5109	0.451	0.88087	0.22666–3.4233	0.855
Cecum	1.1935	0.83360–1.7089	0.334	1.2490	0.7575–2.0592	0.383	0.8856	0.41148–1.9063	0.756	1.1554	0.58274–2.2909	0.679	1.4738	0.73939–2.9376	0.270
Transverse colon	1.3555	0.95149–1.9312	0.092	1.4344	0.87468–2.3525	0.153	1.3924	0.78180–2.4799	0.261	1.3895	0.64779–2.9808	0.398	1.1794	0.61404–2.2656	0.620
Sigmoid colon	0.90444	0.6054–1.3510	0.624	.84511	0.46657–1.5307	0.579	1.2647	0.65211–2.4527	0.487	0.60907	0.21762–1.7046	0.345	0.87001	0.38057–1.9889	0.741
Rectum	0.66842	0.37411–1.1942	0.174	0.6152	0.24751–1.52	0.296	0.29259	0.04743–1.8048	0.186	0.14853	0.01548–1.4248	0.098	1.1893	0.67803–2.0862	0.545
Paneth cells (n per 5 crypts)															
Ileum	1.2155	0.79406–1.8607	0.369	1.4370	0.83549–2.4715	0.190	0.91666	0.21321–3.9410	0.907	1.7817	0.72386–4.3855	0.209	0.58389	0.24628–1.3842	0.222
Cecum	0.89622	0.61389–1.3084	0.570	0.85085	0.45302–1.5980	0.615	1.0298	0.57871–1.8327	0.920	—	—	—	1.0662	0.57511–1.9767	0.839
Transverse colon	0.98008	0.68444–1.4034	0.913	0.85990	0.3323–2.2248	0.756	1.0300	0.60798–1.7452	0.912	—	—	—	1.0817	0.7058–1.6578	0.718
Sigmoid colon	0.68285	0.34943–1.3344	0.264	—	—	—	1.0599	0.5522–2.0340	0.861	—	—	—	—	—	—
Rectum	0.90168	0.5990–1.357	0.620	—	—	—	1.044	0.58617–1.8629	0.881	—	—	—	—	—	—

CI, confidence interval; HPF, high-power fields; IBS, irritable bowel syndrome; IEL, intraepithelial lymphocyte; RRR, relative risk ratio.
^aCell counts standardized so that RRRs are comparable between cell types and localities as a measure of strength of association. Controls are used as the reference.

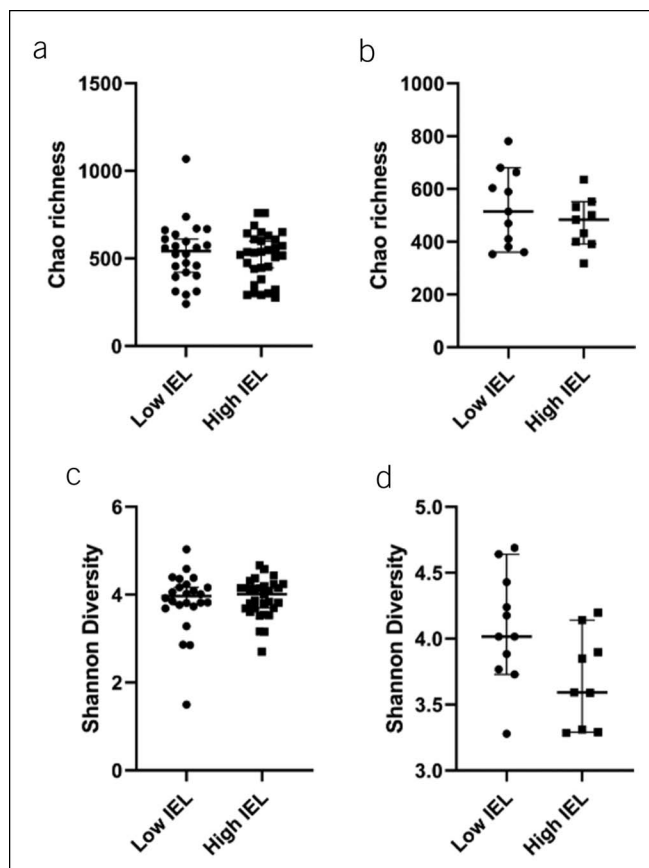


Figure 2. Alpha diversity indices in cases with low intraepithelial lymphocytes (IELs) and high IELs. There was no association between IEL count in the terminal ileum (TI) and Chao richness of (a) the mucosal microbiota ($P = 0.717$) and (b) the fecal microbiota (FM) ($P = 0.503$). There was no association between IEL count in TI with (c) Shannon diversity of the mucosal microbiota ($P = 0.953$). (d) The association between reduced Shannon diversity of the FM and high IEL count in the TI did not reach statistical significance ($P = 0.067$).

subjects had no other obvious risk factor for SIBO (30). PPI and NSAID users were excluded from the analyses, and drugs should not account for distal small intestinal pathology in this study. Chadwick et al. (13) reported increased colonic IELs in IBS in a New Zealand study, and we also observed significantly increased cecal IELs in IBS—diarrhea even when adjusting for mood. The finding of increased terminal ileum IELs is further supported by evidence of circulating CCR9⁺ small intestinal homing T cells in IBS, a marker of small intestinal inflammation (31). Increased IELs are seen in celiac disease, a gluten protein-driven immune response in a genetically primed host (23). Hence, another possibility is increased IELs in the terminal ileum may reflect a food antigen driven process, although the expected changes may often occur more proximally in the small intestine, as has been reported in rumination syndrome where increased IELs in the duodenum have been observed with eosinophils (and rumination syndrome strongly overlaps with FD in terms of gastroduodenal symptom profile) (32). Recent work has also found that in non-IgE food allergy in IBS, IELs become acutely increased in the duodenum after a food challenge consistent with our findings (33).

Mast cells have been implicated in the pathogenesis of IBS. Miner et al. in the United States were the first to report increased mast cells

in the terminal ileum in IBS (34). We have observed increased duodenal mast cells in those with overlapping IBS and FD in another random Swedish population-based cohort who underwent endoscopy (29). However, it is controversial whether increased mast cells are a marker for the disorder (8–12,35). Mast cell activation has been demonstrated in IBS in a number of studies of referred patients through measurement of mast cell mediators in supernatants (36). However, protocols for handling the biopsies and undertaking the supernatant studies have varied, and potential confounders such as current stress or infection may have impacted the results (36). We conclude if increased ileocolonic mast cells occur in IBS, it is likely only in a subgroup of selected referral cases with presumably more severe symptoms, a hypothesis needing to be tested.

Paneth cells have an important role in microbe-host interactions (15,16) but have not been investigated in IBS to the best of our knowledge. In this study, Paneth cells were not altered in the terminal ileum in IBS in contrast to observations in Crohn disease (15). As we did not examine other small intestinal sites, the conclusion there is no change is incomplete. As no dysbiosis was found in this population-based study (24), this may account for the negative findings. As there was no change in Paneth cells in IBS, we did not look for a microbiota Paneth cell link.

Increased colonic eosinophils have been identified in patients with colonic spirochetosis identified in the PopCol study as previously reported (7); those with colonic spirochetes were excluded from the current study. These novel pathological observations have been confirmed in an Australian study (37), and an association between colonic spirochetes and IBS—diarrhea has been observed (7). Furthermore, increased duodenal eosinophilia is found in FD, most notably those with postprandial distress syndrome, observations that have been confirmed worldwide (5,38,39), and FD overlaps with IBS more than expected by chance (1). Because of these previous striking associations, we carefully evaluated for increased ileocolonic eosinophilia in IBS, but no significant changes (or even trends) were observed. Nonceliac gluten sensitivity (NCGS) has also been identified in a subset with IBS (40), and rectal eosinophilia has been observed in NCGS in an Italian study (41). Although these observations of increased rectal eosinophils in NCGS are yet to be confirmed, we did not detect any such changes in the present investigation and suggest this will be uncommon in IBS if it occurs.

Our results suggest that there are modest, discernible differences in the MaM (but not the FM) in the presence of an expanded IEL compartment in the terminal ileum, but this was not significantly associated with IBS status. In particular, we found that the genus *Blautia* and unclassified *Clostridiales* were overexpressed in the MaM of participants with high IEL counts in the terminal ileum. *Blautia* was a prominent genus in the MaM relative to the FM in the wider PopCol study. Although *Blautia* was not associated with IBS in the PopCol study (24), an association was seen in a previous smaller study (42). It is therefore notable that, in our analysis, IELs are increased in the presence of *Blautia*, raising the possibility that there may be transmucosal interaction between these bacteria and the host immune system. *Blautia* species and many other *Clostridiales* are producers of short-chain fatty acids, which are increasingly recognized to be immunomodulatory in nature (43). Bile acid–induced diarrhea is likely an important mechanism in IBS (44), but we did not evaluate bile acids in this study and cannot comment on whether this relates to the microbiome alterations observed; unclassified *Clostridiales* (associated with high IEL count) might well include *Clostridia* with bile acid metabolizing activity (e.g., *Clostridium scindens*), but this is speculative.

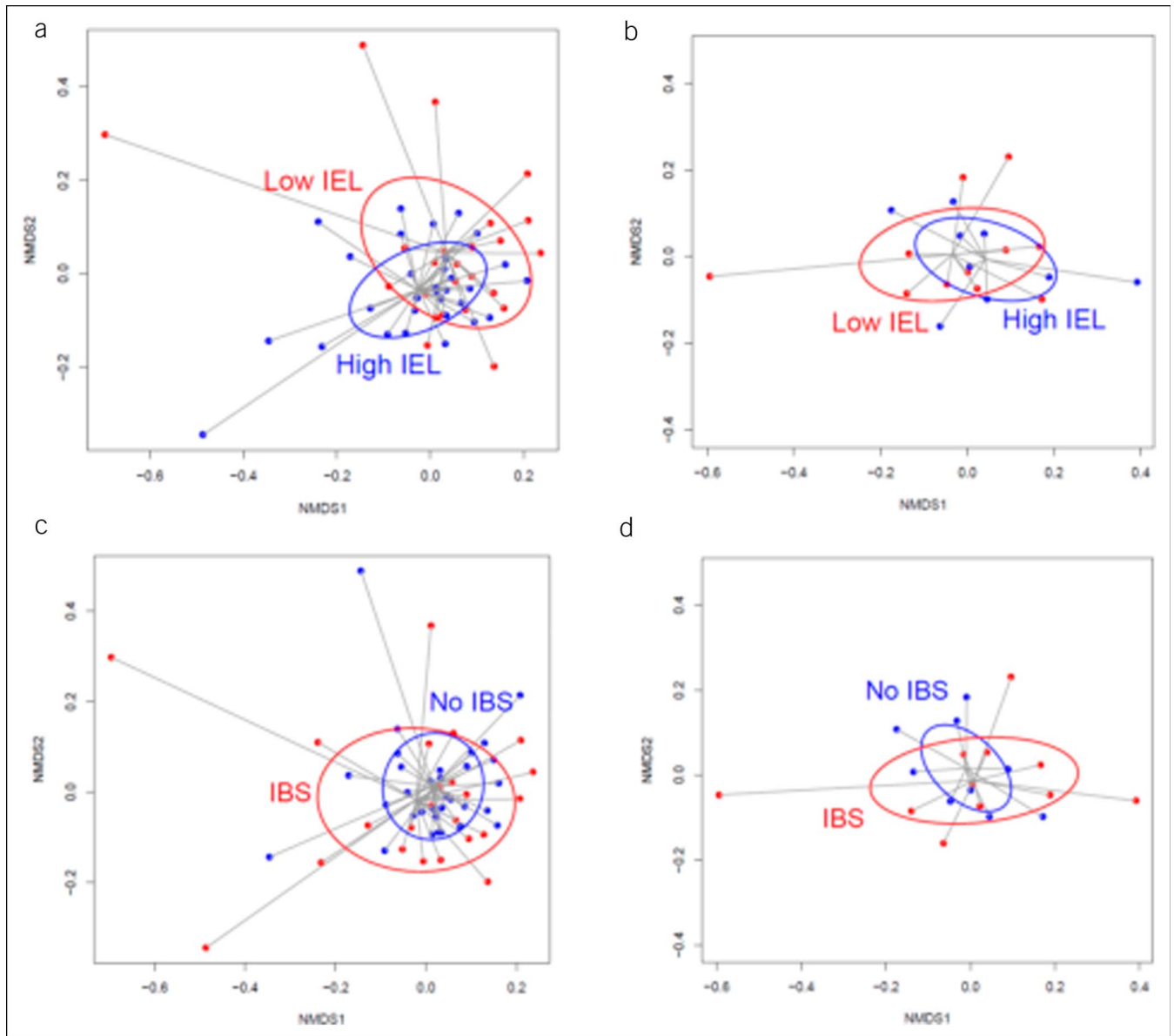


Figure 3. Beta diversity (Bray-Curtis index) of mucosal microbiota in samples with low intraepithelial lymphocytes (IELs) and high IELs in the terminal ileum. Nonmetric dimensional scaling (NMDS) plots comparing subjects with high and low IELs (high IEL samples colored blue; low IEL samples colored red). **(a)** A difference in beta diversity of the mucosa-associated microbiota (MaM) between low and high IEL groups, which does not reach statistical significance (permutational multivariate analysis of variance test; P value = 0.079). There was no difference in fecal microbiome (FM) beta diversity in high and low IEL groups **(b)**; $P = 0.854$). There was no difference in beta diversity of either the MaM or FM between IBS and non-IBS participants **(c and d)**; $P = 0.195$ and 0.544 , respectively).

The novel finding of a distinct microbiome profile in the setting of an expanded IEL compartment in the TI is plausible in the context of emerging evidence of subtle intestinal inflammation in IBS. Nonetheless, this outcome should be interpreted with caution, given the modest numbers of participants included in the current analysis. Our results merit further study and validation in independent cohorts.

This study had limitations. We acknowledge that this is an exploratory analysis of IELs in IBS, and based on the available data, we do not claim “proof” of association; the findings need independent corroboration with prospective follow-up to identify whether there is a temporal relationship which would suggest causality. However, our results were consistent on adjusting for potential confounders. No correction for multiple hypothesis testing was made because of

the exploratory nature of the study. Taking the minimum clinically important effect size to be $RRR = 2.0$, statistical power is approximately 0.9 for the contrast of overall IBS and controls given the sample size available; however, for the smallest IBS subtype, statistical power is approximately 0.4 under the same conditions. The study was constrained by the available sample size. With regard to the paired IEL—microbiome analysis, although our focus was on IEL count in the terminal ileum, samples for microbiome analysis were available only from the sigmoid colon and feces. The histopathology was performed blinded to case-control status, but we could not assess for mast cell activation, which may still play a key role in the pathogenesis of IBS even if absolute mast cell numbers are not increased, and we did not assess enterochromaffin cells and IBS. The ASQ asks for abdominal pain and discomfort, but the

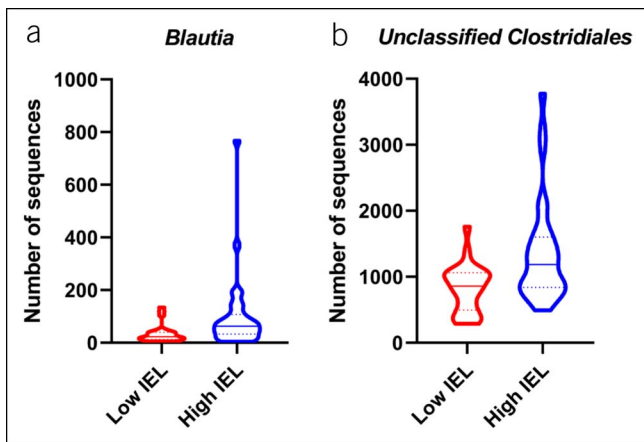


Figure 4. Violin plots of significant taxonomic differences in (a) *Blautia* spp. ($P = 0.024$) and (b) unclassified *Clostridiales* ($P = 0.036$) in the mucosa-associated microbiota between subjects with low and high intraepithelial lymphocyte (IEL) counts. Medians shown as full lines, and quartiles shown as dotted lines.

current Rome IV criteria focus on abdominal pain, not discomfort (2). However, the ASQ includes 11 modalities of abdominal pain and discomfort in Swedish and only 2 of the IBS subject did not specify a pain modality, suggesting that most cases had pain-predominant symptoms consistent with Rome IV criteria (18). Notably, a representative community sample was obtained, and selection bias was minimal as previously reported (18).

In conclusion, in a novel nested case-control study from a representative population, we conclude there is no significant increase in ileocolonic mast cells or eosinophils or changes in Paneth cells in subjects with IBS vs. IBS-free subjects in a Northern European community. Mast cell counts alone seem to be unhelpful as a biomarker for IBS in this part of the world in the terminal ileum or colon. Although increased IELs in the terminal ileum and cecum were identified in IBS, their clinical and pathological significance is unclear and requires further investigation.

CONFLICTS OF INTEREST

Guarantor of the article: Nicholas J. Talley, MD, PhD.

Specific author contributions: A.A., M.M.W., N.P., and N.J.T.: study concept and design. L.E., L.W.H., M.M.W., and L.A.: acquisition of data. A.A., M.P.J., J.L.A., N.P., L.E., L.W.H., and N.J.T.: analysis and interpretation of data. N.J.T., J.L.A., and A.A.: drafting of the manuscript. All authors: critical revision of the manuscript for important intellectual content. A.A. and M.P.J.: statistical analysis. L.A.: obtained funding; administrative, technical, or material support; study supervision.

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Study Highlights

WHAT IS KNOWN

- ✓ Reports on the presence of colonic mucosal immune cells (e.g., mast cells) in IBS have been inconsistent but have been based on case-control studies in selected patients.
- ✓ The normal ileocolonic cell counts are unclear because of a lack of population-based studies.
- ✓ No random population-based colonoscopy studies have investigated ileocolonic mucosal immune cells in IBS and healthy subjects.

WHAT IS NEW HERE

- ✓ Normal values for mast cells, IELs, eosinophils, and Paneth cells in the ileum and colon in healthy subjects and IBS are described.
- ✓ Subtle increased numbers of IELs ($n/100$ epithelial cells) in the terminal ileum in IBS (TI, median 17) and cecum in IBS-diarrhea (median 12) were observed, compared with healthy controls (TI median 15, cecum 9).
- ✓ IELs may be associated with mucosal-associated microbiome alterations, but these alterations are not related to the presence of IBS.

TRANSLATIONAL IMPACT

- ✓ Knowledge of normal cutoffs for immune cell counts in the terminal ileum and colon is likely to assist in identifying subtle low-grade inflammatory diseases currently overlooked.
- ✓ In IBS, IELs may be a marker of disease.

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