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Colonic Mucosal Gene Expression and Genotype in Irritable Bowel Syndrome Patients with Normal or Elevated Fecal Bile Acid Excretion

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Abbreviations: ACTB=Actin, beta; BA=bile acid(s); C4: 7α-hydroxy-4-cholesten-3-one; C4BPA=Complement component 4 binding protein, alpha; CCL20=Chemokine (C-C motif) ligand 20; CLDN1=Claudin 1; CT= colonic transit; FGF19= Fibroblast growth factor 19; FGFR4=Fibroblast growth factor receptor 4; FN1=Fibronectin 1; GAPDH =Glyceraldehyde-3-phosphate dehydrogenase; GPBAR1=G protein-coupled bile acid receptor 1(aka TGR5); GUCA2B=Guanylate cyclase activator 2B; HGDC=Human genomic DNA contamination; IBS=irritable bowel syndrome; IBS-D=diarrhea-predominant IBS; IFIT3=Interferon-induced protein with tetratricopeptide repeats 3; NR1H4=Nuclear receptor subfamily 1, group H, member 4; OCLN=Occludin; P2RY4=Pyrimidinergic receptor P2Y G-protein coupled, 4; PCR=polymerase chain reaction; PDZD3=PDZ domain containing 3; PPC=Positive PCR control; RBP2=Retinol binding protein 2, cellular; RSM=rectosigmoid mucosa; RTC=Reverse transcription control; SLC10A2=Solute carrier family 10 (sodium/bile acid cotransporter family), member 2; SLC6A4=Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4; TFF1=Trefoil factor 1; TGR5=triglyceride receptor 5 (aka GPBAR1); TJP1=Tight junction protein 1; TNFSF15=Tumor necrosis factor (ligand) superfamily, member 15; VIP=Vasoactive intestinal peptide

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ABSTRACT

The mucosal gene expression in rectosigmoid mucosa (RSM) in irritable bowel syndrome with diarrhea (IBS-D) is unknown. Our objectives were: first, to study mRNA expression (by RT^2 PCR of 19 genes pertaining to tight junctions, immune activation, intestinal ion transport and bile acid homeostasis) in RSM in IBS-D patients (n=47) and healthy controls (n=17) and study expression of a selected protein (PDZD3) in 10 IBS-D patients and 4 healthy controls; second, to assess RSM mRNA expression according to genotype and fecal bile acid (BA) excretion (high $\geq$2337mM/48h); and third, to determine whether genotype or mucosal mRNA expression is associated with colonic transit or BA parameters. Fold changes were corrected for false detection rate (FDR) for 19 genes studied ($P<0.00263$). In RSM in IBS-D patients compared to controls, mRNA expression of GUC2AB, PDZD3, and PR2Y4 was increased, whereas, CLDN1 and FN1 was decreased. One immune-related gene was upregulated (C4BP4) and one downregulated (CCL20). There was increased expression of a selected ion transport protein (PDZD3) on immunohistochemistry and Western blot in IBS-D compared to controls ($p=0.02$) . There were no significant differences in mucosal mRNA in 20 IBS-D patients with high, compared to 27 IBS-D patients with normal BA excretion. *GPBAR1* ($p<0.05$) was associated with colonic transit. We concluded that mucosal ion transport mRNA (for several genes and PDZD3 protein) is upregulated and barrier protein mRNA downregulated in IBS-D compared to healthy controls, independent of genotype. There are no differences in gene expression in IBS-D with high compared to normal fecal BA excretion.

Key words: neurotransmitters, ion channels, cytokines, barrier, immune, secretion, PDZD3, GUCA2B

INTRODUCTION

The cause of loose bowel movements in patients with irritable bowel syndrome with diarrhea (IBS-D) is partly attributed to acceleration of colonic transit, documented in ~45% of patients with IBS-D (15), and to intestinal secretory mechanisms (reviewed in refs. 7 and 8). Documented secretory mechanisms include increased duodenal and rectosigmoid expression of secretory transmitters (e.g. 5-HT), reduced expression of the serotonin reuptake protein, and fecal excretion of secretogranins or chromogranins (25,26,46). There is also evidence of reduced expression of pro-absorption mechanisms (e.g. mucosal PYY, somatostatin, NPY).

IBS has also been associated with changes in rectosigmoid mucosal expression of immune factors, barrier function and mucus secretion (1,4,5,9,22,33,51,56,60,63,65,68). In a few instances, altered mucosal gene expression in tissues was associated with the inherited genotype, such as *TNFSF15* gene, which is associated with IBS and has been linked with functional alterations of mucosal immune and protective functions (51,68).

Differences in jejunal mucosal expression (at gene and protein levels) and distribution of apical junction complex proteins between IBS patients and controls support the observed alterations in barrier function in colonic mucosa in patients with IBS-D (36,37). We also demonstrated a borderline difference in the ZO-1 intensity score in the small bowel mucosa ($p=0.06$) of patients with IBS-D compared to healthy controls, with lower intensity in HLA-DQ2/8-positive relative to HLA-DQ2/8-negative patients with IBS-D (56).

In a prior study, based on next generation RNA sequencing and confirmation by reverse transcriptase polymerase chain reaction (RT-PCR), we examined rectosigmoid mucosa from 9 patients with IBS-D and 9 healthy controls and identified differential expression of secretory and barrier genes, suggesting that the transcriptome is different in IBS-D compared to controls (11). The upregulated mechanisms associated with changes in ion transport included PDZD3. PDZ

adapter proteins are involved in multiple ion transport functions in the intestine, including sodium absorption through sodium-hydrogen exchange (NHE3), as well as guanylyl cyclase C receptor (GC-C, or GUCY2C)-induced chloride and water secretion through cGMP signaling that leads to cystic fibrosis transmembrane conductance regulator phosphorylation and chloride-bicarbonate exchange through the SLC26 anion exchanger (35). These ion exchange mechanisms also result in alterations in intestinal fluid transport.

Another upregulated ion transport mechanism identified in our prior study was increased GUCA2B mRNA. GUCA2B encodes uroguanylin, an endogenous ligand for GC-C receptor, increasing cyclic GMP, chloride, and water secretion.

The current study hypotheses were: first, there is upregulation of genes and proteins associated with intestinal secretion in the colonic mucosa of patients with IBS-D, particularly in patients with high fecal bile acid excretion; second, variations in inherited genes are associated with the mRNA expression of the same genes in the colonic mucosa; and third, genetic variation and/or rectosigmoid mucosal expression of the genes are associated with alterations in intermediate phenotype in IBS-D, specifically colonic transit and parameters of bile acid homeostasis.

The aims of this study were: first, to replicate the prior pilot study by targeted mRNA analysis using quantitative RT-PCR of rectosigmoid mucosal biopsies from 47 IBS-D patients and 17 healthy controls and to analyze the protein expression of one of the proteins associated with ion transport, PDZD3, in 10 IBS-D patients and 4 healthy controls; second, to determine whether there are differences in mucosal mRNA expression in subgroups of IBS-D with high compared to normal fecal bile acid (BA) excretion; third, to determine whether genotype was associated with level of expression of mRNA in rectosigmoid mucosa, colonic transit or BA

parameters; and fourth, to assess whether the level of expression of mRNA in rectosigmoid mucosa was associated with colonic transit.

METHODS

Ethical Approval

The study was approved by Mayo Clinic Institutional Review Board on October 28, 2011. Written informed consent was received from participants prior to inclusion in the study.

Study Design

We appraised bowel functions, total fecal BA excretion over 48 hours, fasting serum C4 (7α-hydroxy-4-cholesten-3-one) and FGF19, colonic transit, genotype and rectosigmoid mucosal mRNA expression in 47 patients with IBS-D (by Rome III criteria). Fecal total BA excretion was used to differentiate patients with high or normal BA excretion, suggestive of bile acid diarrhea.

Patient Selection

Patients were recruited by public advertisement or by invitation to participate from a database of ~1200 patients with IBS living in communities within ~120 miles of Mayo Clinic in Rochester, MN. Inclusion criteria were based on symptoms using a validated diary questionnaire that characterized IBS symptoms and, particularly, bowel functions (53). Participants also completed the Hospital Anxiety and Depression Inventory (67). These patients had been evaluated at Mayo Clinic, and alternative diagnoses such as inflammatory bowel disease, cancer and celiac disease were excluded. The main exclusion criteria were intake of medications that could interfere with the study tests, bleeding diathesis (owing to the need for rectosigmoid biopsies), and AST or ALT greater than twice the upper normal limit to avoid interference with the assessment of BA parameters.

Stored Biospecimens

We used stored samples from patients who had consented to the use of biospecimens for future research in prior studies (1,10,57) conducted at Mayo Clinic in Rochester, Minnesota, USA. These samples were obtained in 47 patients with IBS-D and 17 healthy controls. In order to appraise specificity of the observations in IBS-D, we analyzed rectosigmoid mucosal samples from 10 patients with IBS-C previously studied in our laboratory (1).

Participants' rectosigmoid biopsies were preserved in a solution of RNAlater, stored at -80°C. For all the mucosal biopsies, the RNA concentration was 130.3±6.2 (SEM) ng/μL and the RNA integrity number (RIN) ranged from 9.2 to 10 (mean 9.9±0.02).

DNA was similarly stored at -80°C after prior extraction from peripheral venous blood.

Measurement of Colonic Transit by Scintigraphy

Overall colonic transit was measured by validated scintigraphy with ^{111}In-labeled activated charcoal particles delivered to the ileocolonic junction in a methacrylate –coated gelatin capsule. The primary transit endpoints were colonic geometric center (GC) at 24 hours and 48 hours; in addition, ascending colonic emptying $T_{1/2}$ was measured by linear interpolation of radioisotopic content in the ascending colon at 4, 6, 24 and 48 hours (23).

Identification of Subgroups of IBS-D Patients Based on Fecal BA Excretion

Total and main fecal BA excretions (per 48 hours on 100g fat diet) were measured by HPLC/tandem mass spectrometry (48,57,64). This assay was adapted from a method used with serum samples (52).

IBS-D subgroups were identified by fecal BA excretion >2337mM/48h based on 90th percentile of 45 healthy volunteers studied in our laboratory (64). The 90th percentile was used

to define the upper limit of normal range, consistent with the observation that a normal distribution (estimated from 5th and 95th percentiles) requires sampling of 500 normal people (2).

Other Bile Acid Parameters

Fasting serum C4 (a measure of hepatic synthesis rate of BAs sampled in the morning) was measured by HPLC/tandem mass spectrometry (16); serum C4 is a validated method for detecting BA malabsorption (6). Similarly, fasting levels of fibroblast growth factor-19 (FGF19) were measured by a commercial enzyme-linked immunosorbent assay (FGF19 Quantikine Enzyme-Linked Immunosorbent Assay Kit; R&D Systems, Minneapolis, MN) as in prior studies (43,64).

Gene Expression Method by RT^2 PCR Array

Selection of genes of interest

We developed a custom profile including 19 genes to assess the effect of IBS-D on the expression of tight junction proteins, chemokines, markers of innate immunity, ion channels and transmitters that have been demonstrated to be differentially expressed in rectosigmoid mucosa of patients with IBS (11). The custom profile included 2 housekeeping genes for normalization and 3 control genes that check for sample quality and reaction quality (Table 1).

Assay method

For mRNA expression, RNA was purified from human rectosigmoid mucosal biopsies using the Qiagen RNAeasy Kit (Qiagen, Valencia, CA), including on-column DNAse treatment to remove genomic DNA. RNA quality was assessed on the Agilent Bioanalyzer. The resulting RNA (RIN>7) was reverse transcribed using the RT^2 First Strand Kit, (Qiagen), and samples were analyzed for expression by a Custom Profiler RT^2 PCR Array (Qiagen).

Genotyping Method

DNA was extracted from venous blood, and candidate genotype analysis was conducted by established PCR-based methods, as previously detailed in prior publications: rs4795541 [*SLC6A4* (34)]; rs4263839 [*TNFSF15* (68)]; rs11554825 [*GPBAR1* (18)]; rs434434 [*FGFR4* (13)]; rs188096 [*SLC10A2* (18)]; rs17618244 [*KLB* (18)], rs1966265 [*FGFR4* (18)], and rs351855 [*FGFR4* (18)]. FXR SNPs rs17030285 and rs4764980 were also assessed by Taqman assay (catalog #C_34126156_10) (catalog C_3127933_20). Gene names are given according to Hugo Gene Nomenclature.

Briefly, genomic DNA was isolated from whole blood by use of the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA) and stored at −80°C until genotyping. The serotonin transporter protein promoter polymorphism (*SLC6A4* rs4795541), also referred to as 5′-HTTLPR, was determined by PCR-based fragment length. The remaining eight SNPs were analyzed by TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. Following polymerase chain reaction amplification, end reactions were analyzed by using ABI 7300 Real-Time PCR System by Sequence Detection Software (Applied Biosystems).

Confocal Immunofluorescence Microscopy of PDZD3

We chose to study expression of PDZD3 because of its involvement in both sodium absorptive and chloride secretory mechanisms.

Confocal immunofluorescence microscopy was performed as previously described (44). Briefly, unstained rectosigmoid mucosal sections were deparaffinized and rehydrated, boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA), quenched with Image-iT FX signal enhancer (Life Technologies, Grand Island, NY), and blocked with 1%BSA/10%FBS/0.1% Triton X-100 in PBS. Slides were then incubated overnight at 4°C with

primary antibodies PDZD3 (1:1000, LifeSpan BioSciences, Seattle, WA) and cytokeratin 8/18 (1:50, Santa Cruz, Dallas, TX). Fluorescent labeled goat anti-rabbit IgG H&L (Alexa Fluor® 568, Abcam, Cambridge, MA) and donkey anti-mouse IgG H+L (Alexa Fluor 488, Life Technologies) were applied to the slides; the slides were then rinsed and mounted with ProLong Gold with DAPI (Molecular Probes®, Life Technologies) and were visualized on a Zeiss LSM 510 confocal microscope with a 63X magnification oil objective.

PDZD3 Protein Measurements by Western Blots

Whole cell lysates were isolated from human colonic biopsies with the RIPA Lysis Buffer System (Santa Cruz, Dallas, TX), and concentrations were determined using the BCA quantification (Pierce, Rockford, IL). Proteins were separated using 4-15% Mini-PROTEAN® TGX™ gels (BioRad, Hercules, CA) and blotted onto nitrocellulose membranes. The membranes were blocked with 5% milk in phosphate buffered saline (PBS)/0.2% Tween, after which PDZD3 primary antibody (1:4000, LSBio Systems, Seattle, WA) was applied overnight at 40°C.

Vinculin (1:500, Santa Cruz) was used for normalization of protein loading. Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit or donkey anti-goat, Santa Cruz) and visualized with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Pittsburgh, PA) and autoradiography. Band densities were quantified with the Gel Pro Analyzer 6.0.0.349 (Media Cybernetics, Rockville, MD).

Statistical Analysis

All statistical analyses were conducted using SigmaPlot™ (Systat Software, Inc., San Jose, CA 95110). For mRNA expression analysis, the RT^2 Profiler PCR Array software package was used. This package uses $2^{-\Delta\Delta CT}$based fold change calculations and the Student's *t*-test to

calculate two-tail, equal variance p-values. P values for comparison of the whole IBS-D group vs. the healthy control group were corrected for FDR (Bonferroni correction) so that $p<0.00263$ was statistically significant.

Comparison of the protein expression of PDZD3 based on Western blot analysis was performed based on two-tailed Student's t-test.

We used the general genetic model (that is, comparison of the 3 genotypes at each gene SNP) to assess the effect of each genotype separately on the expression of the related gene in terms of mRNA expression in rectosigmoid mucosa; three-group comparisons were conducted using ANOVA on ranks test. The mRNA expression data were also analyzed using the dominant genetic model (major allele homozygous vs. combined heterozygotes and minor allele homozygous groups) for consistency with the rest of the study analysis.

The dominant genetic model was used to assess the associations of genotype with mRNA expression and quantitative traits of interest, such as colonic transit or BA parameters. Group comparisons were performed using the Mann-Whitney rank sum test.

We used Spearman correlations to explore the association of mRNA expression with colonic transit, focusing on ascending colon emptying $T_{1/2}$ which is a continuous variable and has no upper and lower bounds; whereas, colonic transit, based on geometric center at 24 and 48 hours, is limited by values of 1 (all isotope in ascending colon) and 5 (all isotope in stool).

RESULTS

Demographics and Quantitative Bile Acid and Transit Characteristics

Patients with high fecal BA excretion had faster overall colonic transit compared to patients with normal fecal BA excretion (Table 2). Overall, in IBS-D subjects, there were no significant differences in the serum concentrations of FGF19 or C4 in the two groups of IBS-D

patients (Table 2); however, across all the groups studied, there was a significant inverse relationship between serum FGF19 and serum C4 ($r=-0.297$, $p=0.045$), as has been previously documented in the literature, including by our group (58). The lack of significant differences in serum FGF19 or C4 in the two IBS-D groups and controls likely represents a type 2 error, since only 4 of the healthy controls had serum FGF19 and C4 measurements.

The percentages of primary (cholic acid, CA; chenodeoxycholic acid, CDCA) and secondary bile acids (deoxycholic acid, DCA; ursodeoxycholic acid, UDCA; and lithocholic acid, LCA) of the combined secretory bile acids (DCA and CDCA) in the three groups were not different (Table 2). However, given the higher total fecal bile acid in the IBS-D subgroup with high fecal BA excretion, there was an approximately 8-fold higher mass of CDCA in stool in the IBS-D group with high BA excretion, compared to the IBS-D group with normal BA excretion. Thus, on average, there was 8% of mean BA excretion of 5167µmoles per 48 hours or ~0.41mmmol as CDCA in stool of IBS-D with high fecal BA excretion, compared to average 5% of mean BA excretion of 1025µmoles per 48 hours or ~0.05mmmol as CDCA in stool of IBS-D with normal fecal BA excretion.

mRNA Fold Change in Rectosigmoid Mucosa from IBS Relative to Healthy Controls

The mean (and 95% CI) fold changes (based on $2^{-\Delta\Delta CT}$) in IBS-D and subgroups of IBS-D patients relative to healthy controls are illustrated in Table 3 and Figure 1. Using false detection rate (FDR) for 19 gene comparisons, these observations suggest:

a. No significant fold changes in expression of genes associated with absorption (SLC10A2 [ASBT]), effects (GPBAR1[TGR5]) or feedback regulation of synthesis (FGFR4) of bile acids; however, there were borderline changes in expression of FXR ($p=0.0081$);

b. Increased expression of GUCA2B, PDZD3, and P2RY4, reflecting ion transport mechanisms; there was borderline reduction in expression of VIP (p=0.0030);

c. Increased expression of C4BPA and reduced expression of CCL20, reflecting immune functions;

d. Decreased expression of several barrier proteins, claudin-1 and fibronectin-1, and borderline increased expression of retinol binding protein (RBP-2, p=0.0031).

There were no differences in the mucosal expression of any of the genes of interest among IBS-D patients with high compared to normal fecal BA excretion; however, there were numerical (univariate, FDR uncorrected values, $p \leq 0.10$) reductions in expression of the mRNA of tight junction proteins [ZO-1 (TJP1), and FN-1] and decreased expression of TNFSF15 (immune marker).

The mean (and 95% CI) fold changes (based on $2^{-\Delta\Delta CT}$) in IBS-D and subgroups of IBS-D, and IBS-C patients relative to healthy controls are illustrated in Table 3. The only significant change, corrected for FDR, was increased expression of PR2Y4, VIP and OCLN.

PDZD3 Protein Expression by Immunohistochemistry and Western Blot

We used immunofluorescence to assess protein expression of the regulatory protein, PDZD3. Figure 2 shows the increased expression of this ion transport protein in rectosigmoid mucosa of patients with IBS-D compared to normal healthy volunteers by Western blot and immunohistochemistry. Western blots quantitating PDZD3 protein (with vinculin as control) showed increased expression of PDZD3 (p=0.02 by Student's t test) in IBS-D patients (n=10) compared to healthy controls (n=4).

Relationship of Genotype and mRNA Expression (corrected for β-actin) in IBS

a. Using **General Genetic Model:** mRNA expressions (corrected for the expression of

301 β-actin) showed no significant associations with genotypes except for a trend in the expression of
302 *GPBAR1* (Table 4); however, the observation that homozygous genotypes were both associated
303 with higher expression than the heterozygotes questions any biological significance of this
304 finding.

305 b. Using **Dominant Genetic Model:** There was a significant association of *GPBAR1*
306 genotype and mRNA expression (p=0.043) in rectosigmoid mucosa, based on analysis using the
307 dominant genetic model (Table 5).

308 **Spearman Correlation of mRNA Expression with Ascending Colon Emptying $T_{1/2}$**

309 We sought the association of mRNA expression (corrected for β-actin) in rectosigmoid
310 mucosa with ascending colon emptying $T_{1/2}$. There were no significant relationships between
311 mRNA expression of *FGFR4* (r=-0.06, p=0.71), *GPBAR1* (r=-0.08, p=0.63), *GUCA2B* (r=0.22,
312 p=0.13), *P2RY4* (r=0.16, p=0.27), *NR1H4* (r=-0.11, p=0.48), *TNFSF15* (r=-0.63, p=0.673),
313 *SLC10A2* (r=-0.08, p=0.62) and *SLC6A4* (r=0.147, p=0.39) and ascending colon emptying $T_{1/2}$.

314 **Relationship of Genotype with Colonic Transit (CT) in IBS-D (using Dominant Genetic**
315 **Model)**

316 *GPBAR1* was significantly associated with colonic transit at 24 and 48 hours, as well as
317 ascending colon emptying $T_{1/2}$ (Table 6); a borderline association of *5-HTTLPR* with colonic
318 transit at 24 and 48 hours was also observed ($p \leq 0.081$).

319 **Association of Bile Acid Homeostasis Genes (*SLC10A2*, *KLB* and *FGFR4* Genotypes) and**
320 **Bile Acid Measurements in Patients with IBS-D (using Dominant Genetic Model)**

321 In these 47 patients with IBS-D, the variants in the genes associated with BA homeostasis
322 were not significantly associated with serum FGF19, serum C4, and 48-hour fecal BA excretion
323 (Table 7).

DISCUSSION

Our study has provided several novel insights about mucosal pathobiology of IBS by means of studies of rectosigmoid mucosal biopsies from 47 IBS-D patients or controls. The clinical and biochemical characteristics of the participants in this study are consistent with those described recently in a somewhat larger cohort (n=64) that included the same 47 patients. However, the present study could only include the 47 patients who consented to undergo rectosigmoid biopsies. One difference in the current group of patients is that fasting serum FGF19 was not significantly different between IBS-D and healthy controls, in contrast to the original report by Walters et al. (62) and our prior studies (10,64) with larger sample sizes. We perceive that the lack of significant difference in fasting serum FGF19 between IBS-D and healthy controls represents a type 2 error: among the 17 healthy controls who had undergone rectosigmoid biopsies, only 4 had fasting serum FGF19 measurement.

The following observations were made in the present study:

First, targeted mRNA analysis by quantitative RT-PCR replicates our prior results showing generally increased expression of intestinal ion transport mechanisms and generally reduced intestinal barrier and up- or down-regulation of mucosal immune mechansisms. The significance of the observed associations is limited by the relatively small number of biopsies assessed (total 64); however, we present both uncorrected p values and, more importantly, we present the significance of observations corrected for the comparison of 19 selected genes. One of the well-recognized ion transport mechanisms (PDZ) showing increased mRNA expression was also associated with increased expression of PDZD3 protein on immunohistochemistry and Western blot analysis.

346 Second, there were no differences in mucosal expression in IBS-D associated with degree
347 of fecal BA excretion.
348 Our third general finding was that genotype was not significantly associated with the
349 level of expression of mRNA in rectosigmoid mucosa, colonic transit or BA parameters, with the
350 exception of *GPBAR1* gene which is significantly associated with mucosal expression of
351 GPBAR1 and with colonic transit.
352 Fourth, the level of expression of mRNA in rectosigmoid mucosa was not associated with
353 ascending colon emptying time.
354 **Mucosal mRNA Expression in IBS-D**
355 Among the intestinal secretory mechanisms, increased mRNA expression of GUCA2B
356 and P2RY4 could all support fluid and electrolyte secretion through actions on enterocytes or
357 submucosal neurons. On the other hand, the significantly increased expression of PDZD3 is
358 associated with either increased sodium ion and fluid absorption (through effects on NHE3) or
359 increased chloride ion and fluid secretion through CFTR, and borderline decreased expression of
360 VIP ($p=0.003$) may conceivably be associated with reduced intestinal secretion.
361 The largest fold increases in mRNA expression in our IBS-D patients were observed for
362 the purinergic receptor, P2RY4. Purinergic receptors are divided into adenosine P1 [A(1),
363 A(2A), A(2B), A(3)], ionotropic ATP-gated P2X receptors [P2X(1-7) that form ion channels or
364 pores], or metabotropic P2Y(1,2,4,6,11-14) receptors. Metabotropic receptors are indirectly
365 linked with ion channels on the plasma membrane through transduction mechanisms, often G
366 proteins. The purinergic hypothesis is based on ATP (or a related nucleotide, e.g., ADP or AMP)
367 release at the neurotransmitter synapses or on neuromuscular transmission, and these may
368 involve βNAD^+ and ADP ribose in neurotransmission in rodents, primates, and humans

(24,29,30). Mechanically evoked reflex electrogenic chloride secretion in rat distal colon is triggered by endogenous nucleotides acting at P2Y1, P2Y2, and P2Y4 receptors (21). P2Y2, 4, and 6 receptors regulate Cl^-, Na^+, and K^+ secretion in the intestinal tract, and absorption mechanisms, in particular, P2Y4 receptors, are involved in chloride secretion and potassium secretion (20,28,38). Thus, our observation of marked increase in expression of P2RY4 is consistent with the increased expression of secretory mechanisms (in addition to GUCA2B) in IBS-D patients. In addition to the novel mechanisms potentially related to ion transport in the manifestations of IBS-D, the current findings provide the basis for further hypothesis testing and, possibly, testing novel pharmacological approaches to reverse electrolyte secretion (41) in IBS-D. These findings complement the observation of increased small intestine secretion in response to bile acid infusion in IBS-D (42).

The specificity of the observed fold changes in mRNA expression is enhanced by the differences in the observations for IBS-D and the additional control group of IBS-C. The biological significance of the significant associations with IBS-D is discussed in the next section.

Increased mRNA expression of GUCA2B is associated with the endogenous GC-C ligand, uroguanylin, which is secreted by intestinal goblet cells (50). In the same way that guanylate cyclase C agonists are effective in the treatment of IBS-C, it is conceivable that antagonists at the guanylate cyclase receptors may impact the management of IBS-D in the future. Atrial and brain natriuretic peptides are agonists at the guanylate cyclase B receptor (66); moreover, homozygous knockout of the guanylate cyclase B receptor in mice results in a severe phenotype with gastric motor dysfunction and intestinal dilatation (49). These data suggest that further study of expression of guanylate cyclase receptors or GC-C ligands (guanylin and uroguanylin) in IBS may yield important insights into pathobiology or treatment of IBS.

We observed borderline increased mRNA expression of FXR in colonic mucosa in the whole IBS-D group (p=0.0081, relative to significance value with FDR, p=0.00263); this was also observed in both IBS-D subgroups. FXR activation prevents chemically-induced intestinal inflammation, with improvement of colitis symptoms, inhibition of epithelial permeability, and reduced goblet cell loss in a mouse model of inflammatory bowel disease (27). FXR expression is decreased in colonic mucosa of patients with primary sclerosing cholangitis [PSC (54)], and PSC is associated with higher circulating levels of the conjugated primary bile acids (3). These data are consistent with a potential role of colonic mucosal FXR in protecting the colonic mucosal integrity in patients with IBS-D. Increased expression of C4BPA and reduced expression of CCL20 reflect changes in immune functions that may ultimately lead to the immune activation observed in IBS-D (45).

Decreased expression of several barrier proteins, especially claudin-1 and fibronectin-1, may reflect the observed increase in intestinal mucosal permeability in IBS-D (14). The observed borderline increased expression of retinol binding protein (RBP-2) may be a compensatory change to correct the decreased expression of the other tight junction proteins.

On the other hand, there were no significant differences (see Table 3) in the mucosal expression of any of the genes of interest among IBS-D patients with high compared to normal fecal BA excretion. The significance of the increased mucosal expression in P2RY4 and VIP in IBS-C is unclear, although their increased expression may be related to muscle function (e.g. relaxation) rather than ion secretion. The increased OCLN in mucosa from IBS-C patients may reflect greater mucosal barrier functions in these patients; prior work (5) had documented the preservation of occludin and claudin expression in IBS-C in contrast to IBS-D.

There are only a few other papers in the published literature that describe alterations in mucosal mRNA expression of several mechanisms by next generation sequencing methods in patients with IBS-D. Our previous study used next generation RNA sequencing and RT-PCR of rectosigmoid mucosa in 9 patients with IBS-D and 9 healthy controls; we identified differential expression of secretory and barrier genes (11). In the current study in IBS-D patients, we confirmed the fold changes for 8 of the 10 genes evaluated in the prior study: *FN1*, *IFIT3*, *PDZD3*, *TFF1*, GUCA2B, *RBP2*, *C4BPA*, and *P2RY4*. The Barcelona group used combinations of microarray and PCR, focused their studies on mucosal tight junction expression and immune activation in jejunal mucosal biopsies, and identified reduced expression of zonula occludens 1 (ZO-1) in IBS-D at both gene and protein levels (37), as well as higher mucosal immune activity in IBS-D, with upregulation of germline transcripts and immunoglobulin genes (58). A recent combined study from Helsinki and Nottingham explored the association of host rectal mucosal expression and the fecal microbiome in different subgroups of IBS, including IBS-D and post-infectious IBS (31), rather than the differences in mucosal expression between IBS subgroups and healthy controls. However, one of the strongest associations between microbial populations and rectal mucosal mRNA expression in IBS patients pertains to NR1H4 or FXR, which was a change of borderline significance ($p=0.0081$) in our current study.

Relation of mRNA Expression and Genotype and Phenotype in IBS-D

Among the genotypes studied, the only one that is significantly associated with colonic transit is *GPBAR1* genotype. We had previously observed this relationship in studies in several hundred patients (17), but we present it now in only 47 patients with IBS-D and present this unique statistically significant finding in the context of a broad spectrum of genes associated with absorption, feedback regulation and action of bile acids, and selected immune function and

serotonin transporter genes. None of the other genes of interest was associated with alteration in colonic transit. Importantly, we did not observe association with variation in ASBT, which has been reported rarely in familial or sporadic diarrhea (39,40).

We found no relation between genotype and expression for all the candidate genes of interest, and, similarly, we did not find significant associations of bile acid-related genotypes with serum C4, serum FGF19 and fecal BA excretion. This suggests that post-translational modification may be a more relevant mechanism controlling these phenotypic parameters of bile acid control and expression than genotype.

Finally, there was no relation between mRNA expression and ascending colon emptying or transit; we focused on the latter parameter of transit, since it appears to be more closely related to the state of secretion within the colon. Thus, ascending colon emptying is accelerated in carcinoid diarrhea [a classical secretory diathesis (61)], and proximal colon emptying is positively correlated with stool weight (55).

Strengths and Limitations

We have studied >5 times as many IBS-D patients in the current study compared to the prior pilot study, and we evaluated mRNA expression in rectosigmoid mucosa for 19 genes; the statistical analysis was corrected for multiple comparisons. We also replicated the numerically increased or decreased mRNA fold changes (identified as FDR uncorrected changes in the prior study) in 8 of the first 10 genes of interest. Overall, these findings support the potential role of altered mucosal functions in IBS-D.

The limitations include: first, we restricted our study to 19 candidates, for which there was a strong biological rationale for each biological process (e.g. ion transport, immune mechanisms, barrier proteins) in IBS. However, several different potential mechanisms were

explored and, therefore, we did not bias the study, other than using the prior pilot study conducted using RNA sequencing and RT-PCR confirmation of the main findings (11) as a starting point. In designing such studies, one has to balance the need to explore as many potentially relevant mechanisms with the sample size available and the need to correct for false detection rate.

Second, although genetic variation in *GPBAR1* was associated with colonic transit (with $p<0.05$ for colonic transit at 48 hours), the association of genotype with colonic transit appraised 9 variants in 7 genes [*SLC6A4*, *GPBAR1, TNFSF15, KLB, FGFR4, FXR, SLC10A2 (ASBT*)] and three measurements of colonic transit. Therefore, the observation should be regarded as hypothesis-generating and not definitely proven in this study of 64 people. Nevertheless, the current sample of patients is much smaller than the ~650 IBS patients and healthy controls that were used to demonstrate in prior studies the association of *GPBAR1* (17,18) and other genes [e.g. *KLB, FAAH* (summarized in ref. 12)] with small bowel or colonic transit.

Third, we do not know that the parallel expression (mRNA and protein) of PDZD3 is necessarily a good "representation" for the other mRNA changes.

CONCLUSION

In conclusion, the current data demonstrate that mucosal ion transport mechanisms (mRNA for several factors, and PDZD3 protein) are generally upregulated and barrier genes downregulated in IBS-D compared to healthy controls, independent of genotype. There are no differences in gene expression in IBS-D with high compared to normal fecal BA excretion. These pathobiological mechanisms deserve further study to further advance the understanding of pathophysiological mechanisms in patients with IBS and diarrhea.

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783 **Table 1. Genes of interest included in the RT-PCR analysis of rectosigmoid mucosa**

Gene Symbol	Refseq #	Official Full Name
C4BPA	NM_000715	Complement component 4 binding protein, alpha
CCL20	NM_004591	Chemokine (C-C motif) ligand 20
CLDN1	NM_021101	Claudin 1
FGFR4	NM_002011	Fibroblast growth factor receptor 4
FN1	NM_002026	Fibronectin 1
GPBAR1	NM_170699	G protein-coupled bile acid receptor 1 (syn. TGR5)
GUCA2B	NM_007102	Guanylate cyclase activator 2B (uroguanylin)
IFIT3	NM_001549	Interferon-induced protein with tetratricopeptide repeats 3
NR1H4	NM_005123	Nuclear receptor subfamily 1, group H, member 4 (syn. Farnesoid X receptor)
OCLN	NM_002538	Occludin
P2RY4	NM_002565	Pyrimidinergic receptor P2Y, G-protein coupled, 4
PDZD3	NM_024791	PDZ domain containing 3
RBP2	NM_004164	Retinol binding protein 2, cellular
SLC10A2	NM_000452	Solute carrier family 10 (sodium/bile acid cotransporter family), member 2 (syn. Apical Sodium-coupled Bile acid transporter)
SLC6A4	NM_001045	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (syn. Serotonin transporter)
TFF1	NM_003225	Trefoil factor 1
TJP1	NM_175610	Tight junction protein 1 (syn. zonula occludens 1)
TNFSF15	NM_005118	Tumor necrosis factor (ligand) superfamily, member 15
VIP	NM_003381	Vasoactive intestinal peptide
ACTB	NM_001101	Actin, beta (housekeeping gene)
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)
HGDC	SA_00105	Human Genomic DNA Contamination
RTC	SA_00104	Reverse Transcription Control
PPC	SA_00103	Positive PCR Control

784 (syn=synonym)

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786 **Table 2. Demographics and bile acid and colonic transit characteristics**

Parameter (mean±SEM)	Healthy Controls	IBS-C	Whole IBS-D group	IBS-D + high BA excretion	IBS-D + normal BA excretion	p value high vs. normal BA excretion*
N	17	10	47	20	27	
Age, y	38.1 ± 2.7	48.3 ± 4.3	40.7 ± 1.7	40.0 ± 2.4	41.4 ± 2.5	
BMI, kg/m^2	27.3 ± 1.5	26.4 ± 2.1	30.1 ± 1.2	32.3 ± 1.8	28.5 ± 1.6	0.051
Total fecal BA (μmoles/48h)	1322.4 ± 656.8$^{\#}$		2787.6 ± 480.7	5167.2 ± 874.9	1024.9 ± 129.0	<0.001
Mean % fecal LCA/CDCA/DCA/CA/UDCA	38/1/58/1/3		28/6/54/8/4	24/8/51/13/4	32/5/55/5/3	Not tested
Serum C4 (ng/mL)	35.7 ± 5.7$^{\#}$		34.4 ± 3.8	34.5 ± 4.7	34.3 ± 5.7	NS
Serum FGF19 (pg/mL)	162.0 ± 39.4$^{\#\#}$		118.8 ± 10.8	126.7 ± 22.3	117.2 ± 13.9	NS
CT GC24h^	2.2 ± 0.2	2.0 ± 0.3^^	2.9 ± 0.2	3.3 ± 0.3	2.5 ± 0.2	0.017
CT GC48h	3.7 ± 0.5$^{\#}$	2.9 ± 0.4^^	4.2 ± 0.1	4.5 ± 0.2	4.0 ± 0.2	0.022
Asc. colon $T_{1/2}$ (hours)	13.0 ± 2.8$^{\#}$		15.0 ± 1.2	13.1 ± 1.9	16.4± 1.6	0.124

787 CT = colonic transit; Asc. = ascending; * Mann Whitney Rank Sum test; $^{\#}$ based on analysis in 3
788 among these healthy participants; $^{\#\#}$ based on analysis in 4 among these healthy participants;
789 ^ based on data from 12 healthy controls; ^^ based on 7 patients with IBS-C
790 CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid;
791 UDCA = ursodeoxycholic acid; LCA = lithocholic acid

792 **Table 3. mRNA fold change in rectosigmoid mucosa from IBS-D patients relative to healthy controls.**
793 Bolded data show univariately significant fold changes (without correction for false detection resulting from multiple comparisons)
794 relative to healthy controls. The decreased expression is indicated by italics.
795 Bolded p values reflect the comparison of whole IBS-D group vs. control corrected for FDR (Bonferroni correction) so that p<0.00263
796 is statistically significant. There were no significant differences (with only few p values ≤0.10, without FDR correction) in mRNA
797 fold changes in IBS-D patients with high vs. low fecal BA excretion; all other p values were >0.20.
798

Parameter data: mean (95% CI)	IBS-C group	p IBS-C vs. control	Whole IBS-D group	p IBS-D vs. control	IBS-D + high BA excretion (n=20)	IBS-D + normal BA excretion (n=27)	p, high vs. normal BA excr.
Bile acid absorption and effects							
SLC10A2 (ASBT)	1.86 (0.49, 3.22)	0.0759	**2.71 (1.37, 4.06)**	0.0298	**3.27 (0.92, 5.63)**	**2.36 (1.06, 3.66)**	
NR1H4 (FXR)	1.64 (0.86, 2.41)	0.0420	**3.02 (1.95, 4.08)**	0.0081	**2.98 (1.50, 4.46)**	**3.04 (1.90, 4.19)**	
FGFR4	1.14 (0.86, 1.41)	0.2030	***0.82 (0.69, 0.95)***	*0.0463*	***0.82 (0.67, 0.95)***	***0.82 (0.67, 0.98)***	
GPBAR1	1.45 (1.02, 1.88)	0.0178	0.93 (0.74, 1.12)	0.2298	0.90 (0.70, 1.11)	0.95 (0.75, 1.16)	
Ion transport mechanisms							
GUCA2B	1.45 (0.56, 2.34)	0.3359	**3.81 (2.25, 5.36)**	**0.00016**	**3.78 (1.99, 5.56)**	**3.83 (2.23, 5.42)**	
PDZD3	1.14 (0.00, 2.48)	0.0099	**2.51 (1.88, 3.14)**	**0.000001**	**2.49 (1.72, 3.17)**	**2.56 (1.88, 3.23)**	
P2RY4	4.27 (1.45, 7.09)	**0.0016**	**11.34 (4.11, 18.58)**	**0.000001**	**9.30 (2.05, 16.56)**	**13.14 (5.01, 21.26)**	
VIP	3.21 (1.61, 4.80)	**0.0008**	***0.44 (0.24, 0.63)***	*0.0030*	***0.46 (0.20, 0.73)***	***0.42 (0.20, 0.63)***	
SLC6A4	1.97 (0.73, 3.21)	0.0726	**2.75 (1.30, 4.21)**	0.0104	**2.44 (0.71, 4.17)**	**3.01 (1.38, 4.64)**	
Immune functions							
TNFSF15	1.17 (0.50, 1.84)	0.1901	***0.69 (0.48, 0.90)***	*0.0185*	***0.55 (0.33, 0.77)***	***0.81 (0.56, 1.06)***	0.10
C4BPA	1.97 (0.76, 3.17)	0.0189	**3.73 (2.37, 5.08)**	**0.00003**	**4.14 (2.27, 6.00)**	**3.45 (2.02, 4.88)**	
CCL20	1.43 (0.35, 2.51)	0.7339	***0.23 (0.10, 0.36)***	***0.000001***	***0.22 (0.08, 0.35)***	***0.24 (0.10, 0.38)***	
IFIT3	0.92 (0.33, 1.50)	0.3555	0.84 (0.38, 1.30)	**0.0279**	0.90 (0.39, 1.42)	0.79 (0.35, 1.23)	
Barrier functions							
TJP 1 (ZO-1)	1.64 (1.14, 2.14)	0.0070	***0.76 (0.57, 0.96)***	*0.0457*	***0.76 (0.57, 0.96)***	0.92 (0.72, 1.11)	0.06
OCLN	1.77 (1.25, 2.30)	**0.0017**	1.19 (0.87, 1.51)	0.2900	1.12 (0.79, 1.45)	1.25 (0.91, 1.59)	
CLDN1	1.20 (0.69, 1.70)	0.7099	***0.59 (0.39, 0.80)***	***0.0004***	***0.63 (0.40, 0.86)***	***0.57 (0.35, 0.78)***	
RBP2	1.14 (0.04, 2.25)	0.1005	**1.78 (1.16, 2.40)**	0.0031	**1.72 (1.08, 2.36)**	**1.83 (1.13, 2.52)**	
FN1	1.33 (0.77, 1.90)	0.4134	***0.35 (0.23, 0.47)***	***0.00001***	***0.28 (0.16, 0.40)***	***0.40 (0.26, 0.54)***	0.10
TFF1	3.34 (0.00, 7.22)	0.1918	1.17 (0.72, 1.63)	0.8739	1.12 (0.63, 1.61)	1.21 (0.72, 1.70)	

799

800 **Table 4. Relationship of genotype and mRNA expression (median, IQR; corrected for**
801 **β-actin) by general genetic model (comparing expression in 3 genotypes: AA, Ab, bb) in**
802 **IBS (analysis used ANOVA on ranks to compare 3 genotypes).**

Genotype	MAF	N	mRNA expression	p
Serotonin Transporter				
SLC6A4 rs4795541LL	0.447 (L)	7	16.59 (15.47, 17.44)	0.463
SLC6A4 rs4795541LS		28	16.49 (15.19, 17.95)	
SLC6A4 rs4795541SS		12	15.72 (14.87, 16.75)	
Immune Function				
TNFSF15 rs4263839AA	0.38 (A)	6	12.94 (12.11, 13.85)	0.27
TNFSF15 rs4263839AG		24	12.55 (12.11, 13.54)	
TNFSF15 rs4263839GG		17	12.28 (12.07, 12.96)	
Bile Acid Absorption, Homeostasis or Receptor				
GPBAR1 rs11554825CC	0.436 (C)	10	13.05 (12.53, 13.33)	0.082
GPBAR1 rs11554825CT		21	12.86 (12.52, 13.03)	
GPBAR1 rs11554825TT		16	13.18 (12.72, 13.59)	
NR1H4 rs 17030285 CC	0.128 (G)	35	12.80 (11.59, 13.04)	0.30
NR1H4 rs 17030285 CG		12	11.83 (11.15, 12.46)	
NR1H4 rs4764980 AA	0.49 (G)	12	12.30 (11.60, 12.75)	0.98
NR1H4 rs4764980 AG		23	12.03 (11.09, 13.22)	
NR1H4 rs4764980 GG		12	12.18 (11.15, 12.93)	
SLC10A2 rs188096 AC	0.106 (A)	10	16.65 (15.83,17.80)	0.82
SLC10A2 rs188096 CC		37	17.16 (15.11, 17.87)	
FGFR4 rs1966265 AA	0.213 (A)	2	10.15 (9.55, 10.75)	0.18
FGFR4 rs1966265 AG		16	11.06 (10.79, 11.32)	
FGFR4 rs1966265 GG		29	10.85 (10.46, 11.52)	
FGFR4 rs434434 AG	0.191 (A)	18	10.80 (10.52, 11.11)	0.28
FGFR4 rs434434 GG		29	11.03 (10.54, 11.52)	
FGFR4 rs351855 AA	0.287 (A)	2	11.17 (10.51, 11.83)	0.73
FGFR4 rs351855 AG		25	11.03 (10.51, 11.52)	
FGFR4 rs351855 GG		20	10.83 (10.62, 11.17)	

803

804

805 **Table 5. Relationship of genotype and mRNA expression (median, IQR, corrected for**
806 **ß-actin) by dominant genetic model (comparing expression in homozygous major allele**
807 **genotype vs combining heterozygotes and minor allele homozygous) in IBS (comparisons**
808 **with Mann-Whitney test).**
809

Genotype	MAF	N	mRNA expression	p
Serotonin Transporter				
SLC6A4 rs4795541 LL	0.447 (L)	7	16.59 (15.47, 17.44)	0.70
SLC6A4 rs4795541 LS/SS		37	16.22 (14.95, 17.24)	
Immune Function				
TNFSF15 rs4263839 GG	0.38 (A)	17	12.28 (12.07, 12.96)	0.12
TNFSF15 rs4263839 AA/AG		30	12.58 (12.14, 13.62)	
Bile Acid Absorption, Homeostasis or Receptor				
GPBAR1 rs11554825TT	0.436 (C)	16	13.18 (12.72, 13.59)	0.043
GPBAR1 rs11554825 CC/CT		31	12.86 (12.54, 13.17)	
NR1H4 rs 17030285 CC	0.128 (G)	35	12.80 (11.59, 13.04)	0.30
NR1H4 rs 17030285 CG		12	11.83 (11.15, 12.46)	
NR1H4 rs4764980 AA	0.49 (G)	12	12.30 (11.60, 12.75)	0.86
NR1H4 rs4764980 AG/GG		35	12.11 (11.09, 13.12)	
SLC10A2 rs188096AC	0.106 (A)	10	16.65 (15.83,17.80)	0.821
SLC10A2 rs188096CC		37	17.16 (15.11, 17.87)	
FGFR4 rs1966265 GG	0.213 (A)	29	10.85 (10.46, 11.52)	0.50
FGFR4 rs1966265 AG/AA		18	11.01 (10.74, 11.25)	
FGFR4 rs434434 AG	0.191 (A)	18	10.80 (10.52, 11.11)	0.28
FGFR4 rs434434 GG		29	11.03 (10.54, 11.52)	
FGFR4 rs351855 GG	0.287 (A)	20	10.83 (10.62, 11.17)	0.48
FGFR4 rs351855 AA/AG		27	11.03 (10.51, 11.52)	

810 S is minor allele for 5-*HTTLPR*; T is minor allele for *GPBAR1*.

811

812 **Table 6. Relationship of genotype with colonic transit (CT) and ascending colon emptying**
813 **$T_{1/2}$ in IBS-D (data show median, IQR, and analysis is by Mann-Whitney rank sum test).**
814 **Note the significant association with *GPBAR1* genotype and the borderline association with**
815 ***SLC6A4 (*5-HTTLPR polymorphism).**
816

Genotype	N	Colonic GC24	P	Colonic GC48	p	AC emptying $T_{1/2}$ h	p
Serotonin Transporter							
5-HTTLPR rs4795541 LL	7	3.91 (3.01, 4.42)	0.079	5.00 (4.17, 5.00)	0.081	15.14 (5.41, 17.41)	0.362
5-HTTLPR rs4795541 LS/SS	40	2.43 (1.78, 3.89		4.57 (3.33, 4.99)		16.39 (10.46, 19.47)	
Immune Function							
TNFSF15 rs4263839 GG	17	2.97 (1.70,4.56)	0.79	4.67 (3.99, 5.00)	0.40	15.57 (10.67, 18.57)	0.825
TNFSF15 rs4263839 AA/AG	30	2.72 (1.90,3.90)		4.58 (3.16, 5.00)		16.60 (5.30, 20.15)	
Bile Acid Absorption, Homeostasis or Receptor							
GPBAR1 rs11554825 CC	10	3.9 (3.33, 4.49)	**0.010**	5.00 (4.72, 5.00)	**0.005**	9.18 (4.79, 15.58)	**0.034**
GPBAR1 rs11554825CT/TT	37	2.26 (1.78, 3.38)		4.24 (3.02, 4.93)		16.60 (10.49, 19.59)	
KLB rs17618244 AG#	17	2.83 (2.03,4.32)	0.42	4.86 (3.79, 5.00)	0.237	13.1 (7.33, 17.64)	0.514
KLB rs17618244 GG	30	2.26 (1.77,3.90)		4.41 (3.04, 4.93)		16.67 (7.08, 19.59)	
FGFR4 rs1966265 GG	29	2.53 (1.80,4.32)	0.947	4.83 (2.91, 5.00)	0.418	15.57 (6.83, 20.61)	0.897
FGFR4 rs1966265 AG/AA	18	2.78 (1.88,3.89)		4.50 (3.68, 4.95)		16.27 (7.33, 18.67)	
FGFR4 rs 434434 GA	18	2.53 (1.84,4.07)	0.973	4.64 (2.91, 5.00)	0.920	16.30 (9.11, 20.00)	0.622
FGFR4 rs 434434 GG	29	2.78 (1.82,4.20)		4.65 (3.72, 4.98)		14.65 (6.95, 18.39)	
FGFR4 rs 351855 GG	20	2.53 (1.80,4.32)	0.947	4.83 (2.91, 5.00)	0.418	15.57 (6.83, 20.61)	0.897
FGFR4 rs 351855 AA/AG	27	2.78 (1.89,3.89)		4.50 (3.68, 4.95)		16.27 (7.33, 18.67)	
SLC10A2 rs188096 AC	10	2.41 (1.73, 4.47)	0.88	4.66 (2.91, 5.00)	0.95	14.75 (5.12, 19.2)	0.795
SLC10A2 rs188096 CC	37	2.78 (1.94, 3.89)		4.65 (3.64, 5.00)		16.00 (7.49, 19.0)	
FXR rs4764980 AA	12	2.26 (1.87, 3.89)	0.80	4.52 (3.69, 4.93)	0.88	15.62 (6.79, 19.9)	0.96
FXR rs4764980 AG/GG	35	2.80 (1.79, 4.31)		4.66 (3.20, 5.00)		16.00 (7.33, 19.6)	

817 S is minor allele for *5-HTTLPR*; T is minor allele for *GPBAR1*; # is number of AA KLB
818 genotype identified

819

820 **Table 7. Associations of variants in genes (associated with BA absorption and feedback**
821 **regulation of synthesis) with serum FGF-19, serum C4, and 48-hour fecal BA excretion in**
822 **IBS-D**
823

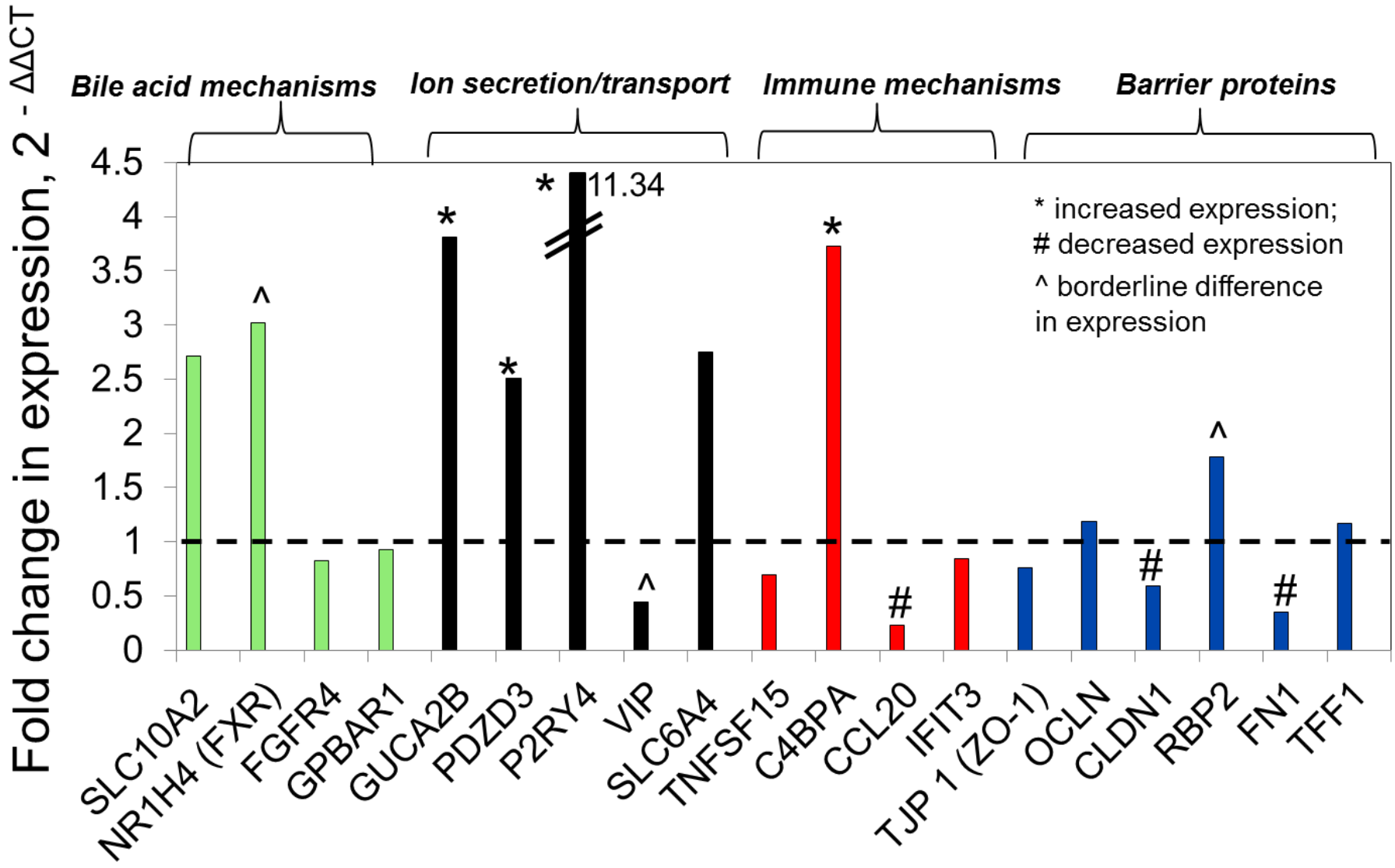
Genotype: (data: median, IQR)	**N**	**Fasting serum FGF-19 (pg/mL)**	**p**	**Fasting serum C4 (ng/mL)**	**p**	**Total fecal BA (µmoles/48h)**	**p**
SLC10A2 rs188096 AC	10	117 (63, 246)	0.13	28 (16, 60)	0.73	2723 (1037, 7766)	0.25
SLC10A2 rs188096 CC	37	86 (47, 135)		26 (14, 41)		1827 (737, 2887)	
KLB rs17618244 AG	17	110 (55, 173)	0.38	26 (13,54)	0.99	2127 (1493, 4056)	0.30
KLB rs17618244 GG	30	81 (48, 138)		26 (15.5, 46)		1559 (694, 3055)	
FGFR4 rs351855GG	20	74.2 (46.5, 157.8)	0.45	39 (13,61)	0.13	1850 (1073, 2916)	0.87
FGFR4 rs351855 AG/AA	27	99.8 (57.7, 144.7)		23 (16, 36)		1829 (733, 3442)	
FGFR4 rs1966265 GG	29	94.3 (49.3, 154.2)	1.00	27 (16, 59)	0.37	2127 (903, 4543)	0.16
FGFR4 rs1966265AG/AA	18	101.7 (49.1,140.1)		23 (14, 38)		1559 (666, 2741)	
FGFR4 rs434434 GG	29	77.9 (44.6, 132.8)	0.11	26 (16, 38)	0.92	1829 (676, 3652)	0.99
FGFR4 rs434434 GA	18	107.2 (63.9,182.4)		26 (12, 59)		1890 (1078, 2894)	

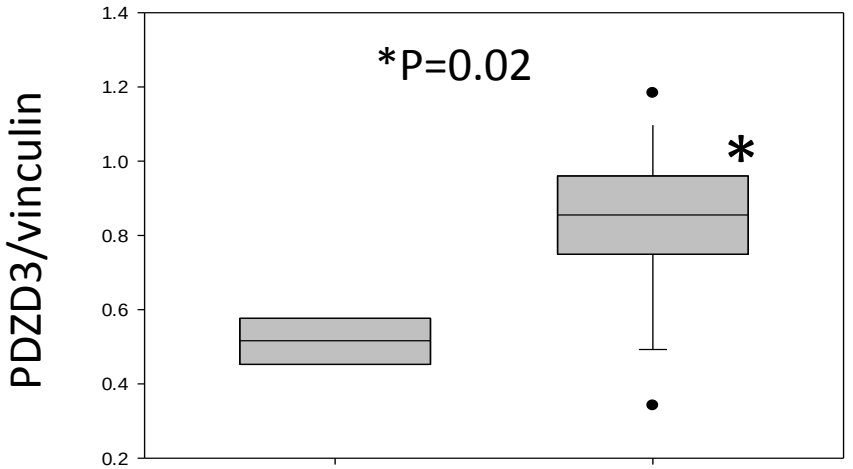
824

825

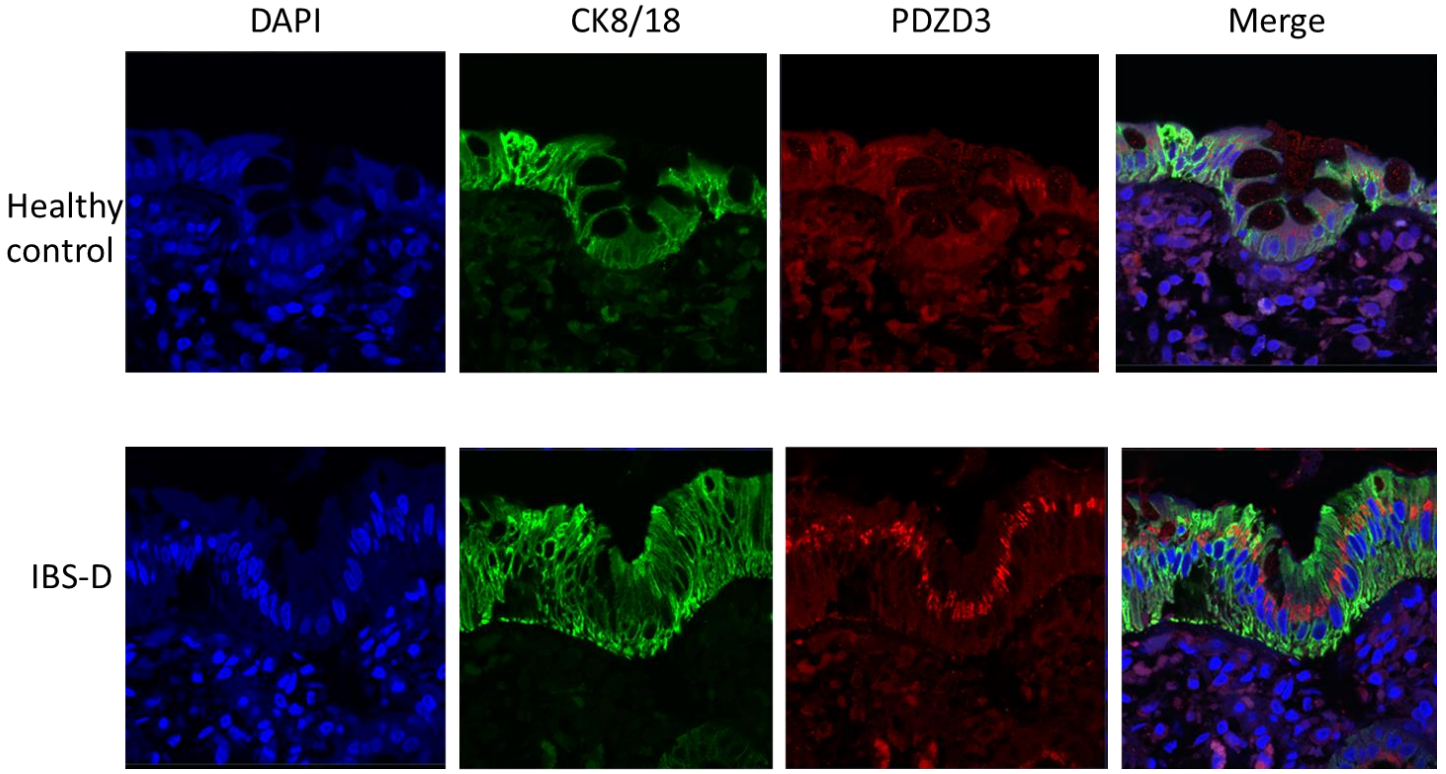
826 **Figure 1. Pictorial summary of mean fold changes in mRNA expression of candidate**
827 **mechanisms in IBS-D patients (n=47) compared to healthy controls (n=17), plotted from**
828 **data in Table 3 (which includes 95% CI for all fold changes in expression).**
829
830 **Figure 2. Upper panel shows Western blots quantitating PDZD3 protein (with vinculin as**
831 **control). Note the increased expression (p=0.02) of PDZD3 in IBS-D patients (n=10)**
832 **compared to healthy controls (n=4). Lower panel shows immunofluorescence for PDZD3 in**
833 **rectosigmoid colon mucosa of a patient with IBS-D compared to a normal, healthy control**
834 **(63X magnification). Stains show nuclear staining (DAPI), cytokeratin (C8/18, marker of**
835 **epithelial cells), PDZD3 and merge. Note the localization of increased PDZD3 protein in**
836 **epithelial cells in mucosa from the patient with IBS-D compared to the healthy control.**
837

838


Bile acid mechanisms
Ion secretion/transport
Immune mechanisms
Barrier proteins
Fold change in expression, 2 - ΔΔCT
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
* increased expression;
# decreased expression
^ borderline difference in expression
11.34
SLC10A2
NR1H4 (FXR)
FGFR4
GPBAR1
GUCA2B
PDZD3
P2RY4
VIP
SLC6A4
TNFSF15
C4BPA
CCL20
IFIT3
TJP 1 (ZO-1)
OCLN
CLDN1
RBP2
FN1
TFF1


*P=0.02
PDZD3/vinculin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
Healthy controls
IBS-D


PDZD3
vinculin


DAPI
CK8/18
PDZD3
Merge
Healthy control
IBS-D