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Molecular Mechanisms Underlying Loss of Vascular and Epithelial Integrity in Irritable Bowel Syndrome

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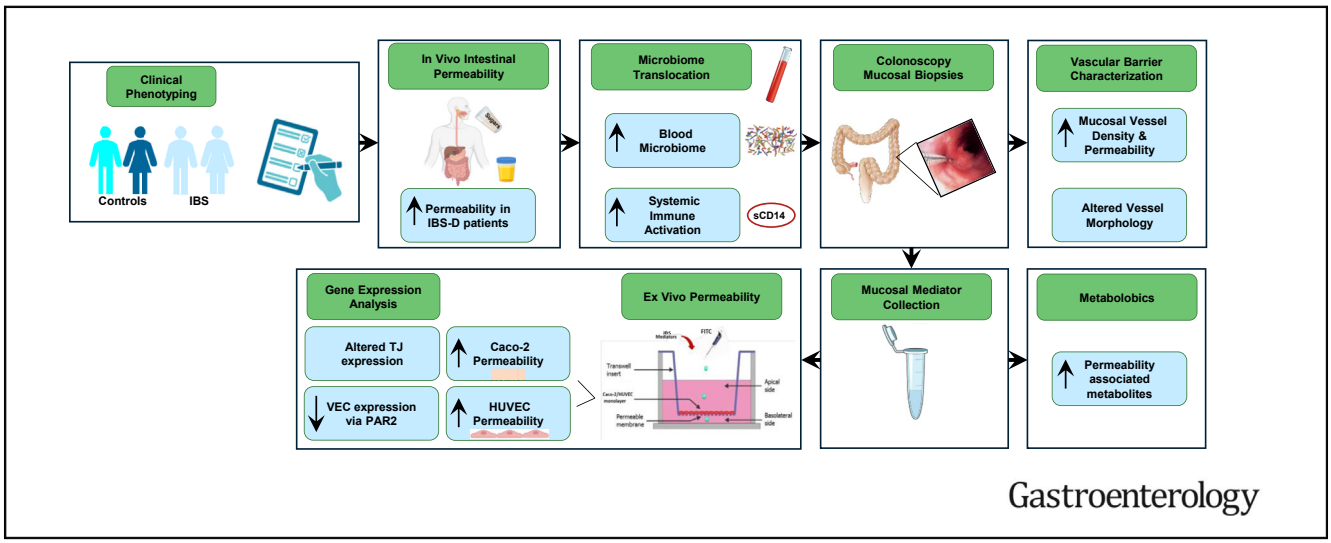

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BACKGROUND & AIMS: The pathophysiology of irritable bowel syndrome (IBS) is multifactorial and includes epithelial barrier dysfunction, a key element at the interface between the gut lumen and the deeper intestinal layers. Beneath the epithelial barrier there is the vascular one representing the last barrier to avoid luminal antigen dissemination The aims of this study were to correlate morpho-functional aspects of epithelial and vascular barriers with symptom perception in IBS. **METHODS:** Seventy-eight healthy subjects (controls) and 223 patients with IBS were enrolled in the study and phenotyped according to validated questionnaires. Sugar test was used to evaluate in vivo permeability. Immunohistochemistry, western blot, and electron microscopy were used to characterize the vascular barrier. Vascular permeability was evaluated by assessing the mucosal expression of plasmalemma vesicle–associated protein-1 and vascular endothelial cadherin. Caco-2 or human umbilical vein endothelial cell monolayers were incubated with soluble mediators released by mucosal biopsies to highlight the mechanisms involved in permeability alteration. Correlation analyses have been performed among experimental and clinical data. **RESULTS:** The intestinal epithelial barrier was compromised in patients with IBS throughout the gastrointestinal tract. IBS-soluble mediators increased Caco-2 permeability via a downregulation of tight junction gene expression. Blood vessel density and vascular permeability were increased in the IBS colonic mucosa. IBS mucosal mediators increased permeability in human umbilical vein endothelial cell monolayers through the activation of protease-activated receptor-2 and histone deacetylase 11, resulting in vascular endothelial cadherin downregulation. Permeability changes correlated with intestinal and behavioral symptoms and health-related quality of life of patients with IBS. **CONCLUSIONS:** Epithelial and vascular barriers are compromised in patients with IBS and contribute to clinical manifestations.

Keywords: Gut Vascular Barrier Permeability; Intestinal Barrier Permeability; Plasmalemma Vesicle–Associated Protein 1; Protease-Activated Receptor 2; Vascular Endothelial Cadherin; Histone Deacetylase.

Irritable bowel syndrome (IBS) is a disorder of gut-brain interaction[1] characterized by recurrent abdominal pain associated with defecation or a change in bowel habits.[2] Although IBS is not a life-threatening disease, it is associated with a reduction of health-related quality of life (HRQOL) of patients, a high worldwide prevalence of 4.1%[3] and an important economic burden on society.[4] According to Rome IV criteria, IBS can be subcategorized into IBS with predominant diarrhea (IBS-D), IBS with predominant constipation (IBS-C), IBS with mixed bowel habits (IBS-M), and unclassified IBS (IBS-U).[2] The pathophysiology of IBS is multifactorial and includes a combination of dysfunctions within the gut-brain axis, including gut motility, visceral sensitivity, mucosal immune system, epithelial barrier, and intestinal microbiota.[5]

The intestinal mucosal barrier is the largest interface between the environment and the human body and encompasses primary roles of gut microbiota, mucus, and the epithelium.[6] The epithelial barrier contributes to human homeostasis allowing the absorption of nutrients, water, and electrolytes but at the same time limiting the passage of harmful antigens and microorganisms. Increased intestinal permeability[7] was reported in 37% to 62% of patients with IBS-D,[7] although present also in the other subgroups. Genetic predisposition, stress, and adverse food reaction, as well as bile acid malabsorbtion[8] and the excessive release of proteolytic mediators, may also participate in permeability alterations.[6]

There is a meaningful potential implication of increased intestinal permeability for symptom generation in patients with IBS. Indeed, increased intestinal permeability exposes the mucosa to an abnormal challenge of luminal antigens and microbiota and their metabolites promoting and maintaining mucosal immune activation and visceral hypersensitivity, a concept further corroborated by evidence that in IBS increased intestinal permeability correlates with the severity of abdominal pain.[9] Conversely, an attenuation of barrier dysfunction[10] improves abdominal pain,[11] and visceral hypersensitivity.[12]

Although epithelial barrier defects may suffice to evoke local tissue damage and dysfunction, effects on distant organs requires breaching of the vascular endothelial barrier leading to the systemic spread of noxious luminal antigens, metabolites, cytokines, chemokines, and microbiota metabolites. Blood capillaries are strategically located immediately beneath the epithelial barrier to efficiently receive and distribute nutrients and signals throughout the body. Maintaining the integrity of the epithelium as well as the gut vascular barrier (GVB) is essential to avoid the systemic spread of harmful antigens and bacterial particles.[13] Vascular endothelial cadherin (VEC) plays a key role in the maintenance of endothelial barrier integrity as demonstrated in in vitro studies of VEC silencing[14] and considering the evidence that VEC knockout in embryo mice is lethal.[15]

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Irritable bowel syndrome is characterized by intestinal and extra-intestinal symptoms contributing to impaired quality of life. This study aimed to correlate morpho-functional aspects of the epithelial and vascular barriers with symptom perception in irritable bowel syndrome.

NEW FINDINGS

Intestinal epithelial barrier integrity was compromised in patients with irritable bowel syndrome. This was accompanied by mucosal vascular dysfunction, as shown by a quantitative increase in mucosal blood vessels and increased vascular permeability.

LIMITATIONS

Whether epithelial and vascular barrier dysfunction is reversible and amenable to specific treatments requires further investigation.

CLINICAL RESEARCH RELEVANCE

This study demonstrates that increased vascular and epithelial permeability correlates with intestinal and extra-intestinal symptoms, providing targets to identify novel therapeutic targets.

BASIC RESEARCH RELEVANCE

This study elucidates morphological changes and molecular mechanisms underlying increased vascular endothelial permeability in irritable bowel syndrome. Irritable bowel syndrome mucosal mediators induced increased permeability in human vascular endothelial monolayers by the activation of protease-activated receptor-2 and histone deacetylase 11, resulting in vascular endothelial cadherin downregulation.

One of the pathways involved in endothelial and epithelial barrier dysfunction relates to the activation of protease-activated receptor 2 (PAR2). PAR2 is widely expressed in the colonic mucosa and on endothelial cells.[16,17] PAR2 is activated by proteases, including tryptase and trypsin-3 that are both markedly increased in the colonic mucosa of patients with IBS.[18] This occurs through the cleavage of the N-terminal extracellular domain, and subsequent interaction of this newly released domain that acts as a tethered ligand binding the second extracellular loop of the receptor, thereby initiating signal transduction.[19]

The aims of the present study were to characterize the gut epithelial and vascular endothelial barriers in IBS by

Abbreviations used in this paper: **ALT, alanine aminotransferase; AST, aspartate aminotransferase; CD, Crohn's disease; GVB, gut vascular barrier; HDAC11, histone deacetylase 11; HRQOL, health-related quality of life; HUVEC, human umbilical vein endothelial cell; IBS, irritable bowel syndrome; IBS-C, IBS with predominant constipation; IBS-D, IBS with predominant diarrhea; IBS-M, IBS with mixed bowel habits; iPAR, PAR2 antagonist I-191; L/M, lactulose/mannitol; PAR2, protease-activated receptor 2; PV1, plasmalemma vesicle–associated protein-1; UC, ulcerative colitis; VEC, vascular endothelial cadherin.**

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using in vitro and in vivo analyses; to investigate the molecular mechanisms underlying barrier dysfunctions in patients with IBS compared with asymptomatic controls; and to evaluate the relationships between experimental data and clinical symptoms.

Materials and Methods

The study design is summarized in Figure 1.

Subjects and Patients

The population included 78 healthy subjects (controls) and 223 patients with IBS (IBS-D = 112, IBS-C = 58, IBS-M = 53) (Supplementary Table 8). Controls were recruited from asymptomatic subjects undergoing colonoscopy for colorectal cancer screening or polypectomy follow-up after exclusion of gastrointestinal symptoms. Controls had a score of 3 to 4 according to the Bristol Stool Form Scale, having no symptoms in most cases or mild and occasional abdominal symptoms falling under the 90th percentile of normality and belonging to the normal frequency of occurrence of gastrointestinal symptoms in the general population. Patients with IBS were diagnosed according to Rome IV criteria. In some experiments, patients with inflammatory bowel disease were also enrolled.[20,21] Eighteen patients with Crohn's disease (CD) undergoing in vivo permeability test were recruited at the University of Padova (see "Subjects and patients" in the Supplementary Material).

Symptom Questionnaires

Each subject completed a full series of questionnaires within 2 weeks before enrollment (see "Symptom questionnaires" in the Supplementary Material).

Gastrointestinal Permeability Testing

Gastrointestinal permeability was assessed by means of a multi-saccharide test through the detection of the saccharides in the urine following oral administration using the Mass-Q GASTROPACK I test (AB-Analitica, Padova, Italy) according to the manufacturer's instructions. The test is based on the urinary quantification of 4 sugars ingested by the subject. The abnormal presence of these sugars in the urine indicates alterations in the morphology or functioning of a specific gastrointestinal site. Precisely, mannitol was used as an internal control as a marker of small bowel epithelial surface and integrity; sucrose, lactulose, lactulose/mannitol (L/M), and sucralose were used as markers of gastro-duodenal, small intestinal, small intestinal normalized for the total small intestinal absorbing surface, and colonic permeability, respectively (see "Sugar intestinal permeability test" in the Supplementary Material).[22]

Histology and Immunohistochemistry

During colonoscopy, mucosal biopsies were taken from the descending colon and used for histology and immunohistochemistry. Histological sections were analyzed for exclusion of microscopic colitis or overt mucosal inflammation by an expert pathologist who was unaware of the diagnosis (see "Histology and immunohistochemistry" in the Supplementary Material).

Electron Microscopy

For ultrastructural analyses, specimens of controls and patients with IBS were harvested and fixed in 2.5% buffered glutaraldehyde. The following most common ultrastructural changes in capillary morphology were assessed: intercellular junction leaks, which sometimes were associated with disruption of endothelial continuity and widening of the space between the endothelium and pericytes. (see "Electron microscopy" in the Supplementary Material).

Western Blotting

Total proteins were extracted from biopsies using a tissue protein extraction reagent with the addition of a protease inhibitor cocktail (Thermo Scientific) according to the manufacturer's instructions. Details are reported in "Western blot" in the Supplementary Material.

Mucosal Mediator Release

The spontaneous release of mediators from colonic biopsies was carried out following a previously validated method[23] (see "Mucosal mediator release" in the Supplementary Material).

Caco-2 and Human Umbilical Vein Endothelial Cell Cultures

The human intestinal epithelial cell line Caco-2 (EATCC, Port Down, UK) was used as an in vitro model of intestinal epithelial barrier as previously described.[23] The human umbilical vein endothelial cells (HUVECs) were used as an in vitro model of endothelial barrier. Details are reported in "HUVEC cell cultures" in the Supplementary Material.

In Vitro Permeability Assays

Caco-2 or HUVECs were seeded onto porous filters (12-well Transwell Clear, 0.40 μm porosity, 1.1 cm of diameter; Corning, Milan, Italy), at a density of 200,000 cells/filter. The volume of growth medium on the apical side was 500 μL, whereas on the basolateral side it was 1000 μL (see "In vitro permeability assays" in the Supplementary Material).

qPCR Assay

Cells stored in RNAlater were thawed and washed 2 times with phosphate-buffered saline, the RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germania) according to the manufacturer's instructions, and the complementary DNA was obtained with the QuantiTect Reverse Transcription Kit (Qiagen) that was used according to the manufacturer's instructions. Amplification condition details are reported in "qPCR" in the Supplementary Material.

Metabolomics

Untargeted metabolomic analyses were performed (see "Metabolomics" in the Supplementary Material).

Blood Bacterial DNA

Bacterial DNA in blood samples was quantified according to the method previously described by Gargari et al,[24] with a few modifications (see "Blood bacterial DNA" in the Supplementary Material).

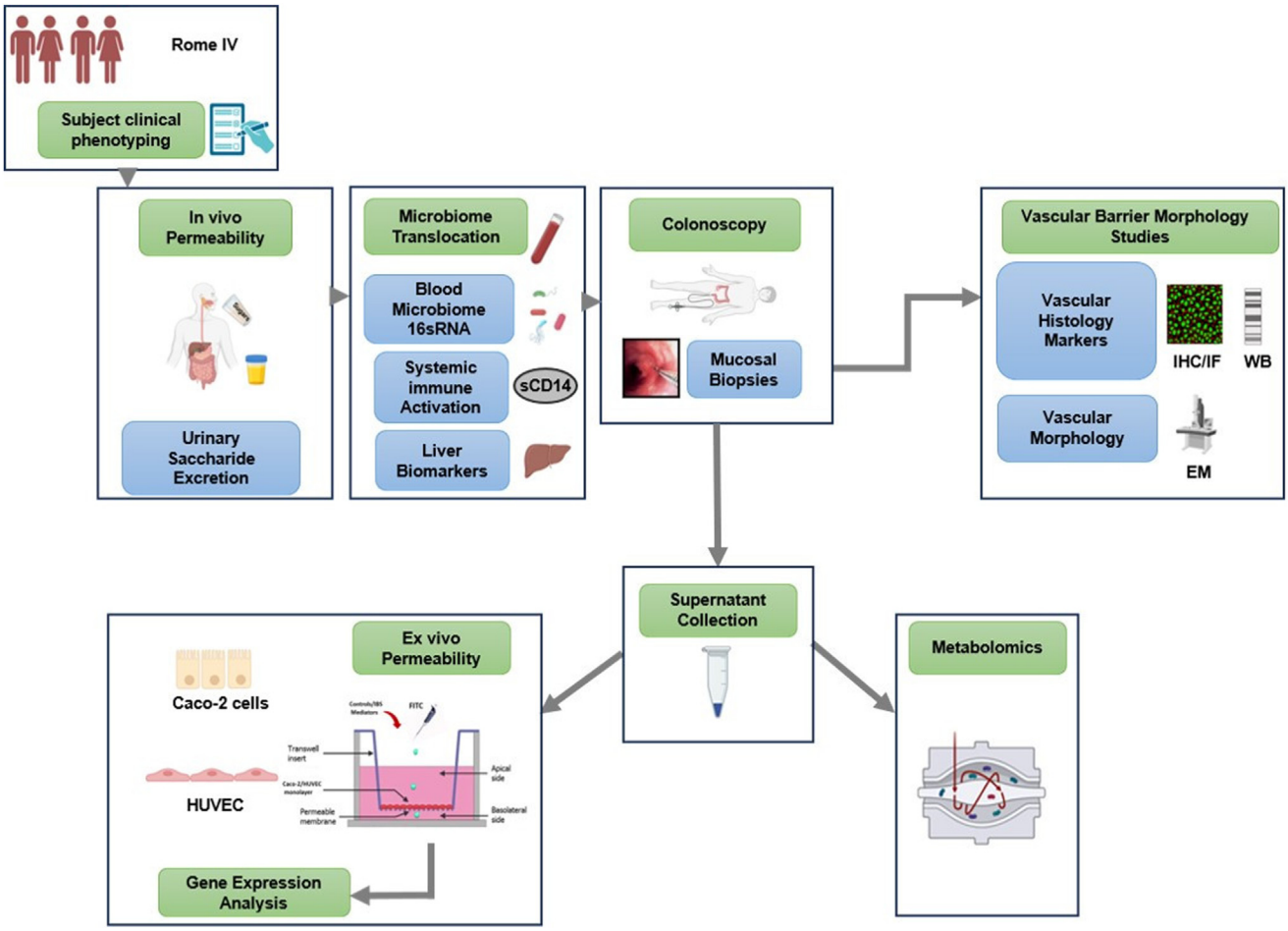


Figure 1. Study design. All subjects were phenotyped by using validated questionnaires. The experimental analyses were conducted on urine (in vivo permeability test), biopsies (translational experiments and morphological analyses), and blood to evaluate bacterial translocation, immune activation, and liver functionality. EM, electron microscopy; IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blot.

Liver Enzymes

Serum samples were used to evaluate alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Values <25 U/L were considered normal.

Systemic Innate Immune Activation

Serum levels of soluble CD14 (sCD14; R&D systems) were determined by enzyme-linked immunosorbent assay, according to the manufacturer's protocol.

Data Expression and Statistical Analysis

Data were reported as mean and standard deviation or standard error of the mean for continuous variables and numbers and percentages for categorical variables. Comparisons of clinical and demographic characteristics were assessed by Fisher, χ^2, χ^2 for trend, Kruskal-Wallis, or Mann-Whitney tests. The relationships between experimental and clinical data were assessed using Spearman's rho. Corrections for multiple testing were not applied as this approach can be overly conservative. Finally, receiver operating characteristic (ROC) curves were built and compared for testing the diagnostic accuracy of each sugar excretion test for the diagnosis of IBS-D. Partial least square discriminant analysis (PLSDA) was performed using R software. *P* values less than .05 were considered statistically significant. Statistical analyses were performed using Stata/SE (Version 17; Stata Corp, College Station, TX) (Supplementary Tables 9–14).

Results

Study Subjects

Demographic data and symptomatic scores for the recruited population for gut epithelial barrier studies are shown in Supplementary Table 1. Both anxiety and depression were significantly higher in the IBS-D group compared with controls, whereas there were no differences in patients with CD.

Regarding HRQOL, all 8 items were significantly lower in patients with IBS-D compared with controls. Supplementary Table 2 details demographic data and symptomatic scores for the endothelial study. The IBS group was notably younger and had more female patients than the controls. Specifically, IBS-D and IBS-M subgroups were significantly younger, whereas IBS-C and IBS-M subgroups had a higher

percentage of female patients. Anxiety and depression scores were significantly higher in both the IBS group and its subgroups compared with controls. Sub-analyses stratifying patients by gender and median age were also performed (Supplementary Tables 9–14).

In Vivo and In Vitro Permeability Testing Shows Increased Gut Permeability in IBS via Downregulation of Tight Junction Gene Expression

Patients with CD showed a marked increase in sucrose and sucralose and borderline increase ($P = 0.052$) of L/M ratio compared with controls, indicating increased intestinal permeability throughout the gastrointestinal tract and were therefore used as positive internal reference (Figure 2). Patients with IBS-D demonstrated a significant increase of sucralose (1.55 ± 0.10 vs 1.25 ± 0.16, $P < .01$, Figure 2*F*) and sucrose excretion (0.05 ± 0.005 vs 0.03 ± 0.004, $P < .01$, Figure 2*C*), lactulose (0.33 ± 0.03 vs 0.25 ± 0.03, $P < .05$, Figure 2*D*) as well the L/M ratio (0.02 ± 0.001 vs 0.01 ± 0.002, $P < .001$, Figure 2*E*) compared with controls, suggesting an increase in gastric, small intestinal, and colonic permeability. Mannitol excretion (Figure 2*B*) in IBS-D was not statistically different compared with controls (18.4 ± 0.85 vs 20.3 ± 1.01, respectively). Compared with IBS-D and controls, patients with CD showed reduced excretion of mannitol, suggesting a reduction of total epithelial surface.

The diagnostic accuracy of sugar excretion in discriminating controls from patients with IBS-D was determined with ROC curves. The highest level of accuracy was associated to the L/M ratio (area under the ROC: 0.773), with the best cutoff value of 0.0105 (sensitivity 97%, specificity 56%, Supplementary Figure 1).

Supernatants of patients with IBS evoked a time-dependent increase in paracellular permeability of Caco-2 cells with the highest values at 6 hours of incubation and were therefore used in the following experiments as a referral time point. Compared with controls, supernatants of patients with IBS induced a significant increase in Caco-2 paracellular permeability. Each of the 3 IBS subgroups induced a significant increase in paracellular permeability (Figure 2*A* and *B*). Supernatants of patients with IBS induced a downregulation in zonula occludens-1, claudin-1, occludin, and junctional associated molecule-A gene expression in Caco-2 cells (Figure 2*C*–*G*).

Mucosal Vessel Density Is Increased Along With Higher Expression of Mucosal Permeability Markers in IBS

Mucosal blood vessel structure and density were assessed in controls and patients with IBS, whereas UC and CD were used as positive controls.[25] Compared with controls (4.88 ± 0.53), blood vessel density was significantly increased in UC (2-fold, 9.94 ± 0.77, $P < .001$), CD (2.7-fold, 12.96 ± 0.45, $P < .0001$), and IBS (1.5-fold, 7.39 ± 0.43, $P < .01$) (Figure 3*B*). Blood vessel density was increased only in patients with IBS-D (1.7-fold, 8.19 ± 0.69) and IBS-M (1.4-fold, 6.96 ± 0.76), but not in patients with IBS-C (6.87 ± 0.77) compared with controls ($P < .01$ and $P < .05$, respectively). Western blot analysis (Supplementary Figure 2) of CD34 protein expression in colonic mucosa homogenates of patients with IBS showed a significant increase compared with samples from controls ($P < .05$). The most striking significant effect was detected in IBS-D compared with controls ($P < .05$). To confirm the increased mucosal blood vessel density in patients with IBS, a second marker (ie, CD31) was used. Compared with controls (11.03 ± 4.16), CD31 expression was increased in the mucosa of patients with UC (23.27 ± 4.98) and CD (85.86 ± 31.30, Figure 3*D*) as well as in IBS (42 ± 5.32, including all the 3-bowel habit IBS subcategories. Compared with controls, vessels with a diameter >20 and <30 μm were significantly increased in patients with IBS ($P < .05$). Patients with IBS-C showed increased number of vessels with a diameter >20 and <30 μm ($P < .05$). No other significant difference emerged.

Vascular permeability was assessed by measuring mucosal expression of VEC and plasmalemma vesicle–associated protein-1 (PV1). The expression of VEC was decreased in IBS (25.60 ± 3.16) and in the subgroups IBS-D (26.01 ± 4.95) and IBS-C (20.35 ± 3.24) compared with controls (64.37 ± 20.03, Figure 3*F*, $P < .05$). PV1 expression (Figure 3*H*) was increased in patients with IBS (4.21 ± 0.35, $P < .05$) and in the subgroups IBS-D (4.71 ± 0.46, $P < .01$) and IBS-M (4.11 ± 0.44, $P < .05$) compared with controls (2.79 ± 0.48).

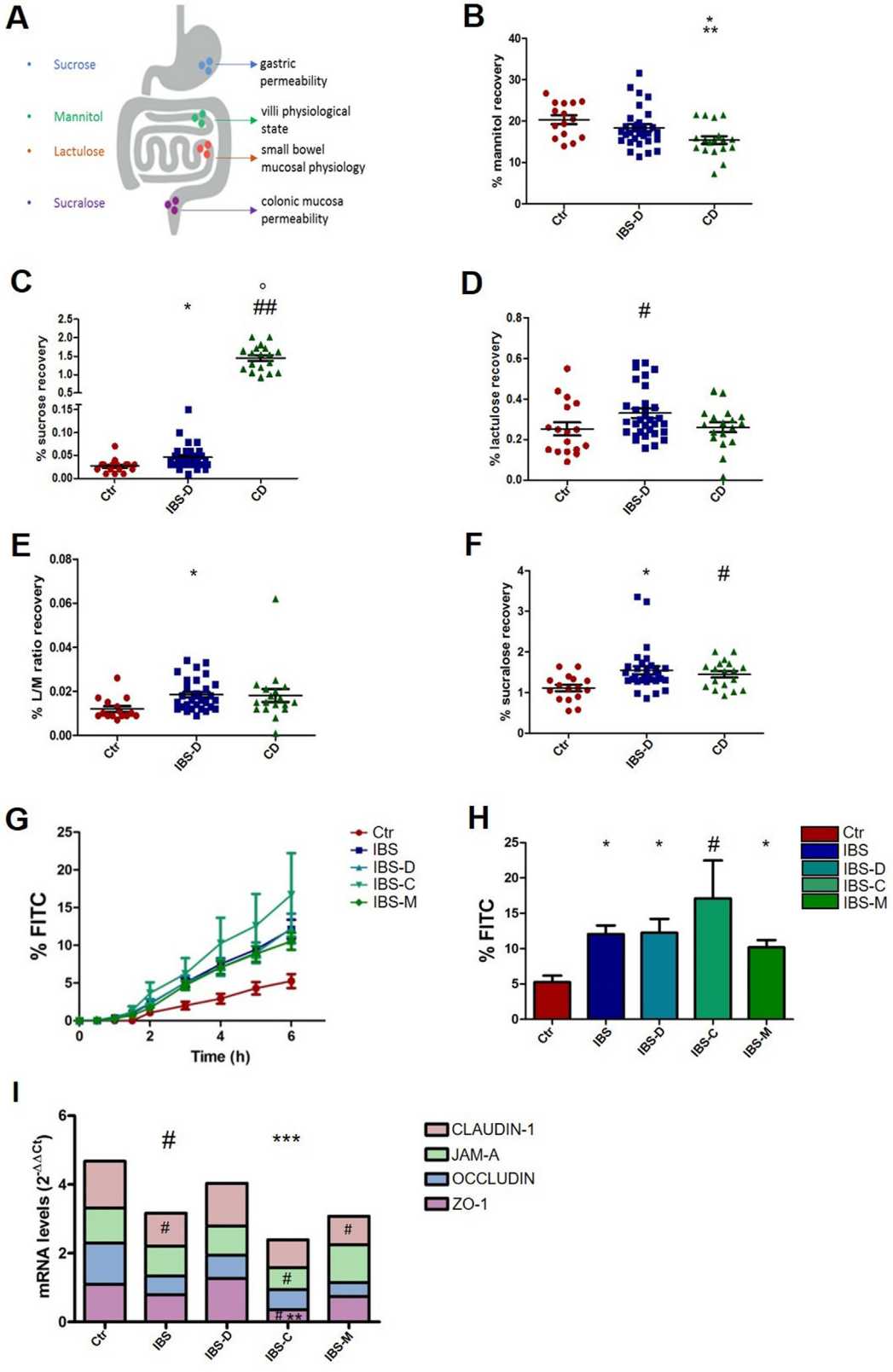
A visual comparison of normal and altered capillary morphology is provided in Figure 3*I*–*J*. The percentage of abnormal vs normal capillaries was 48% in the IBS group and 29% in the control group (Figure 3*K*). The distance of the lamina propria capillaries from the epithelial interface (Figure 3*L*) was significantly reduced in the IBS group (5.93 ± 4.1 μm) compared with controls (8.43 ± 4.29 μm) ($P < .01$) (Figure 3*M*).

Mucosal Mediators Increase Endothelial Permeability via PAR2 in IBS

Compared with HUVECs incubated with growth medium alone, IBS supernatants induced a significant increase of endothelial permeability ($P < .01$, Figure 4). Stratification of this effect by bowel habit showed that all IBS subgroups induced a significant increase in permeability compared with cells incubated with growth medium alone (IBS-D, $P < .01$; IBS-C, $P < .01$; IBS-M, $P < .01$).

Stratification of patients with IBS by severity of abdominal pain showed that IBS supernatants obtained from both patients with severe ($P < .01$) and mild abdominal pain ($P < .01$) determined a marked and significant increase in endothelial permeability compared with cells incubated with the growth medium alone. Compared with control supernatants, HUVECs incubated with IBS supernatants showed a trend toward increased permeability although statistical significance was not reached. Interestingly, the subgroup of patients with IBS with severe pain significantly increased HUVEC permeability compared with controls ($P < .05$) and with patients with IBS with mild pain severity ($P < .01$).

To evaluate the contribution of PAR2 in the observed IBS-dependent increase in endothelial permeability (Figure 4*B*), we assessed the effect of the PAR2 antagonist (iPAR2). As expected, the PAR2 activating peptide SLIGRL induced a 6-fold increase in endothelial permeability ($P < .01$), which was blunted by iPAR2. The incubation of



HUVECs with iPAR2 had no effect on basal endothelial permeability. In HUVECs, iPAR2 co-incubated with IBS supernatants significantly reduced fluorescein isothiocyanate passage through the monolayer compared with HUVECs incubated with IBS supernatants alone ($P < .001$), regardless of bowel habit. These data indicated a key role of PAR2 agonists in the IBS supernatant on increased endothelial permeability. A significant improvement of HUVEC permeability was observed when iPAR2 was co-incubated with supernatants obtained from patients with severe pain ($P < .01$) but not with mild pain patients with IBS with higher frequency of severe abdominal pain and bloating induced more pronounced increased permeability compared with patients with lower pain and bloating frequency (data not shown).

IBS Mediators Downregulate the Expression of VEC via PAR2

One of the key pathways of PAR2-mediated increase of endothelial permeability is the activation of histone deacetylase 11 (HDAC11) leading to reduction of VEC expression (Figure 4*C*).

The incubation of HUVECs with controls and IBS supernatants induced an increase in HDAC11 expression compared with cells incubated with medium alone. Supernatants obtained from patients with severe pain induced an increase in HDAC11 expression compared with controls and with supernatants from patients with IBS with mild pain. The incubation of IBS supernatants with iPAR2 induced a decrease in the expression of HDAC11, compared with cells incubated with IBS supernatants alone ($P < .05$). The co-incubation of iPAR2 and IBS-D supernatants induced a significant reduction in HDAC11 expression compared with cells incubated with IBS-D supernatants alone ($P < .01$). The co-incubation of SLIGRL-NH2 PAR2 agonist (SLIGRL) and iPAR2 induced a reduction of HDAC11 expression ($P < .05$) (Figure 4*D*).

The incubation of HUVECs with IBS supernatants induced a borderline significant decrease of VEC mRNA expression compared with control supernatants. Both IBS-D and IBS-C induced a reduction in VEC expression compared with control supernatants. Interestingly, supernatants obtained from patients with severe pain induced a reduction in VEC expression compared with supernatants from patients with mild pain. The co-incubation of IBS supernatants with iPAR2 induced an increase in VEC expression, and an opposite effect in cells incubated with control supernatants (Figure 4*E*). Taken together, these data suggest that IBS supernatants increase endothelial permeability downregulating the expression of VEC via a PAR2- and an HDAC11-dependent mechanism.

Mucosal Metabolome of IBS Differs From That of Controls and Contains Mediators With Potential to Increase Epithelial and Vascular Permeability

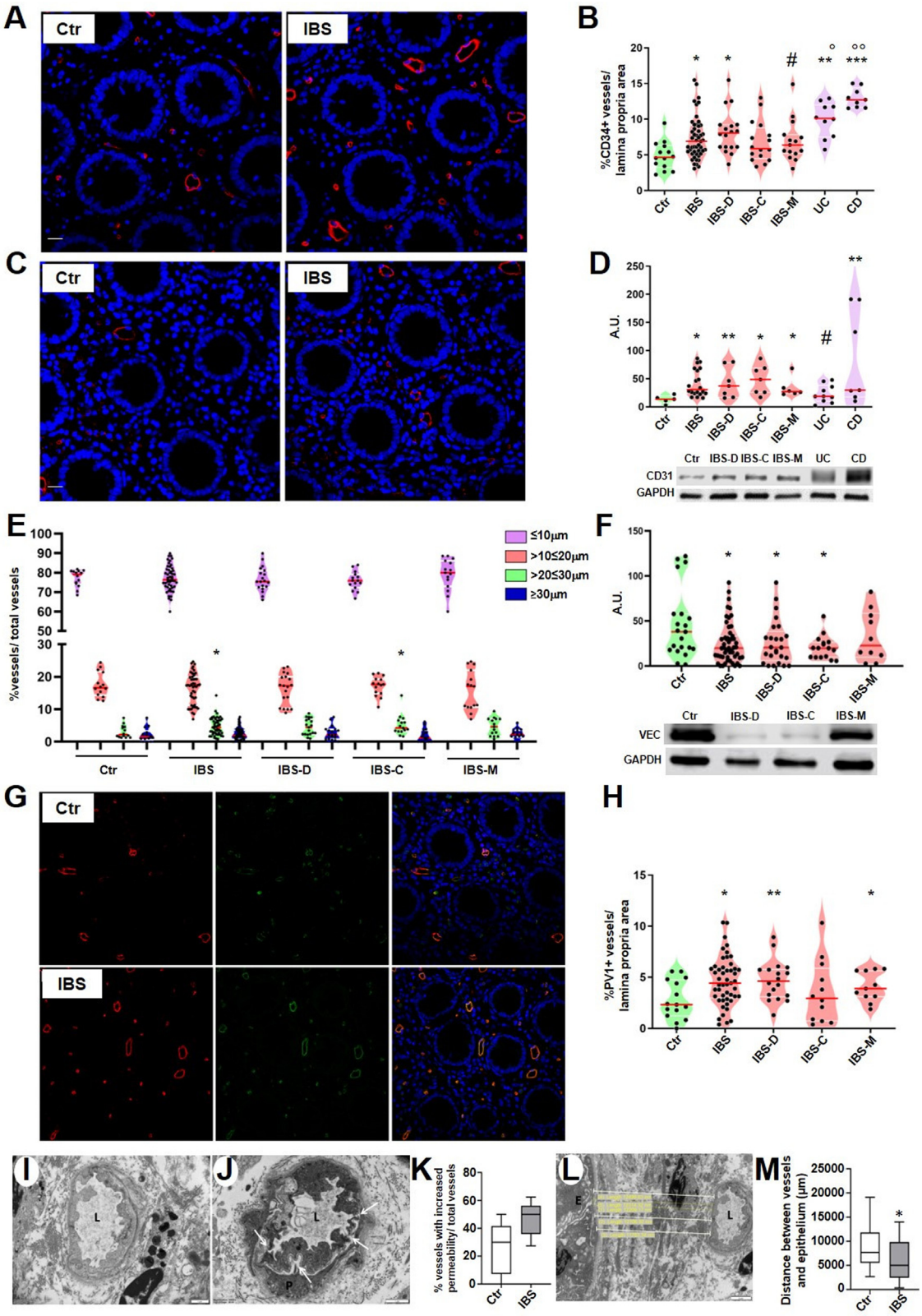
PLSDA analysis of the entire metabolome revealed a distinct composition of mucosal mediators between patients with IBS and controls (Figure 5). Figure 5*B* shows the distribution of metabolome in the IBS subgroups. The cross-validation test (Figure 5*C*) with 1 principal component showed a 57% accuracy. In addition, the positive Q2 confirms the robustness of the test (Figure 5*C*). Finally, permutation test demonstrated the absence of over fitting (Supplementary Figure 3). Figure 5*D* shows the top 25 metabolites different between controls and IBS. Metabolites increased in IBS compared with controls included histamine, urea, and 8-hydroxydeoxyguanosine (8-OHdG).

Increased Vascular Permeability Is Associated With Systemic Microbiome Translocation and Gut-Liver Axis Alteration

Because increased vascular permeability may lead to systemic spread of microbiota-derived particles, including bacterial genetic material or full bacterial cells, we evaluated bacterial DNA in the blood of controls and patients with IBS as a surrogate marker of microbiota translocation. Our results showed that bacterial DNA was markedly and significantly increased in patients with IBS compared with controls ($P < .01$, Figure 6*A*). In line with this result, we found a significant increase in the serum levels of sCD14, a systemic marker of innate immune response to microbial components ($P < .05$, Figure 6*B*).[26] Based on this evidence, and the fact that the liver represents the first station encountered by luminal factors absorbed by the intestine through the portal vein, we assessed AST and ALT liver enzymes. Although both enzymes were within the normal range in both controls and IBS, the L/M ratio significantly correlated with AST serum levels ($P = .048$, r: 0.3229) and were borderline correlated with ALT serum levels ($P = .093$). Interestingly, we observed a borderline correlation between ALT and the density of PV1 positive vessels

Figure 2. In vivo and in vitro epithelial permeability testing. (*A*) Gastrointestinal-tract permeability mapped by orally administered saccharides. The % of saccharide urinary recovery is reported (upper part of the picture). Data are reported as mean ± SE. Mannitol (*B*), controls, n = 16; IBS-D, n = 31; CD, n = 18. Sucrose (*C*), controls, n = 16; IBS-D, n = 31; CD, n = 18. Lactulose (*D*), controls, n = 17; IBS-D, n = 31; CD, n = 18. L/M ratio (*E*), controls, n = 16; IBS-D, n = 31; CD, n = 18. Sucralose (*F*), controls, n = 17; IBS-D, n = 31; CD, n = 18. (*G*) Permeability changes during 6 hours of Caco-2 incubation with controls/IBS supernatants. (*H*) Focus on permeability changes after 6 hours of Caco-2 incubation with controls/IBS supernatants. Controls, n = 13; IBS-D, n = 21; IBS-C, n = 7; IBS-M, n = 21. (*I*) Gene expression of zonula occludens-1 (ZO-1), occludin, junctional associated molecule-A (JAM-A), and claudin-1 in Caco-2 treated with controls/IBS. Results are reported as relative expression ($2^{-\Delta\Delta Ct}$), calibrator group: controls. *$P < .01$ vs controls; **$P < .05$ vs IBS-D; #$P < .05$ vs controls; ***$P < .001$ vs controls; ##$P < .0001$ vs controls; °$P < .0001$ vs IBS-D. Mann-Whitney test was used to determine statistical significance.

($P = .052$) in the overall population (controls and IBS together). In the IBS group alone, ALT levels were significantly correlated with PV1 positive density ($P = .0137$, r: 0.4771, Figure 6*C*). Taken together, these data suggest that increased vascular permeability could lead to mild liver enzyme activation.



IBS Symptoms Correlated With Epithelial and Endothelial Permeability Markers

We found a significant correlation between in vivo permeability, as assessed with sugar urinary excretion, and symptoms perceived by patients with IBS. The L/M ratio showed the highest number of significant relationships with key abdominal (eg, abdominal pain and bloating, Figure 7*A*) and psychological (ie, anxiety and depression, Figure 7*B* and *C*) symptoms. Accordingly, L/M ratio was negatively correlated with HRQOL (both physical and mental component score, Figure 7*D* and *E*). In the IBS group alone, L/M ratio strongly correlated with depression ($P = .0041$, r: 0.5007) (Supplementary Figure 4). Gut vascular barrier markers strongly correlated with psychological symptoms (Figure 7*A*). Mucosal vessel density correlated positively with anxiety and negatively with the mental component score of HRQOL (Figure 7*A*). The density of PV1 positive vessels was positively correlated with of anxiety and depression scores (Figure 7*F* and *G*, respectively). In addition, PV1-positive vessels negatively correlated with the mental component score (Figure 7*H*). A borderline correlation emerged between the density of PV1 positive vessels and ALT level ($P = .052$, Figure 7*I*). Taken together these data suggest a role for vascular endothelial permeability in psychological comormidity and poor HRQOL. Increased HUVEC permeability induced by IBS supernatants correlated with both abdominal pain severity and frequency ($P = .0184$, r: 0.4349 and $P = .0009$, r: 0.5908, respectively, Supplementary Figure 4). In addition, in the IBS group alone, the density of PV1 positive vessels strongly correlated with anxiety ($P = .0067$, r: 0.5724, Supplementary Figure 4) and with ALT level (Figure 7*B*).

All correlation analysis results are reported in the Supplementary Material (Supplementary Tables 3–7).

Discussion

In this study, we demonstrated that epithelial barrier integrity is compromised throughout the entire gastrointestinal tract, particularly in patients with IBS-D. The GVB is affected via a PAR2-mediated mechanism, potentially leading to systemic spread of potentially harmful luminal substances, including bacterial elements as shown by increased blood bacterial ribosomal RNA and increased levels of sCD14, a marker of systemic innate immune activation, although other causes cannot be excluded. A mild increase in transaminases may also suggest increased portal flow to the livel of intestinal substances escaping the intestinal barrier filter. One of the most interesting findings was the correlation of markers of epithelial and vascular dysfunction with the severity of abdominal pain and bloating, anxiety and depression, and reduced HRQOL. Taken together, these data suggest that defective epithelial and vascular barriers may participate in gut-brain axis dysfunction in IBS.

A novel finding of our study is the characterization of intestinal permeability throughout the gastrointestinal tract. We found that the strongest discriminator between IBS and controls was the L/M ratio, although no difference emerged comparing the diagnostic accuracy of L/M ratio and that of sucrose and sucralose, confirming a widespread alteration of permeability throughout the entire gastrointestinal tract. To note we used a 6-hour urine collection and choose sucralose as a marker to evaluate colonic permeability, in line with some previous study and in contrast with others.[7]

The intestinal mucosa of patients with IBS is more densely populated by capillaries that were located more closely to the epithelium and showed a higher percentage of structural abnormalities including intercellular junction leaks and occasional disruption of endothelial continuity along with increased expression of the endothelial transmembrane glycoprotein PV1, a marker of vascular permeability, that previous studies showed to be increased during GVB failure.[13] In parallel, we observed a decreased expression of VEC, whose integrity is a major determinant of permeability.[27] PV1 expression was significantly higher in female compared with male individuals, whereas in patients with IBS, CD34- positive vessels were increased in male

Figure 3. Characterization of the gut vascular barrier and its permeability. (*A*) Confocal images of cross section of the colonic mucosa from 2 representative samples of controls (Ctr) and patients with IBS stained with the blood vessel marker CD34. (*B*) Quantitative analysis of CD34-stained blood vessels. Controls, n = 14; IBS, n = 49; IBS-D, n = 18; IBS-C, n = 16; IBS-M, n = 15; UC, n = 10; CD, n = 9. *$P < .01$ vs controls; **$P < .001$ vs controls; ***$P < .0001$ vs controls; #$P < .05$ vs controls; °$P < .01$ vs IBS; °°$P < .001$ vs IBS. Data are presented as percentage of blood vessels on total area of lamina propria ± SE. *Scale bar:* 50 μm. (*C*) Representative pictures of CD31 immunostaining of the colonic mucosa. (*D*) Quantification of CD31 expression in the colonic mucosa by Western blot (upper part) and representative picture of CD31 immunoblotting (lower part). Controls, n = 5; IBS, n = 20; IBS-D, n = 7; IBS-C, n = 7; IBS-M, n = 6; UC, n = 10; CD, n = 7. *$P < .01$ vs controls; **$P < .05$ vs controls; #$P < .05$ vs IBS-C. A.U., arbitrary units. (*E*) Blood vessel diameter in the colonic mucosa. Data are reported as percentage of vessels over total vessel number. Controls, n = 14; IBS, n = 49; IBS-D, n = 18; IBS-C, n = 16; IBS-M, n = 15. *$P < .05$ vs controls. (*F*) Quantification of VEC expression in the colonic mucosa by Western blot (upper part) and representative picture of VEC immunoblotting (lower part) (controls, n = 11; IBS, n = 21; IBS-D, n = 7; IBS-C, n = 9; IBS-M, n = 5). *$P < .05$ vs controls. (*G*) Representative pictures of CD34, PV1 expression, and their colocalization in the colonic mucosa. (*H*) Quantitative analysis of PV1-stained blood vessels (controls, n = 15; IBS, n = 41; IBS-D, n = 18; IBS-C, n = 12; IBS-M, n = 11). *$P < .05$ vs controls, **$P < .01$ vs controls. *Scale bar:* 50 μm. (*I*) Electron micrograph showing normal capillary morphology from controls. (*J*) Electron micrograph showing multiple altered capillary morphology (arrows) from patient with IBS. L, vascular lumen; P, pericyte. (*K*) Quantification of altered capillaries in the colonic mucosa of controls and patients with IBS. *Scale bar:* 1 μM. (*L*) Representative picture of the distance measurement of the lamina propria capillaries from the epithelial interface. E, epithelium, L, vascular lumen. *Scale bar:* 2 μM. (*M*) Quantification of the distance between lamina propria capillaries and epithelium. $P < .01$. Mann-Whitney test was used to determine statistical significance.

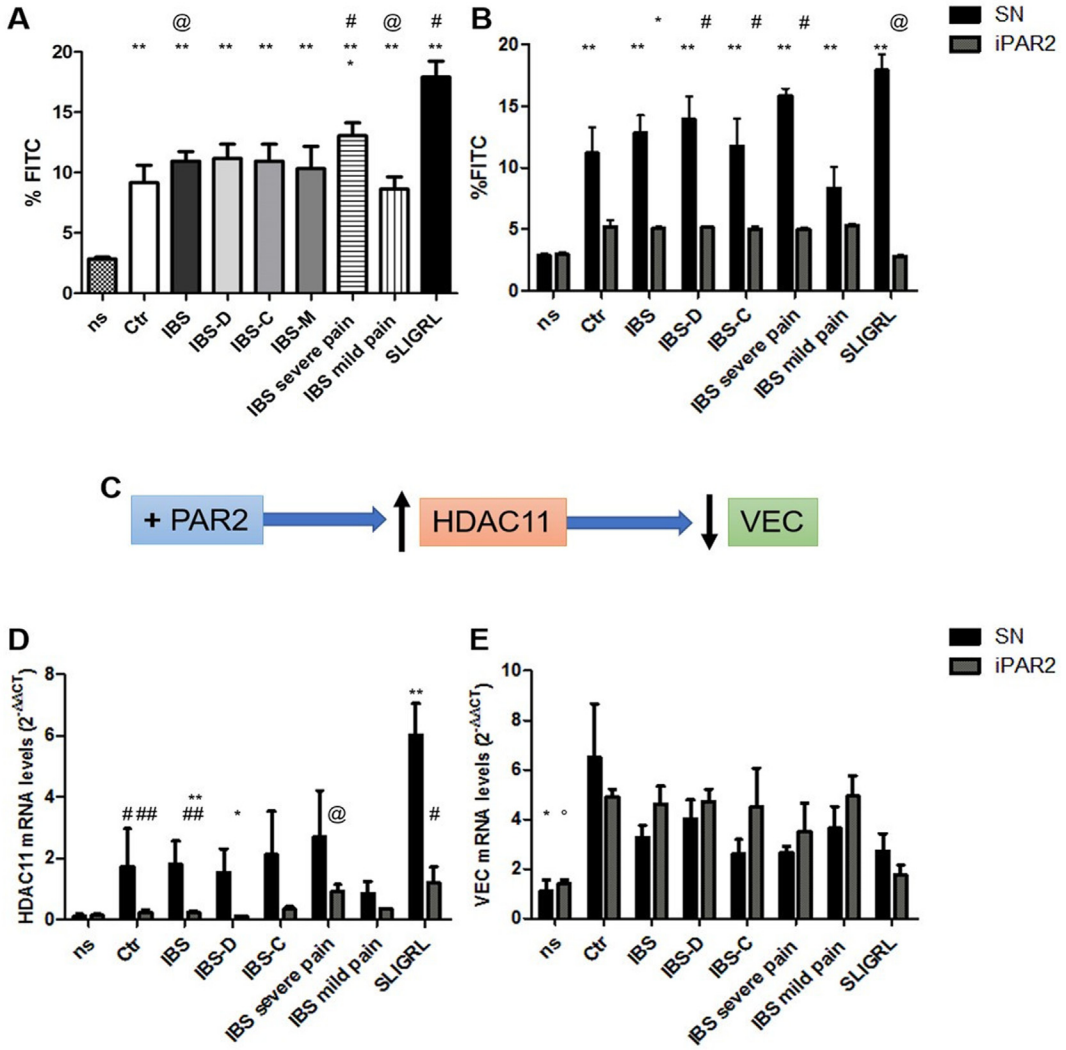


Figure 4. IBS mediators increase endothelial permeability via a PAR2-mediated mechanism. (*A*) Effect of IBS supernatants stratified according to pain severity on HUVEC. Results are reported as % of fluorescein isothiocyanate (FITC) passed through the HUVEC monolayer. ns, HUVECs incubated with growth medium alone. ns: 4; controls (Ctr): 7; IBS: 33; IBS-D: 16; IBS-C: 11; IBS-M: 6; IBS with severe pain: 18; IBS with mild pain: 11; SLIGRL-NH2 Protease-Activated Receptor 2 (PAR2) Agonist (SLIGRL): 3. **$P < .01$ vs ns; #$P < .05$ vs controls; @$P < .05$ vs SLIGRL; *$P < .01$ vs IBS with mild pain. (*B*) Effect of PAR2 inhibitor on HUVEC permeability in the presence of IBS supernatants. ns: 4; controls: 4; controls + iPAR2: 4; IBS: 10; IBS + iPAR2: 10; IBS-D: 5; IBS-D + iPAR2: 5; IBS-C: 5; IBS-C + iPAR2: 5; IBS with severe pain: 6; IBS with mild pain: 4; SLIGRL: 4. **vs ns $P < .01$; *vs IBS $P < .001$; #vs the corresponding condition without iPAR2 $P < .01$; @vs SLIGRL $P < .05$. (*C*) Representative scheme of the molecular mechanism investigated. (*D*) HDAC11 mRNA expression in HUVECs incubated with IBS supernatants PAR2 inhibitor. ns: 3; ns + iPAR2: 4; controls: 6; controls + iPAR2: 7; IBS: 14; IBS + iPAR2: 13; IBS pain severity >2: 7; IBS with severe pain + iPAR2: 5; IBS with mild pain: 8; IBS with mild pain + iPAR2: 1; IBS-D: 7; IBS-D + iPAR2: 6; IBS-D with mild pain: 5; IBS-C: 7; IBS-C + iPAR2: 7; SLIGRL: 8; SLIGRL + iPAR2: 3. *vs IBS-D $P < .01$; **vs IBS $P < .05$; #vs SLIGRL $P < .05$; ##vs SLIGRL + iPAR2 $P < .05$; @vs controls + iPAR2 $P < .05$. (*E*) VEC mRNA expression in HUVECs incubated with IBS supernatants PAR2 inhibitor. ns: 3; ns + iPAR2: 4; controls: 7; controls + iPAR2: 6; IBS: 13; IBS + iPAR2: 10; IBS with severe pain: 5; IBS with mild pain: 7; IBS-D: 6; IBS-D + iPAR2: 6; IBS-C: 7; IBS-C + iPAR2: 5; SLIGRL: 6; SLIGRL + iPAR2: 3. *vs controls, IBS, IBS-D, $P < .05$; °vs controls + iPAR2, $P < .05$. SN, supernatants.

compared with female individuals, suggesting that gender may have a role in the GVB dysfunction. Our in vitro model of HUVEC permeability allowed us to investigate molecular mechanisms underlying vascular hyperpermeability. Mediators released by IBS biopsies, particularly those of patients reporting severe pain, induced a greater increase in permeability compared with controls. By using PAR2 antagonists, we demonstrate the role of this receptor and proteases in the observed permeability changes. This result is corroborated, on one hand, by previous evidence of increased protease activity in IBS supernatants[28] and on the other hand, by the evidence that proteases can actively increase endothelial permeability via PAR2.[16,29] Interestingly, PAR2 activation in microvessels is also known to induce leukocyte rolling, adhesion, and extravasation,[16] suggesting that recruitment of inflammatory cells could also occur to a

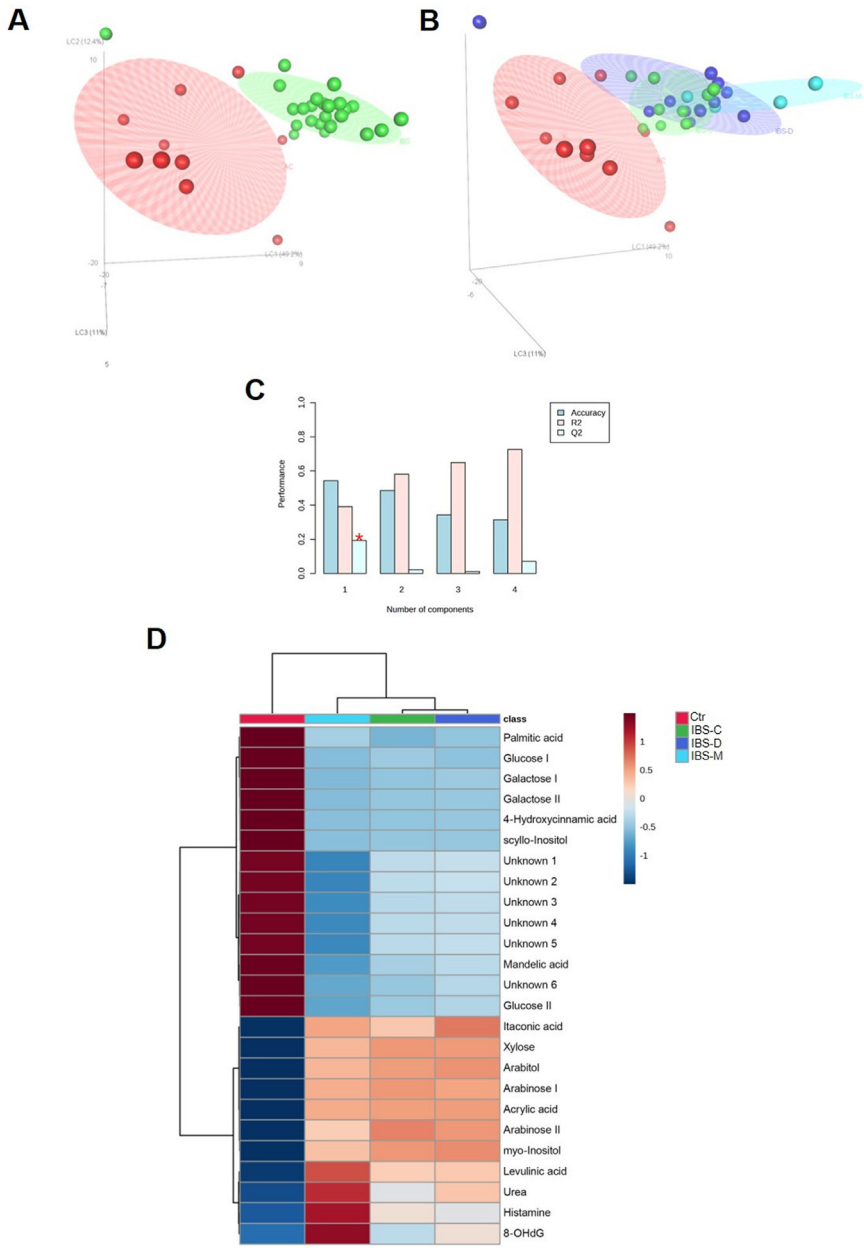


Figure 5. Metabololomics analysis of mucosal mediators. (*A*) 3D-PLSDA of metabolites from controls (Ctr, *red*) and patients with IBS (*green*). (*B*) PLSDA of metabolites from controls (*red*) and patients with IBS classified according to bowel habits (*green*: IBS-C, *blue*: IBS-D, *light blue*: IBS-M). (*C*) Cross-validation test associated with PLSDA. (*D*) Heat map of the 25 top metabolites discriminating controls, IBS-D, IBS-C, and IBS-M according to *P* value.

certain extent in tissues of patients with IBS.[30] Previous studies have also reported that PAR2 activation stimulates angiogenesis,[31] suggesting that PAR2 signaling could also be involved in dense capillary population we report here in intestinal mucosa of patients with IBS.

To further elucidate the PAR2 role in permeability changes in IBS, we evaluated a mechanism involving HDAC11 and VEC.[29] We demonstrate that the incubation of HUVECs with IBS mediators induced an increase in the expression of HDAC11 and a concomitant decrease in VEC expression. The effect on VEC expression did not reach statistical significance likely because of the activation of compensation mechanisms, which are reasonable to hypothesize considering the pivotal role of VEC in the control of vascular permeability. The effect of IBS supernatants was abolished by the co-incubation with the PAR2 antagonist. Taken together our data demonstrate that, via PAR2 activation, proteases regulate, at least in part, the increase of endothelial permeability in IBS. Thus, we have isolated the vascular endothelial mechanism and mediators from the previously published changes using confocal laser endomicroscopy and this adds to existing knowledge.[32,33] It is likely that also other mediators are involved in the observed permeability changes. Consistent with this hypothesis, we found the metabolome released by IBS colonic biopsies is different from that of controls and includes metabolites known to increase intestinal permeability, such as histamine,[34,35] urea,[36] and 8-OHdG.[37] As the functional unit of the GVB comprises also pericytes and enteric glial cells, our HUVEC in vitro model cannot reproduce the complexity of

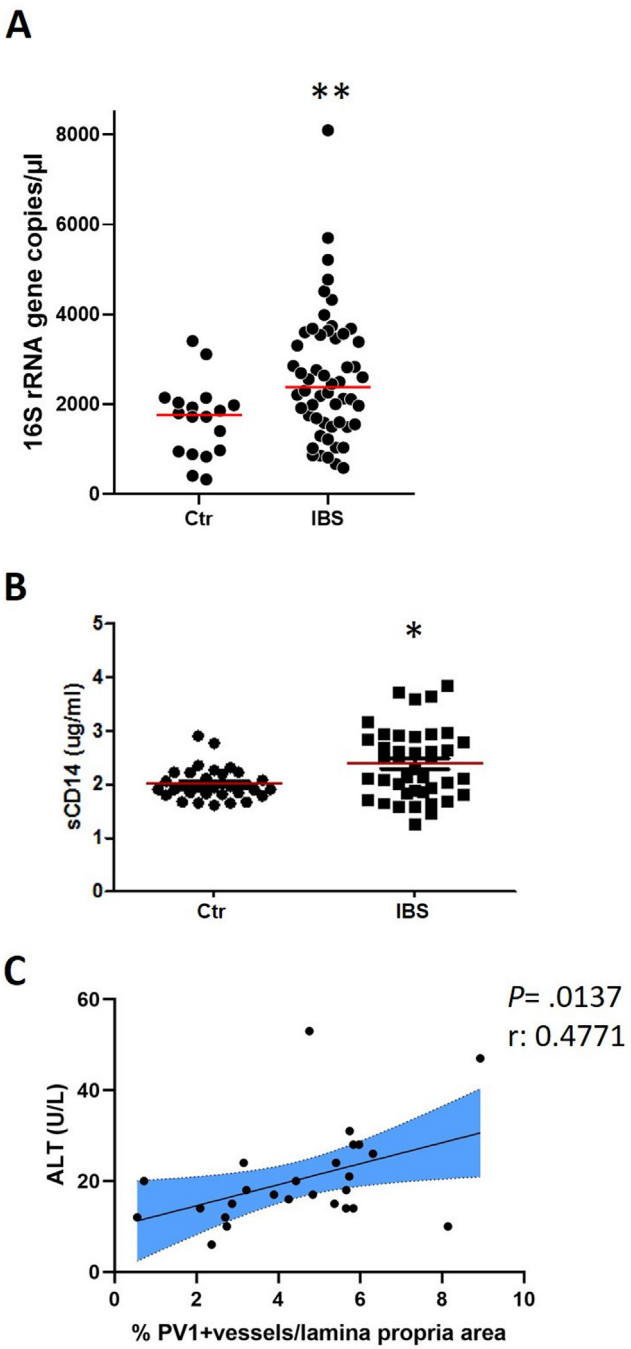


Figure 6. Systemic bacteria translocation, immune response, and gut-liver axis correlation. (*A*) Quantification of 16S rRNA copies/μL of blood in controls (Ctr) and patients with IBS. Controls, n = 18; IBS, n = 54. $^{**}P < .01$, according to Mann-Whitney test. (*B*) Serum levels of sCD14. Controls, n = 32; IBS, n = 41, according to Mann-Whitney test. $^{*}P < .05$. (*C*) Correlation between the density of PV1-positive vessels and ALT level in the blood. The Spearman correlation test was used to determine statistical significance.

the GVB and future studies are needed to assess the potential role of players other than endothelial cells in this context.

Although human blood is conventionally considered a sterile environment, the existence of a blood microbiome axis is gaining attention.[38] A recent large-scale study showed that microbial species can be found in the blood in only 16% of 9770 healthy individuals. Blood bacteria were primarily gut commensals.[39] We found that bacterial RNA was increased in the blood of patients with IBS along with increased levels of sCD14. The binding of sCD14 to lipopolysaccharide is necessary to activate Toll-like receptor 4 which was demonstrated to be increased in the colonic mucosa of patients with IBS, particularly in the IBS-D subtype.[40] We cannot define the origin of this bacterial genetic material and at this point we can only speculate that the involvement of a disrupted GVB is involved.

The portal system is involved in several diseases, including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis. Direct evidence of the role of GVB on liver dysfunction comes from patients with celiac disease following a gluten-free diet and with high levels of ALT. This subgroup of patients was characterized by increased expression of PV1 in the duodenal biopsies compared with healthy subjects and to patients with celiac disease with low ALT levels. Interestingly, the administration of concanavalin A to mice induces liver inflammation that is not paired to PV-1 upregulation.[13] Taken together, these data suggest that the GVB has a key role in maintaining liver homeostasis regulating the dissemination of bacteria and/or their products to the liver. Gut microbiota has itself a direct effect on GVB dysfunction and consequently on liver,[41] giving rise to the so-called microbiota-gut-liver axis defined as the complex network.[42] We show a correlation between PV1 mucosal expression and ALT levels in patients with IBS. This result, together with the evidence of microbiome diffusion into the blood, would suggest a role for the microbiota-gut-liver axis in IBS. In line with this hypothesis: first, IBS is associated with gut dysbiosis[43]; second, a retrospective, case-control study showed a higher prevalence of elevated ALT in patients with IBS compared with control subjects[44]; third, NAFLD is associated with increased risk of IBS[45]; and fourth, a recent work reported that IBS is highly prevalent in patients with NAFLD and is associated to reduced HRQOL and increased anxiety and depression.[46] Finally, we show the disruption of the intestinal epithelial barrier and GVB, characterized by the increase of PV1 expression, have been demonstrated to be early events in the development of liver dysfunction.[47] As in our study the levels of ALT were in the normal range, future studies should corroborate our preliminary results.

Consistent with previous data, we found an association between sugar excretion and abdominal symptoms, psychological traits, and poor quality of life,[9] but with the advantage of having all clinical and biological data recorded in a single well-characterized cohort, thus overcoming the limitation of having data from different and methodologically noncomparable studies. Interestingly, the markers of epithelial permeability showed a stronger association with abdominal symptoms, whereas PV1 (a marker of vascular permeability) correlated the strongest with psychological symptoms, in line with previously published data.[48]

In conclusion, our data showed increased epithelial/vascular pereability, the molecular underlying mechanisms,

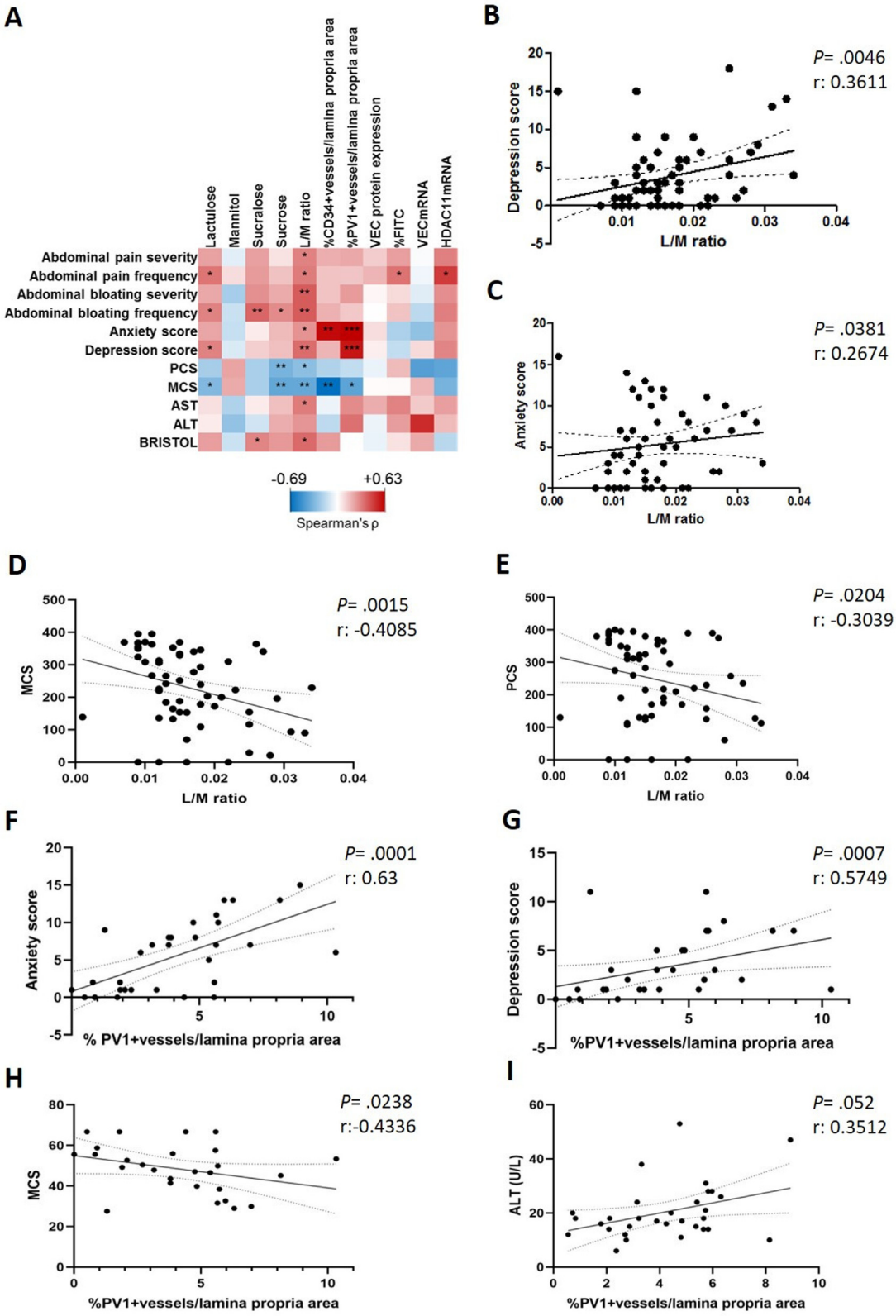


Figure 7. Relationships between epithelial and endothelial permeability markers and symptoms. (*A*) The heatmap represents the *r* coefficient of the Spearman's correlation. *Asterisks* indicate significance in the Spearman's correlation. **P* < .05; ***P* < .01; ****P* < .001. (*B–E*) Relationships between L/M ratio and anxiety and depression and HRQOL (physical and mental component scores, PCS and MCS, respectively). Graphs report mean ± error and 95% CI. (*F–I*) Relationships between PV1-positive vessels and anxiety and depression, MCS, and ALT levels.

and their association with abdominal and psychological comorbidity in IBS (Supplementary Figure 5). These novel findings further strengthen the concept of micro-organic bowel alterations and their potential participation in gut and systemic comorbidity in IBS.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2024.07.004.

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Conflicts of interest
The authors disclose no conflicts.

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Data Availability
All methods, study materials, and data associated with this study can be found in the article or in the Supplementary Material.