Constipation-Predominant Irritable Bowel Syndrome Females Have Normal Colonic Barrier and Secretory Function

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OBJECTIVES: **The objective of this study was to determine whether constipation-predominant irritable bowel syndrome (IBS-C) is associated with changes in intestinal barrier and secretory function.**

METHODS: **A total of 19 IBS-C patients and 18 healthy volunteers (all females) underwent saccharide excretion assay (0.1 g ^{13}C mannitol and 1 g lactulose), measurements of duodenal and colonic mucosal barrier (transmucosal resistance (TMR), macromolecular and *Escherichia coli* Bio-Particle translocation), mucosal secretion (basal and acetylcholine (Ach)-evoked short-circuit current (Isc)), *in vivo* duodenal mucosal impedance, circulating endotoxins, and colonic tight junction gene expression.**

RESULTS: **There were no differences in the *in vivo* measurements of barrier function between IBS-C patients and healthy controls: cumulative excretion of ^{13}C mannitol (0–2 h mean (s.e.m.); IBS-C: 12.1 (0.9) mg vs. healthy: 13.2 (0.8) mg) and lactulose (8–24 h; IBS-C: 0.9 (0.5) mg vs. healthy: 0.5 (0.2) mg); duodenal impedance IBS-C: 729 (65) Ω vs. healthy: 706 (43) Ω; plasma mean endotoxin activity level IBS-C: 0.36 (0.03) vs. healthy: 0.35 (0.02); and in colonic mRNA expression of occludin, zonula occludens (ZO) 1–3, and claudins 1–12 and 14–19. The *ex vivo* findings were consistent, with no group differences: duodenal TMR (IBS-C: 28.2 (1.9) $\Omega\,cm^2$ vs. healthy: 29.8 (1.9) $\Omega\,cm^2$) and colonic TMR (IBS-C: 19.1 (1.1) $\Omega\,cm^2$ vs. healthy: 17.6 (1.7) $\Omega\,cm^2$); fluorescein isothiocyanate (FITC)–dextran (4 kDa) and *E. coli* Bio-Particle flux. Colonic basal Isc was similar, but duodenal basal Isc was lower in IBS-C (43.5 (4.5) $\mu A\,cm^{-2}$) vs. healthy (56.9 (4.9) $\mu A\,cm^{-2}$), P=0.05. Ach-evoked $\triangle$Isc was similar.**

CONCLUSIONS: **Females with IBS-C have normal colonic barrier and secretory function. Basal duodenal secretion is decreased in IBS-C.**

INTRODUCTION

Irritable bowel syndrome (IBS) is a common and chronic gastrointestinal disorder that results in significant morbidity and health-care utilization. Although several peripheral and central mechanisms have been shown to play a role, the pathophysiology of IBS remains to be clarified (1). More recently, an increase in

intestinal permeability has been shown to be present in a subset of patients with diarrhea-predominant IBS and was found to correlate with visceral sensitivity and symptom severity (2).

Paracellular permeability is primarily regulated by the tight junctions (TJs) composed of integral membrane (claudins, occludin) and structural binding proteins (zonula occludens (ZO)).

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Most of the existing literature reporting increased intestinal permeability in IBS focuses on diarrhea-predominant IBS (IBS-D) or postinfectious IBS that is usually IBS-D or mixed IBS. Little is known about intestinal permeability alterations in IBS-C. Two studies reported increased flux of fluorescein-5.6 sulfonic acid (478 Dalton) across colonic biopsies of IBS-C patients (number of subjects: 3 and 10) as compared with healthy subjects (3,4). Another study showed that colonic biopsy supernatant of all IBS subtypes reduced transepithelial resistance and increased flux of fluorescein isothiocyanate (FITC)–dextran (4 kDa) across Caco-2 monolayers (3). Colonic epithelial protein expression of occludin was found to be decreased in small studies of patients with IBS-C (5,6). Another study showed unchanged claudin-1 protein expression in IBS-C as compared with healthy individuals (7). The *in vivo* measurements of intestinal permeability using urinary excretion of orally ingested, poorly absorbable saccharides have also shown variable results in IBS-C. One study has shown increased permeability using polyethylene glycol 3350/400 (ref. 8), another using polyethylene glycol 400/1500/4000 showed no difference (9), and one using ^{51}Cr-EDTA showed decreased permeability (10). Variability in timing of urine collection and dietary contamination often limits interpretation from these *in vivo* assays.

In a validation study using lactulose and mannitol, cumulative collections between 0 and 2 h and between 8 and 24 h were found to be most reflective of small bowel and colonic permeability, respectively (11). In addition, we have noted that up to 30% of participants can have a high baseline (before administration of the test dose) presence of mannitol that can interfere with the test performance. Our studies showed that ^{13}C mannitol has better performance characteristics and can be used as a novel saccharide for measurement of intestinal permeability (12). Additionally, mucosal secretory function has not been studied in IBS-C.

The overall objective of this study was to use a comprehensive and comparative methodological approach to determine changes in mucosal barrier and secretory functions in females patients with IBS-C compared with healthy volunteers. Specific goals were to compare *in vivo* permeability, *in vivo* mucosal impedance, *ex vivo* duodenal and colonic mucosal barrier function, mucosal bacterial translocation, and circulating endotoxemia in females with IBS-C in comparison with healthy volunteers. In addition, we investigated baseline and acetylcholine (ACh)-evoked mucosal secretory properties and colonic TJ gene expression in IBS-C in comparison with healthy volunteers.

METHODS
Subjects
The study population consisted of 19 Rome III IBS-C patients and 18 healthy volunteers (without a history of IBS or any other gastrointestinal disorder). All participants were females and the controls were age-matched to IBS-C cases. Neither population had history of abdominal surgery (except appendectomy or cholecystectomy), inflammatory bowel disease, microscopic colitis, or celiac disease nor were they pregnant at the time of the study. Additional exclusion criteria included use of tobacco (within 6 months of the

study), nonsteroidal anti-inflammatory drugs, systemic antibiotics (within 1 week of the study), or oral corticosteroids (within 6 weeks of the study). In addition, any prescription, over the counter, or herbal medications that can affect gastrointestinal transit were prohibited 7 days before study start (e.g., osmotic laxatives, antiemetics, prokinetics, anticholinergics, narcotics, peppermint oil, or antidepressants with known effects on intestinal transit). Participants were also excluded if they scored higher than 8 for anxiety or depression on a Hospital Anxiety and Depression Scale (HADS). None of the participants were on a type 2 chloride channel activator, guanylate cyclase C agonist, or 5-HT4 agonist at the time of the study. The IBS-C participants completed IBS-symptom severity scale (13), IBS-quality of life (14), and somatic symptom checklist. The study was approved by the institutional review board at Mayo Clinic and all subjects provided written informed consent before participation. The study was listed on ClinicalTrials.gov (NCT02246647).

In vivo intestinal permeability
Saccharide excretion assay. We used a recently developed assay for *in vivo* measurement of intestinal permeability using ^{13}C mannitol and lactulose (12). Volunteers were instructed to not consume artificial sweeteners such as Splenda (sucralose), Nutrasweet (aspartame), lactulose, or mannitol for 2 days before permeability testing. They were orally administered 1,000 mg lactulose, 100 mg ^{12}C (regular) mannitol, and 100 mg ^{13}C mannitol dissolved in 250 ml of water. Urine samples were collected at baseline (before test saccharide administration) and 0–2, 2–8, and 8–24 h after saccharide administration. High-performance liquid chromatography-tandem mass spectrometry was used to measure saccharide concentrations as previously described (12). Cumulative concentrations were calculated using the overall urine volume excreted in the individual intervals. Concentrations of ^{13}C were adjusted for the percentage of ^{13}C in ^{12}C mannitol (4.98% of ^{12}C mannitol excreted was subtracted from ^{13}C mannitol values; determined by analyzing replicate samples of control urine). Cumulative lactulose or ^{13}C mannitol concentrations between 0 and 2 h and between 8 and 24 h were used for determination of small bowel and colonic permeability, respectively. Respective lactulose to ^{13}C mannitol excretion ratios, as a measure of dose of saccharide administered, were calculated for the 0–2 h and 8–24 h intervals.

Duodenal impedance. All participants fasted for 8 h and underwent sedated esophagogastroduodenoscopy. Duodenal impedance measurements were performed using a recently designed endoscopically passed catheter that measures electrical impedance of the duodenal epithelium by mucosal contact under direct visualization (15). A 2 mm diameter catheter was used with two 360° circumferential sensors placed at a separation of 2 mm with the end of the distal ring being 1 mm away from the catheter tip. The electrodes were connected to an impedance voltage transducer via thin wires that ran the length of the catheter traversing through the working channel of the upper endoscope. The voltage generated by the transducer was limited to produce 10 μA current

at a frequency of 2 kHz. Impedance measurements were expressed in Ω as the ratio of voltage to the current. Data were acquired with a stationary impedance data acquisition system (InSight; Sandhill Scientific) and were analyzed using BioView analysis software (Sandhill Scientific, Denver, CO). Impedance was measured every 90° in duodenal circumference with a decompressed lumen after suctioning of all fluid from the lumen. The catheter was embedded in the mucosa parallel to the two sensors and measurements were taken from a steady baseline for >10 s. Average of the four values was calculated for each patient.

Endotoxemia activity assay (EAA)

Fasting, EDTA anticoagulated, whole blood was collected from all participants. Samples were analyzed using an EAA kit (Spectral Diagnostic, Toronto, ON, Canada) that utilizes a monoclonal IgM antibody specific for Gram-negative bacterial lipopolysaccharide. Samples were tested in duplicate within 1 h of collection using a Berthold SmartLine luminometer (Bad Wildbad, Germany) (photon-counting). The endotoxin activity level was calculated as follows: chemiluminescence (test sample−negative control)/chemiluminescence (positive−negative control). Average values for each participant were expressed as absolute units.

Ex vivo mucosal barrier function

Biopsy collection. Ten duodenal biopsies were obtained from the second portion of the duodenum. At the same setting, all participants underwent a flexible sigmoidoscopy and 10 biopsies were obtained from the sigmoid colon ~30 cm from the anal verge. All endoscopic procedures were done by a single endoscopist (M.G.) and biopsies were collected using a large-capacity (2.8 mm) biopsy forceps (no pin). Three biopsies from each site were placed in RNAlater stabilization solution (Life Technologies, Carlsbad, CA) and flash frozen in liquid nitrogen. Two were placed in fixatives (formalin, 4% paraformaldehyde). The rest of the biopsies were placed in Krebs solution, on ice, for Ussing chamber studies. Biopsies were immediately transported from the Clinical Research and Trials Unit to the laboratory.

Ussing chamber studies. Ussing chamber studies were performed to measure duodenal and colonic mucosal barrier function, bacterial translocation, and secretory responses. Four biopsies from each site were mounted in 4 ml Ussing chambers (Physiologic Instruments, San Diego, CA) exposing 0.031 cm² area, within 45 min of collection. Chambers were filled with Krebs with 10 mM mannitol (mucosal side) and Krebs with 10 mM glucose (submucosal side). Further details on drugs and solutions are provided in **Supplementary Methods** online. Baseline transmucosal resistance (TMR) and short circuit current (Isc) of each tissue was measured using a pair of Ag/AgCl electrodes with agar-salt bridges and a pair of current-giving platinum electrodes to maintain voltage clamp conditions. Average TMR and Isc across 3–4 biopsies/subject were calculated. Secretory responses to basolateral ACh (0.01–300 µM) was measured as the change in Isc (ΔIsc) in 1–2 biopsies/site for each subject. A positive ΔIsc indicated an anion flux into the lumen or a cation flux from luminal to serosal side. Biopsies with baseline TMR <10 Ω cm², Isc drift of >100 µA cm⁻² over the experimental course, or high flux within 30 min of mounting were excluded from the analysis.

Paracellular flux across biopsies was measured using 4 kDa FITC–dextran administered on the mucosal side (1 mg/ml chamber concentration). Transcellular transport was studied using translocation of the fluorescently labeled *Escherichia coli* K-12 Bio-Particles (10^7 colony-forming unit (CFUs)/ml mucosal chamber concentration). Sampling was done from the submucosal side every 30 min for a total of 3 h and cumulative fluorescence was measured using a Synergy Multi-Mode Microplate Reader (BioTek, VT) and converted to concentration using standard curves. Cumulative flux at the end of 3 h and rate of flux was calculated for FITC–dextran and *E. coli* K-12 Bio-Particles. At the end of the experiment, tissue was treated with 10 µM forskolin (activates adenylyl cyclase and increases intracellular cyclic adenosine monophosphate) on apical side to check for tissue responsiveness.

Mucosal cellular changes

A representative duodenal and colonic biopsy from each participant was stained with hematoxylin and eosin and examined by a pathologist in a blinded manner (T.C.S.) to count eosinophils/high-power field (HPF). Immunostaining was done for mast cells using tryptase (1° rabbit anti-human (polyclonal), Santa Cruz, 1:800 dilution (Santa Cruz, CA, USA); 2° donkey anti-rabbit CY3, Millipore, 1:800 dilution (Darmstadt, Germany)) and for CD3⁺ cells (Mouse anti-human CD3 (monoclonal), Bio-Rad, 1:4,800 dilution (Hercules, CA, USA); 2° Donkey anti-mouse CY3, Millipore, 1:800 dilution) on 1–2 biopsies from each site (duodenum and sigmoid colon) on all participants. These were graded by two independent blinded reviewers (S.E. and M.G.) as normal (0–25% increase or decrease from controls) or increased (>25% over normal).

TJ gene expression

RNA was extracted from freshly frozen colonic tissue using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) from all IBS-C patients and 10 healthy volunteers. Synthesis of complementary DNA (cDNA) from RNA was performed with an RT² First-Strand cDNA Kit (Qiagen). PCR was performed using an RT² Profiler PCR Array for 84 human TJ genes (Qiagen). Gene expression was normalized by arithmetic mean to the control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As the array tested for 84 genes, statistical significance level was corrected for multiple comparisons using Bonferroni correction and a *P* value of <0.0006 was considered significant. All samples tested had a RNA integrity value of >8.

Statistical analysis

Means and s.e.m. are reported for continuous variables, whereas frequencies and percentages are reported for categorical variables. Two-sided Mann–Whitney *U*-test assuming non-Gaussian distribution was used to compare variables between the two groups. Spearman's correlations (nonparametric) were used to analyze relationship between the TMR and FITC–dextran flux. All analyses

Table 1. Demographic characteristics of IBS-C patients and healthy volunteers

	IBS-C	Healthy
Age, years: mean±s.e.m.	45.37±2.82	43.06±2.78
IBS-SSS	206.74±18.05	—
HADS Anxiety Score, mean (s.e.m.)	3.37±0.49	2.61±0.52
HADS Depression Score, mean (s.e.m.)	1.05±0.40	0.39±0.14
IBS-QOL	32.29±2.62	—
Somatization score	0.61±0.11	—
Medications (number of subjects)		
Thyroid replacement	3	5
Antidepressants	4	0
Vitamin replacements	8	4
Proton pump inhibitors	5	1
Birth control	3	1

HADS, Hospital Anxiety and Depression Scale; IBS, irritable bowel syndrome; IBS-C, constipation-predominant IBS; IBS-QOL, IBS-quality of life; IBS-SSS; IBS-symptom severity scale.

were done using the GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered statistically significant unless otherwise specified. The sample size calculations were based on *in vivo* permeability parameters as primary end point. Based upon pooled data from IBS patients and healthy volunteers, 18 subjects/group allow 17% effect size detection for 0–2 h cumulative excretion of mannitol, 19% effect size detection for 8–24 h cumulative excretion of mannitol, and 30% for 8–24 h cumulative excretion of lactulose. Effect size is the difference between two groups as a percentage of the listed mean value, based on a two-sample t-test using a two-sided α-level of 0.05 and all estimates were based on 80% power.

RESULTS

Participant characteristics

Table 1 shows demographic characteristics of study participants. All subjects were white. Anxiety and depression scores were low and comparable between the two groups. The mean (s.e.m.) IBS-symptom severity scale was 206 (18) on a 0–500 scale (with 500 being most severe symptoms, 175–300 categorized as moderate severity). The IBS-C cohort also scored low to moderate for quality-of-life impairment (mean IBS-quality of life 32, 0–100 scale) and somatization (mean somatic symptom checklist score 0.6, 0–4 scale).

In vivo permeability

Baseline lactulose was undetectable in both IBS-C and healthy. Cumulative baseline excretions for ^{13}C mannitol were significantly lower than ^{12}C mannitol in both healthy (^{13}C mannitol: 0.06 (0.03) mg vs. ^{12}C mannitol: 6.1 (1.8) mg, $P<0.0001$; **Supplementary Figure S1a**) and IBS-C (^{13}C mannitol: 0.009 (0.007) mg vs. ^{12}C

mannitol: 3.87 (1.09) mg, $P<0.0001$; **Supplementary Figure S1b**). Of note, there were 5 participants in IBS-C and 6 participants in the control group with a baseline urinary quantity of >5 mg of ^{12}C mannitol. Similar observations were made in our previously published study (12). Hence, we used ^{13}C mannitol excretions as our primary measurement.

Cumulative excretion of probe molecules 0–2 h after ingestion.
The cumulative 0–2 h excretion of lactulose (IBS-C: 1.17 (0.18) mg vs. healthy: 0.95 (0.07) mg, $P=0.53$), ^{13}C mannitol (IBS-C: 12.12 (0.97) mg vs. healthy: 13.20 (0.79) mg, $P=0.39$), and lactulose/^{13}C mannitol excretion ratio (IBS-C: 0.01 (0.001) vs. healthy: 0.007 (0.0004), $P=0.25$) were similar between IBS-C and healthy (**Figure 1a–c**). Therefore, small bowel permeability was unchanged in IBS-C.

Cumulative excretion of probe molecules 8–24 h after ingestion.
The cumulative 8–24 h excretion of lactulose (IBS-C: 0.92 (0.47) mg vs. healthy: 0.51 (0.16) mg, $P=0.75$), ^{13}C mannitol (IBS-C: 3.12 (0.70) mg vs. healthy: 3.88 (0.61) mg, $P=0.08$), and lactulose/^{13}C mannitol excretion ratio (IBS-C: 0.02 (0.005) vs. healthy: 0.01 (0.004), $P=0.87$) was similar between IBS-C and healthy (**Figure 1d–f**). Therefore, colonic permeability in IBS-C was also unchanged.

The 2–8 h and the overall 0–24 h cumulative excretions were also similar between the two groups (**Supplementary Figure S2**).

Ex vivo mucosal barrier function

Duodenal TMR was similar between IBS and healthy (IBS-C: 28.16 (1.96) Ω cm² vs. healthy: 29.8 (1.94) Ω cm², $P=0.56$; **Figure 2a**). Subsequent to mucosal chamber administration of 4 kDa FITC–dextran, similar 3 h cumulative submucosal concentrations were obtained (IBS-C: 156.8 (33.73) ng/ml vs. healthy: 123.3 (24.29) ng/ml, $P=0.61$; **Figure 2b**) and the rate of flux (IBS-C: 6,528 (1,584) ng/h/cm² vs. healthy: 4,980 (1,044) ng/h/cm², $P=0.73$; **Figure 2c**) were also similar. Translocation of *E. coli* K12 Bio-Particles measured as 3 h cumulative submucosal concentration (IBS-C: 1.82×10^4 (6.0×10^3) CFU/ml vs. healthy: 1.48×10^4 (2.6×10^3) CFU/ml, $P=0.96$; **Figure 3a**) or rate of flux (IBS-C: 5.70×10^5 (1.90×10^5) CFU/h/cm² vs. healthy: 5.42×10^5 (1.2×10^5) CFU/h/cm², $P=0.34$; **Figure 3b**) were also similar among the two groups.

Colonic TMR was similar between IBS and healthy (IBS-C: 19.06 (1.10) Ω cm² vs. healthy: 17.60 (1.70) Ω cm², $P=0.24$; **Figure 2d**). Subsequent to mucosal chamber administration of 4 kDa FITC–dextran, similar 3 h cumulative submucosal concentrations were obtained (IBS-C: 122.5 (27.44) ng/ml vs. healthy: 132.2 (24.23) ng/ml, $P=0.66$; **Figure 2e**) and the rate of flux were also similar (IBS-C: 6,069 (1,030) ng/h/cm² vs. healthy: 4,931 (1,156) ng/h/cm², $P=0.36$; **Figure 2f**). Translocation of *E. coli* K12 Bio-Particles measured as 3 h cumulative submucosal concentration (IBS-C: 2.09×10^4 (6.8×10^3) CFU/ml vs. healthy: 1.07×10^4 (1.8×10^3) CFU/ml, $P=0.32$; **Figure 3c**) and the rate of flux (IBS-C: 7.67×10^5 (4.19×10^5) CFU/h/cm² vs. healthy: 4.26×10^5 (5.3×10^4) CFU/h/cm², $P=0.49$) were also similar among the two groups (**Figure 3d**).
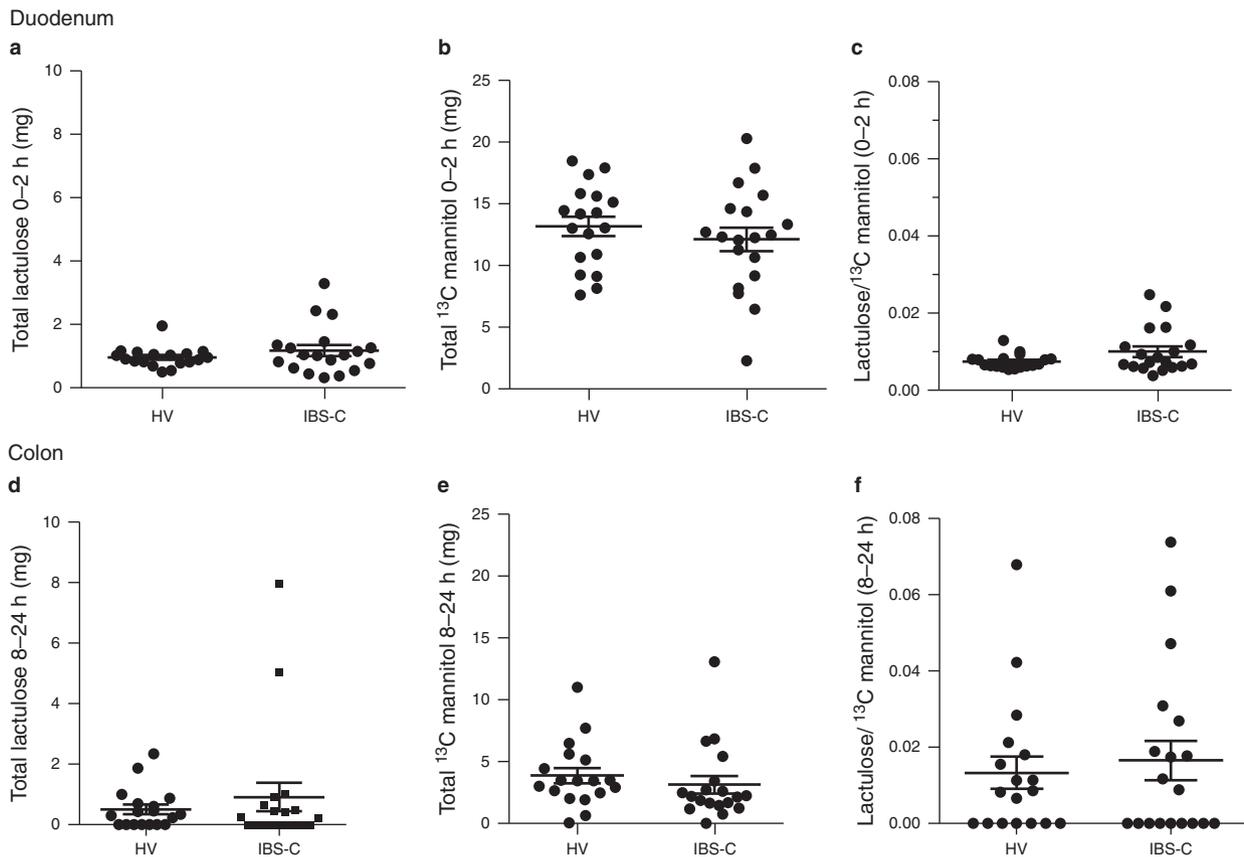
Duodenum

Colon

Figure 1. *In vivo* permeability assessment using saccharide excretion assay. (**a–c**) Small intestine (0–2 h posttest saccharide administration) and (**d–f**) colon (8–24 h posttest saccharide administration) are shown. (**a**) Cumulative lactulose excretion. (**b**) Cumulative ^{13}C mannitol excretion. (**c**) Lactulose/^{13}C mannitol ratio expressed as percentage of administered dose for each volunteer. (**d–f**) Corresponding cumulative excretion values and lactulose/^{13}C mannitol ratio for colon. Data are presented as mean±s.e.m. Healthy volunteers (HV) *n*=18 and constipation-predominant irritable bowel syndrome (IBS-C) *n*=19.

There was statistically significant correlation between the 4 kDa FITC–dextran flux and TMR tested on the same biopsy in duodenum (*r*=−0.57, *P*=0.0006; **Figure 4a**) and colon (*r*=−0.58, *P*=0.0012; **Figure 4b**).

Duodenal impedance

The duodenal impedance measurement catheter (**Figure 5a**) and a representative impedance tracing is shown (**Figure 5b**). The impedance values across the duodenal circumference were averaged for each subject. The mean impedance for IBS-C was 729.5 (64.85) Ω vs. 705.9 (42.73) Ω for healthy, *P*=0.71 (**Figure 5c**). The impedance values on the four duodenal walls ranged from 420.0 to 1,043.6 Ω in IBS-C cohort and 424.4 to 1,060.9 Ω in the healthy.

Serum endotoxemia

IBS-C patients had mean endotoxin levels of 0.36 (0.03) vs. a mean of 0.35 (0.02) in healthy, *P*>0.99 (**Figure 6**). Out of 18 IBS-C patients, 3 had endotoxemia levels of >0.5 (considered high) and these 3 patients also had high colonic mucosal flux of FITC–dextran.

Secretory responses

Baseline Isc was lower in the IBS-C group in duodenum (IBS-C: 43.53 (4.55) μA cm^{-2} vs. healthy: 56.89 (4.93) μA cm^{-2}, *P*=0.05; **Figure 7a**). Baseline colon Isc was similar (IBS-C: 86.41 (5.20) μA cm^{-2} vs. healthy: 96.78 (8.07) μA cm^{-2}, *P*=0.30; **Figure 7c**). The duodenum ΔIsc in response to ACh (IBS-C: 15.44 (5.26) μA cm^{-2} vs. healthy: 15.76 (3.62) μA cm^{-2}, *P*=0.79) was similar between the two groups (**Figure 7b**). The colon ΔIsc in response to ACh (IBS-C: 80.81 (13.73) μA cm^{-2} vs. healthy: 82.08 (13.34) μA cm^{-2}, *P*=0.99) was similar between the two groups (**Figure 7d**).

Mucosal cellular changes

The eosinophil counts were similar in IBS-C and healthy in duodenum (IBS-C: 16.26 (1.59)/HPF vs. healthy: 18.5 (1.73)/HPF, *P*=0.33) and colon (IBS-C: 4.47 (1.08)/HPF vs. healthy: 3.39 (0.49)/HPF, *P*=0.75). Duodenal mast cells were normal in IBS-C patients. However, colonic mast cells were increased in two IBS-C patients. Duodenal and colonic CD3^{+} cell grading was normal in IBS-C patients.
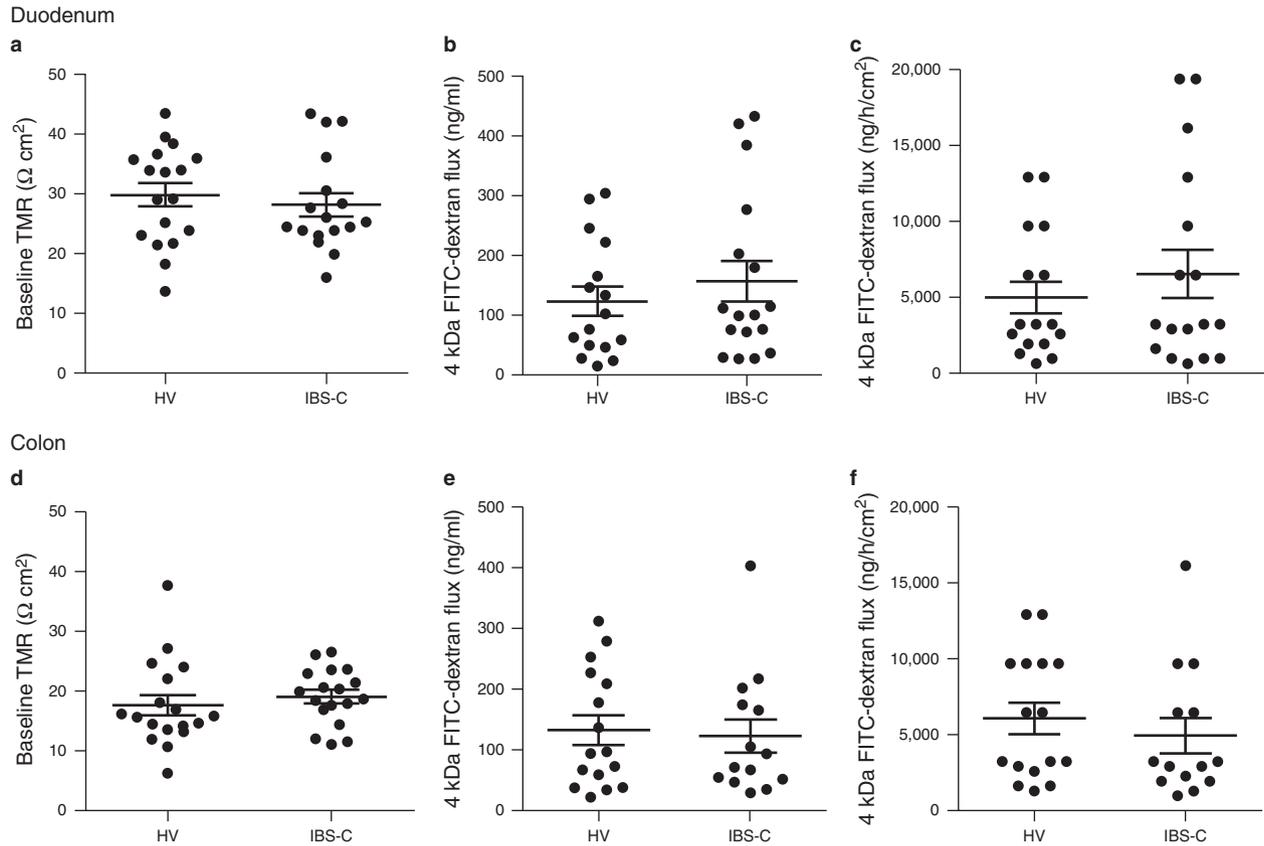
Duodenum

Colon

Figure 2. *Ex vivo* permeability assessment. (**a**) Baseline transmucosal resistance (TMR) of duodenal mucosa. (**b**) Cumulative fluorescein isothiocyanate (FITC)–dextran (4 kDa) concentration across duodenal mucosa measured after 180 min. (**c**) Rate of FITC–dextran (4 kDa) flux across duodenal mucosa, per exposed biopsy area, over time (**d**–**f**) Corresponding values for colonic mucosa. Data are presented as mean±s.e.m. Duodenum healthy volunteers (HV) *n*=18 and constipation-predominant irritable bowel syndrome (IBS-C) *n*=17 (**a**); HV *n*=16 and IBS-C *n*=17 (**b**, **c**). Colon HV and IBS-C *n*=18 (**d**); HV *n*=16 and IBS-C *n*=14 (**e**, **f**).

TJ gene expression

The colonic mucosal expression of transmembrane proteins occludin and claudins (1–12 and 14–19) was unchanged in IBS-C. Similarly, the expression of ZO 1–3 was unchanged (**Figure 8**). The remainder of the TJ genes tested in the array were also unchanged (**Supplementary Table S1**).

DISCUSSION

Disruption of mucosal barrier has been implicated in the pathophysiology of IBS (16). *In vivo* and *ex vivo* measures have shown an impaired barrier function in a subset of IBS patients, mostly in IBS-D. In addition, transcriptional and protein expression of TJ proteins have been found to be altered in IBS. Finally, parameters of impaired barrier function like increased lactulose/mannitol excretion ratio (17), increased mucosal paracellular permeability (4), and increased paracellular permeability of cellular monolayers in response to biopsy supernatants (3) have been found to correlate with the abdominal pain severity and visceral sensitivity in IBS-D. A comprehensive assessment of barrier function in IBS-C was lacking. Our results show that in this cohort of females with IBS-C who underwent detailed studies for assessment of

duodenal and colonic barrier properties, *in vivo* and *ex vivo* barrier function was preserved. There was no evidence of increased mucosal bacterial translocation and circulating endotoxemia in IBS-C patients. Upon assessment of secretion, IBS-C patients trended to have lower duodenal mucosal secretion as compared with healthy volunteers. In addition to helping understand pathophysiology of IBS, modulation of barrier and secretory properties is of interest for drug development in IBS-C. This study provides assessment of these fundamental epithelial functions in females with IBS-C and suggests that intestinal permeability and mucosal bacterial translocation are unchanged in females with IBS-C and therefore should not be targeted.

In vivo measurements of intestinal permeability are often done by measuring urinary excretion of molecules that are absorbed and poorly metabolized in the gut (e.g., lactulose, mannitol, rhamnose, sucralose, ^{51}Cr EDTA, polyethylene glycol, and so on) (18–20). Increased lactulose/mannitol excretion ratio has been shown in postinfectious IBS following *Campylobacter jejuni* (21) and mixed infections (*C. jejuni* and *E. coli* O157:H7) (22). However, there is significant interday variability in excretion of those sugars (23) and about a third of the subjects excrete significant amounts of mannitol at baseline (12). This could be because of inadvertent
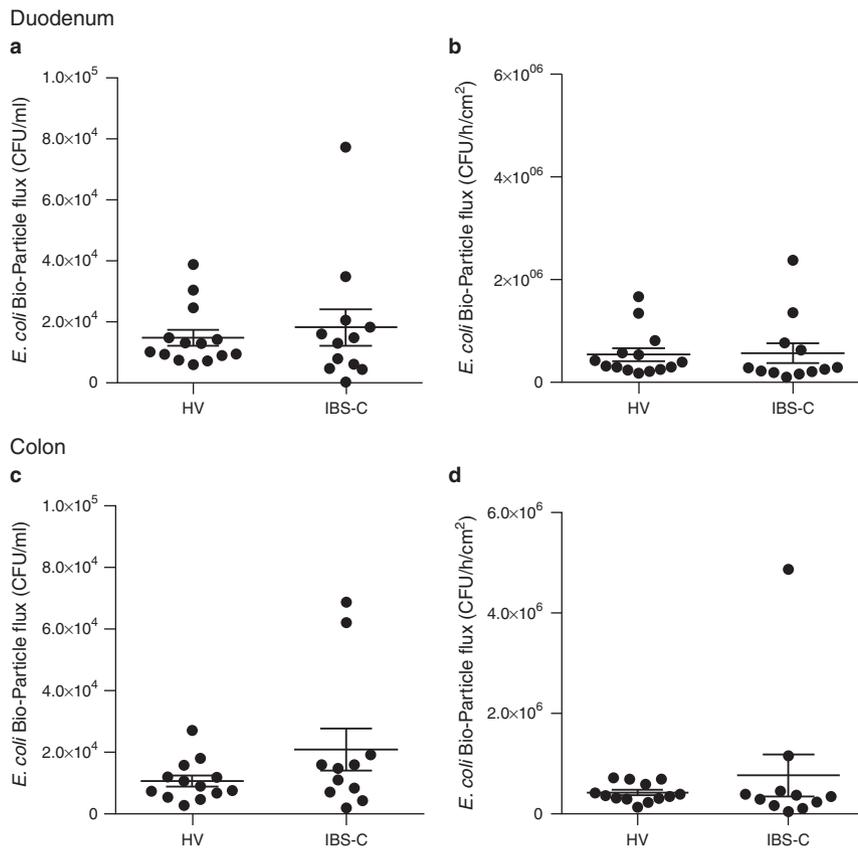
Duodenum

Colon

Figure 3. *Ex vivo* bacterial translocation. (**a**) Cumulative fluorescein conjugated *Escherichia coli* Bio-Particle concentration in colony-forming units (CFUs) across duodenal mucosa, measured after 180 min. Healthy volunteers (HV) *n*=14 and constipation-predominant irritable bowel syndrome (IBS-C) *n*=12. (**b**) Rate of *E. coli* Bio-Particle Flux in CFUs across duodenal biopsies, per exposed area, per time. (**c**, **d**) Corresponding values for colonic mucosa. HV *n*=16 and IBS-C *n*=14.

Duodenum   Colon
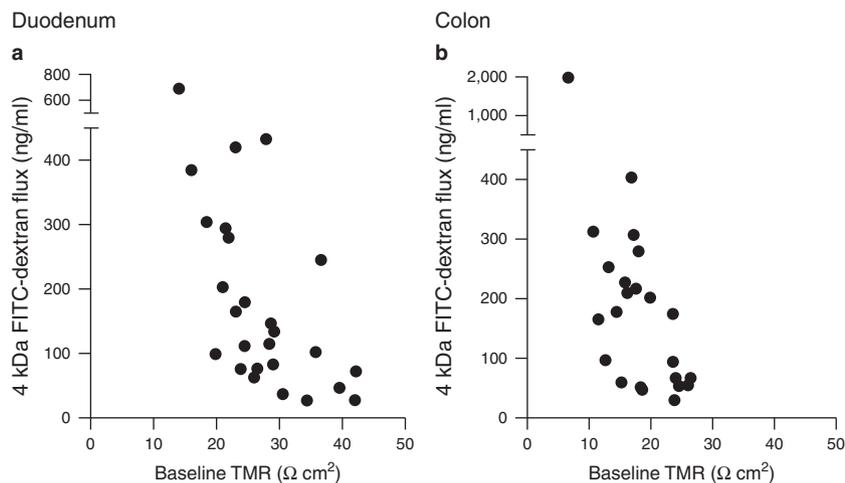
Figure 4. Correlations between baseline transmucosal resistance (TMR) and fluorescein isothiocyanate (FITC)–dextran Flux (4 kDa) across the same biopsies. (**a**) Correlation between baseline duodenal TMR and 3 h cumulative submucosal FITC–dextran (4 kDa) concentration. Pearson's correlation coefficient=−0.57, *P*<0.0006. Number of XY pairs=32. (**b**) Correlation between baseline colonic TMR and 3 h cumulative submucosal FITC–dextran (4 kDa) concentration. Pearson's correlation coefficient=−0.58, *P*<0.0012. Number of XY pairs=28. Healthy volunteer (HV) and constipation-predominant irritable bowel syndrome (IBS-C) biopsy data were combined. A *P* value of <0.05 was considered significant.

contamination during the study (either from diet, medications, or other sources), baseline sugar competing with absorption, or the production of ^{12}C mannitol by colonic microbiota during the testing (24). ^{13}C constitutes only 1% of naturally occurring carbon and is a stable (nonradioactive) isotope. We show in this study that ^{13}C mannitol can be separated from ^{12}C mannitol on
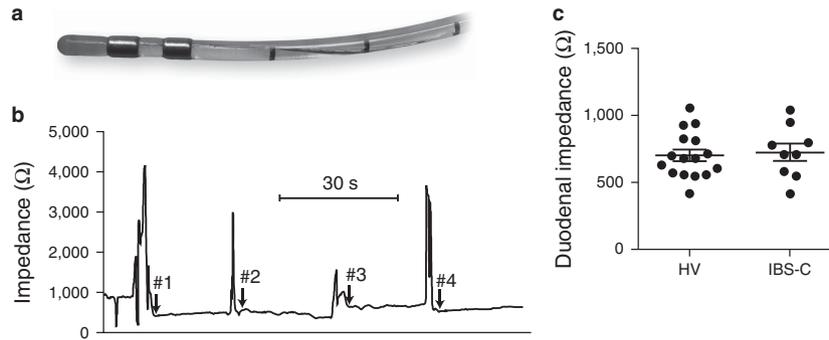
Figure 5. Duodenal impedance. (**a**) Mucosal impedance catheter tip. (**b**) Representative tracing with arrows representing start of impedance recordings on medial, superior, lateral, and inferior duodenal walls with direct mucosal contact under endoscopic visualization. (**c**) Four measurements obtained for each subject and plotted values represent averages of the four recordings. Data are presented as mean±s.e.m. Healthy volunteers (HV) *n*=16 and constipation-predominant irritable bowel syndrome (IBS-C) *n*=9.
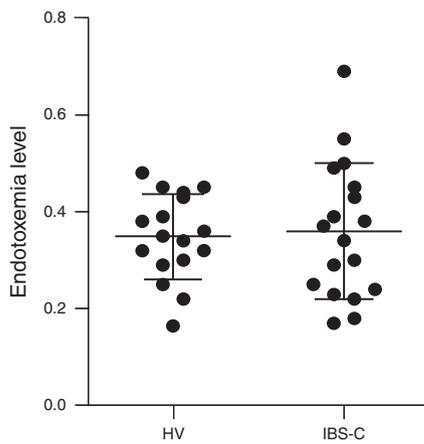
Figure 6. Endotoxemia levels. Bacterial lipopolysaccharide levels in anticoagulated whole blood. Data are presented as mean±s.e.m. Healthy volunteers (HV) *n*=17 and constipation-predominant irritable bowel syndrome (IBS-C) *n*=18.

high-performance liquid chromatography-tandem mass spectrometry and is present in extremely small concentrations in baseline urine sample, offering a novel molecule for measurement of intestinal permeability. Our results indicate no difference in *in vivo* duodenal or colonic permeability using the ^{13}C mannitol excretion assay in this cohort of IBS-C females. Subsequent studies using ^{13}C mannitol in other IBS phenotypes (IBS-D or mixed IBS) should be able to provide a more accurate assessment of intestinal permeability than ^{12}C mannitol.

Soluble factors have been proposed to mediate changes in mucosal barrier function and visceral sensitivity. Fecal cysteine proteases were previously found to be increased in a subset of IBS-C patients and their activity correlated with symptom severity, especially abdominal pain score. Application of IBS-C fecal supernatants on mouse colonic mucosa *in vivo* or cultured monolayers (T84) *in vitro* caused enzymatic degradation of occludin *in vitro*, increased permeability *in vivo*, and increased sensitivity to colorectal distension *in vivo* (5). Two previous studies assessing colonic

mucosal paracellular permeability used fluorescein-5.6 sulfonic acid (478 Da) that is likely to have greater flux as compared with the FITC–dextran (4,000 Da) that we used. However, most of the dietary antigens are >600 Da, and this makes studying a larger molecule more useful for investigating barrier in disease states (25). We also studied *E. coli* K-12 Bio-Particle translocation that is mediated by transcellular pathways and found no differences between IBS-C and health. Several factors can play a role in modulating intestinal barrier function in IBS. Psychosocial comorbidities are common in IBS and associate with symptom severity. Corticotrophin-releasing factor has been shown to increase permeability (26,27) and TJ proteins have a rapid exchange at the junctions (28). Thus, it is plausible that specific dietary or environmental triggers, such as stress, may cause intermittent disruption of barrier function mediated by the soluble factors (29). Previous studies assessing intestinal barrier function in IBS had patients with greater levels of comorbid anxiety and depression and higher symptom severity, whereas our cohort of females with IBS-C scored fairly low on anxiety, depression, and symptom severity. In addition, environmental factors can affect intestinal permeability. An allergic background correlates with a more severe disease and diarrhea predominance with suggestive links with increased paracellular permeability (4). Recently, house dust mite, a common environmental trigger, was shown to increase FITC-sulfonic acid flux in a dose-dependent manner across human colonic mucosa, decrease ZO-1 and occludin expression, and cause damage to the TJ ultrastructure (30).

We also studied endotoxemia levels in our cohort. The precise pathways that allow lipopolysaccharide entry through the gastrointestinal mucosal and defense systems into the portosystemic circulation are unknown and both trans and paracellular routes have been proposed (31). Immune dysregulation and low-grade inflammation have been found to be present in a subset of IBS patients. Bacterial lipopolysaccharide has been proposed to mediate and reflect low-grade inflammation in conditions such as obesity (33); however, it is not known whether IBS patients have elevated circulating endotoxins. Although, overall our IBS-C patients did not have higher endotoxemia levels than controls, 3/18 IBS-C patients had high endotoxemia levels (>0.5) that have been associated with
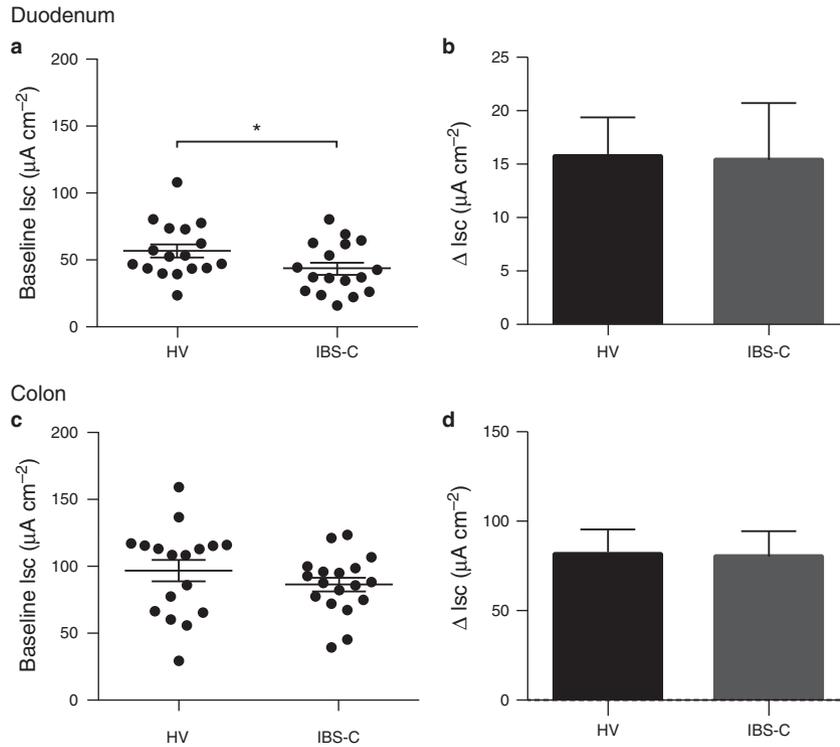
Duodenum

Colon

Figure 7. Mucosal secretory responses. (a) Baseline duodenal short circuit current (Isc) of healthy and constipation-predominant irritable bowel syndrome (IBS-C) participants. Healthy volunteers (HV) $n=17$ and IBS-C $n=17$. (b) Duodenal ΔIsc in response to 0.0003 M submucosal acetylcholine (Ach). HV $n=17$ and IBS-C $n=18$. (c) HV $n=14$ and IBS-C $n=12$ and (d) HV $n=18$ and IBS-C $n=17$ corresponding values for colonic mucosa.
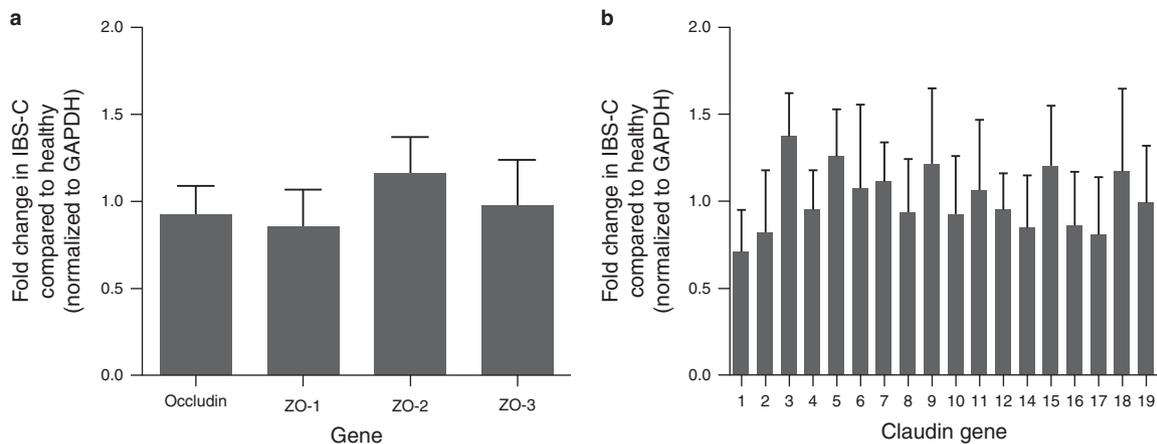

Figure 8. Colonic mucosal gene expression (a) occludin, zonula occludens (ZO)-1, -2, and -3, and (b) claudins 1–12 and 14–19 in constipation-predominant irritable bowel syndrome (IBS-C) in comparison with healthy. Healthy volunteers (HV) $n=10$ and IBS-C $n=18$. Genes were normalized by arithmetic mean to the control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Error bars represent 95% confidence interval (CI).

systemic inflammation. These three patients also had high colonic FITC–dextran flux, suggesting that impaired paracellular barrier may be allowing bacterial lipopolysaccharide to access systemic circulation.

A number of drugs for IBS-C function as secretagogues. However, the baseline mucosal secretory properties in IBS-C are unknown. Isc measured under voltage clamp conditions in Ussing

chambers allows assessment of electrogenic ionic fluxes at baseline and in response to pharmacological and electrical stimulation. Although ΔIsc in response to such stimulation can be a result of complex ionic transport, luminal secretion of Cl^- or HCO_3^- and Na^+ absorption are the predominant processes involved. ACh primarily activates epithelial ion channels via an increase of intracellular calcium levels. We show that the baseline duodenal Isc

was lower in IBS-C as compared with the healthy. This can result in lower luminal anionic secretion and associated luminal transport of water that can result in slower intestinal transit seen in IBS-C (34). Although ionic (Cl^-) and water secretion in colon has been studied in greater detail in animal models, mucosal secretory mechanisms in IBS-C patients have not been studied in either colon or small bowel. One study looking at transmural potential difference as a surrogate for intestinal secretion found elevated potential difference in jejunum but not duodenum in patients with IBS-C (35). However, it is unclear whether perfusion system-based measurement of *in vivo* potential difference reflects mucosal secretory response or correlates with transit. A recent study using surgical specimens provided detailed description of baseline secretory and barrier properties of human intestinal mucosa–submucosa preparations from a variety of disease conditions except IBS (36). Although baseline duodenal and colonic Isc values described were lower than ours, duodenal Isc was lower than rectosigmoid colonic Isc, similar to our observations. This study also showed that ongoing Ach release is present in small intestine but not colon, and both Cl^- and HCO_3^- secretion can play an important role in baseline Isc. Further studies should delineate *ex vivo* and *in vivo* small intestinal secretory mechanisms in IBS-C.

In summary, in this largest cohort of IBS-C females studied to date using complementary *in vivo* and *ex vivo* techniques and simultaneous assessment of small and large intestine, barrier function was not found to be altered. This is important considering an emerging interest for peripheral mechanisms in the pathophysiology of IBS. It is plausible that although IBS-C and IBS-D are diagnosed and conceptualized using similar mechanistic framework, the peripheral mechanisms of these two subtypes are quite different. In addition, although from mechanistic and pharmacological standpoint, secretory mechanisms are better studied and targeted in colon, secretion in the small bowel probably plays an important role in pathophysiology of IBS-C and should be further studied.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

Study Highlights

WHAT IS CURRENT KNOWLEDGE

✓ Disrupted intestinal barrier is present in a subset of irritable bowel syndrome (IBS) patients and possibly plays a role in symptom generation.

✓ Most of the studies have focused on diarrhea-predominant IBS.

✓ Intestinal barrier and secretory function in constipation-predominant IBS (IBS-C) are poorly understood.

WHAT IS NEW HERE

✓ *In vivo* duodenal and colonic permeability, duodenal impedance, *ex vivo* duodenal and colonic mucosal barrier function, and colonic tight junction gene expression were unchanged in IBS-C females.

✓ There was no evidence of increased duodenal or colonic mucosal bacterial translocation or circulating endotoxemia in IBS-C females.

✓ Basal duodenal mucosal secretion was lower in IBS-C.

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