Published in final edited form as:

Benef Microbes. 2018 April 25; 9(3): 345–355. doi:10.3920/BM2017.0059.

Stool and urine trefoil factor 3 levels: associations with symptoms, intestinal permeability, and microbial diversity in irritable bowel syndrome

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Abstract

Background/Aims: Previously we showed that urine trefoil factor 3 (TFF3) levels were higher in females with irritable bowel syndrome (IBS) compared to non-IBS females. To assess if TFF3 is associated with symptoms and/or reflect alterations in gastrointestinal (GI) permeability and gut microbiota in an IBS population, we correlated stool and urine TFF3 levels with IBS symptoms, intestinal permeability, stool microbial diversity and relative abundance of predominant bacterial families and genera. We also tested the relationship of stool TFF3 to urine TFF3, and compared results based on hormone contraception use.

Methods: Samples were obtained from 93 females meeting Rome III IBS criteria and completing 4-week symptom diaries. TFF3 levels were measured by ELISA. Permeability was assessed with the urine lactulose/mannitol (L/M) ratio. Stool microbiota was assessed using 16S rRNA.

Results: Stool TFF3, but not urine TFF3, was associated positively with diarrhea and loose stool consistency. Higher stool TFF3 was also associated with lower L/M ratio and microbial diversity. Of the 20 most abundant bacterial families *Mogibacteriaceae* and *Christensenellaceae* were inversely related to stool TFF3, with only *Christensenellaceae* remaining significant after multiple comparison adjustment. There were no significant relationships between stool or urine TFF3 levels

and other symptoms, nor between stool and urine levels. In premenopausal females, urine TFF3 levels were higher in those reporting hormone contraception.

Conclusions: Collectively these results suggest that higher stool TFF3 levels are associated with IBS symptoms (loose/diarrheal stools), lower gut permeability, and altered stool bacteria composition (decreased diversity and decreased *Christensenellaceae*), which further suggests that TFF3 may be an important marker of host-bacteria interaction.

Keywords

hormone contraception; diarrhea; *Christensenellaceae*; anti-microbial peptide

Introduction

Irritable bowel syndrome (IBS) is a common health care problem in the United States, as well as around the world. It is a functional condition characterized by abdominal pain and alterations in bowel function - diarrhea, constipation or both. IBS symptom severity can range from mild to severe. In the United States, a diagnosis of IBS is associated with significant use of health care resources and reductions in quality of life in those affected (Johansson *et al.*, 2010). It has been suggested that subgroups of patients with IBS may be best characterized by pathophysiological biomarkers that reflect central nervous system, gut-brain axis, and/or gut-specific alterations (Bennet *et al.*, 2016). Gut-specific factors could include both altered gut barrier integrity and microbial population composition. (Saulnier *et al.*, 2011). To date, biomarkers that reflect the common symptoms of IBS (i.e., abdominal pain and diarrhea/constipation) have been elusive.

Previously we showed, using a non-targeted urine proteomic approach in female IBS patients and healthy controls, that 18 proteins demonstrated 1- to 3-fold differences between the two groups. One of these, trefoil factor 3 (TFF3; also called intestinal trefoil factor) was higher in females with IBS relative to healthy controls, as determined by enzyme-linked immunosorbent assay (ELISA) (Goo *et al.*, 2012).

TFF3 is a stable secretory protein produced by gut epithelial goblet cells of the small intestine and colon, as well as by other tissues, including kidney, endometrium, and breast (Mhaweche-Fauceglia *et al.*, 2013; Podolsky *et al.*, 2009; Taupin and Podolsky, 2003). Under conditions of injury and/or inflammation in animal models, expression of gut tissue levels of TFF3 increase (Chang *et al.*, 2014; Renes *et al.*, 2002; Srivastava *et al.*, 2015). In humans, there is some evidence that serum TFF3 levels are higher in patients with inflammatory bowel disease (Gronbaek *et al.*, 2006). As such, urine and stool TFF3 levels could be reflective of a subclinical inflammatory process and/or alteration in barrier function in a subgroup of persons with IBS.

In the GI tract, TFF3 functions to protect the mucosa from insults, stabilize the mucus layer, and enhance healing of the epithelium (Beck *et al.*, 2010). TFF3 is primarily co-released with mucin and adds to the viscoelastic properties of mucus gel. Along with beta-defensin, it is considered an antimicrobial peptide. In one study, TFF3 derived from human milk induced intracellular signaling in cultured colonic cells (HF-29) through activation of protease

activated receptor-2 (PAR-2) (Bennet *et al.*, 2016). This activation resulted in increased expression of defensins, which are small host proteins important for mucosal defense. TFF3 also has been shown to upregulate the expression of tight junction-associated protein claudin-1 and participate in the redistribution of ZO-1 (zonulin) (Buda *et al.*, 2012). Combined, these studies suggest that tissue levels of TFF3 contribute to gut integrity, and ultimately, to the prevention of barrier disruption (Fu *et al.*, 2015; Gronbaek *et al.*, 2006; Verey *et al.*, 2011). Whether our observed increase in urine TFF3 in females with IBS is reflective of barrier function and/or a robust response to luminal stimulus is unknown.

Evidence from an animal model suggests that TFF3 gene expression in the GI tract may occur in a microbiota-dependent manner. For example, neonatal rats with necrotizing enterocolitis treated with *Bifidobacterium bifidum* exhibited a decrease in ileal TFF3 positively stained cells as compared to non-treated animals (Comelli *et al.*, 2008; Khailova *et al.*, 2009). In humans, less is known about the relationship between specific bacterial constituents and TFF3. In the oral cavity, investigators found that salivary TFF3 is inversely related to the number of *Porphyromonas gingivalis* and *Tannerella forsythia* in individuals with chronic periodontitis (Chaiyarit *et al.*, 2012). The results emerging from studies of the microbiota and IBS are conflicted, in that some investigators find microbiota-based (e.g. diversity) differences between IBS and healthy controls, while others do not (Jeffery *et al.*, 2012; Khailova *et al.*, 2009; Tap *et al.*, 2016). Tap *et al.* (Tap *et al.*, 2016) recently reported that while there were no intestinal microbial differences between IBS and healthy controls, specific microbial signatures identified a subgroup of IBS participants with severe IBS symptoms. To date, no studies have been done to examine the relationship of intestinal bacteria composition or diversity to stool TFF3 levels in humans.

Increased gut permeability has been postulated to contribute to IBS pathophysiology in some patients. In a prior study, IBS participants with increased intestinal permeability also had greater abdominal pain and greater impact of symptoms on daily activities (Shulman *et al.*, 2014a). It can be conjectured that if TFF3 levels as reflected in either urine or stool are elevated, this may portend an ability to mount a protective response and maintain intestinal barrier function.

As noted above, TFF3 can be expressed in other tissues such as endometrium and breast, where TFF3 expression is influenced by estrogen (May and Westley, 2015) and menstrual cycle phase (Henze *et al.*, 2016). However, it is not known whether sex hormones influence urine and/or stool levels of TFF3 in IBS. Further, it remains to be determined whether urine TFF3 levels are consistent with levels found in feces, a sample site less likely to be confounded by TFF3 originating from other organs.

The primary aim of this study was to determine if stool and/or urine TFF3 levels correlate with IBS symptom measures (e.g. daily abdominal pain, stool characteristics). Secondly, given the relationship between TFF3 and gut barrier function, we also sought to examine the potential relationship between TFF3 concentrations and gut permeability, as measured by the urinary lactulose/mannitol (L/M) ratio. Finally, we sought to investigate the potential relationships between TFF3 concentrations and stool microbial composition and diversity.

Our female IBS cohort provided an opportunity to explore whether or not the use of hormonal contraceptives affected the above relationships.

Materials and methods

Design

IBS symptom data and specimens (stool and urine) from a randomized controlled trial of behavioral therapy were used for this study (Jarrett *et al.*, 2016).

Participants and setting

As previously described, potential participants with IBS were recruited through general advertisement (flyers, newspapers, public radio, posters on city buses, and targeted mailings to gastroenterology clinic patients) in a metropolitan area in the Pacific Northwest (United States) (Jarrett *et al.*, 2016). Interested adults were screened over the phone. Eligibility was determined across the 5-week baseline assessment (initial interview and 4-week diary) (Jarrett *et al.*, 2016). During the last two weeks of this assessment period, stool, urine, and serum samples for the preselected candidate biomarkers were obtained.

Inclusion criteria specified women 18–70 years of age with a history of IBS symptoms for at least 6 months prior to their IBS diagnosis, and that they meet the current IBS criteria for the prior three months. All were diagnosed by a healthcare provider using Rome III research criteria (Drossman and Dumitrascu, 2006). Women age 50 or older had to have a negative colonoscopy, sigmoidoscopy, abdominal ultrasonography, or barium enema. Potential participants with a ‘red flag’ symptom (e.g., involuntary weight loss, blood in stool) were referred to their healthcare provider for further evaluation and not included in the study.

Potential participants were excluded if they were currently taking antibiotics and/or had taken them in the past 2 months, were using corticosteroids, or were daily using anticholinergics, tricyclic antidepressants, or calcium-channel blockers; had a medical history of abdominal surgery (except appendectomy, Caesarian section, tubal ligation, laparoscopic cholecystectomy, hysterectomy, or abdominal wall hernia repair); had organic GI disease or a moderate to severe pain condition (e.g., low back pain, chronic bladder syndrome); had diabetes, had a current mental health disorder (psychosis, bipolar disorder, or moderate to severe depressive episodes, recent suicide attempt or drug or alcohol abuse or dependence); had an immune-compromised disorder (e.g., autoimmune conditions); or were pregnant, breast feeding, or planning to get pregnant in the next year. This study was approved and reviewed annually by the University of Washington’s institutional review board.

Procedures

At the initial visit, participants gave written informed consent, returned completed questionnaires, and were oriented to the study. They completed a daily diary each evening (e.g., symptoms, stool type, medications) for either one menstrual cycle, or 28 days for those using hormone contraception or who were postmenopausal. For those menstruating, women

started the 28-day diary regardless of where they were in their menstrual cycle, but were asked to record if they were menstruating.

Measures

Demographic and clinical characteristics—Demographic data included age, marital status, years of education, ethnic affiliation, occupation, body mass index (BMI), age at onset of IBS, and regular prescription and over-the-counter medication use. The Rome III Diagnostic Questionnaire was used to retrospectively assess symptoms and stool characteristics (Drossman and Dumitrascu, 2006). Participants rated abdominal pain and discomfort by how often they occurred in the last 3 months (never [0] to every day [6]), while change in stool frequency and appearance were rated from never or rarely (0) to always (4).

Prospective GI symptoms—GI symptoms, as well as stool consistency, were measured using a daily diary over the four weeks. Daily GI symptoms including abdominal pain, diarrhea, constipation, intestinal gas, and urgency were part of a 26-symptom record. All women rated the symptoms on a scale from 0 (*not present*) to 4 (*very severe*). Stool consistency was rated on a 5-point scale (*watery* to *very hard*).

TFF3 in stool and urine—TFF3 in urine and stool was measured with an ELISA assay (BioPorto Diagnostics, Karasek, Czech Republic) that used a recombinant form of TFF3 antibody. Detection range was 0.2–2.5 ng/mL; sensitivity was 0.007ng/ml; and the inter- and intra-assay variance was 12.2% and 10.5%, respectively. Fecal protein content was analyzed using an assay which involved the binding of Coomassie 1 Brilliant Blue G-250 dye to proteins (Bradford, 1976) and spectrophotometric detection. Urine creatinine was measured by spectrophotometric optical density analysis.

Intestinal permeability—The procedures used for permeability testing followed the same procedures as previously described (Shulman *et al.*, 2014b). Briefly, women were asked to refrain from taking non-steroidal anti-inflammatory drugs for at least two weeks prior to testing. They were asked to refrain from alcohol ingestion for at least two days prior to testing. Following their evening meal, participants fasted for four hours, urinated, then drank a 127.5 mL solution containing sucrose (10 g), lactulose (5 g) and mannitol (1 g), followed by 240 mL of water. Urine was collected for the next 24 hours. The urine was placed into a container containing either thimerosal or chlorhexidine to inhibit bacterial growth and kept in the freezer until the participant returned to the laboratory. In the laboratory, samples were stored at −70 °C until analysed, as previously described (Catassi *et al.*, 1991; Shulman *et al.*, 1998). Lactulose and mannitol results were based on the complete 24-hour urine collection. The L/M ratio was calculated using the fractional excretion of each sugar (McOmber *et al.*, 2010).

Stool microbial diversity—Participants collected stools using a container that sealed and served as its own storage system, so stool handling was not required (Saulnier *et al.*, 2011; Thim *et al.*, 2002). Participants kept the specimens stored in their freezers until they returned to the laboratory. Bacterial DNA was extracted using the MOBIO PowerSoil DNA Isolation

kit with the Human Microbiome Project modifications (Aagaard *et al.*, 2013; Caporaso *et al.*, 2010; DeSantis *et al.*, 2006; Edgar, 2010; Haas *et al.*, 2011). 16S ribosomal RNA gene amplicons (V4) (Caporaso *et al.*, 2011) were generated and sequenced on the MiSeq platform (Illumina, Inc, San Diego, CA). Primer sequences were removed from demultiplied reads using fastq-mcf (Aronesty, 2011), and reads were further quality filtered and clustered into operational taxonomic units (OTUs) using the LotuS platform (version 1.462) (Hildebrand *et al.*, 2014) under default settings. Briefly, sequences were quality-filtered to remove those with average quality scores less than 20, containing greater than one ambiguous base call, with homopolymer runs exceeding eight bases, and/or those shorter than 170 base pairs in length. Reads were clustered into OTUs at a 97% similarity threshold using the UPARSE algorithm (Edgar, 2013), chimera-checked using VSEARCH (Rognes *et al.*, 2016), and assigned taxonomic identities using the Ribosomal Database Project Classifier trained on the HIT-db (Ritani *et al.*, 2015) reference database. Community diversity was characterized using the Shannon Index and the number of unique OTUs in each sample as a measure of OUT richness, and values were calculated using QIIME (version 1.9.1) (Caporaso *et al.*, 2010).

Data analysis

Scatterplots were used to show the relationship of stool TFF3 to clinical measures and biomarkers. Pearson correlation was used to quantify the strength of these relationships. Partial correlation was used to examine whether controlling for hormone contraceptive use leads to significantly different results. Correlations were also presented separately for those using, and those not using, hormone contraceptives. Since stool TFF3 had a highly skewed distribution, the log of stool TFF3 was used. For analysis of microbiome diversity, partial correlation controlling for total read count was used because total read count differed across samples. Microbiome data includes many taxa at different levels such as family, genus, species. Analysis of taxa data should incorporate adjustment for multiple comparisons. Our approach was to focus on the 20 most abundant families, and use a Bonferonni adjustment so that only those families with $p < .0025$ are considered significant after adjustment. A logit transform was applied to the relative abundance of each family prior to analysis, since values are constrained to be between 0 and 1 and most have a skewed distribution. Other than stool microbiota measures, no formal adjustments were made for multiple comparisons and hence the results should be interpreted cautiously with this in mind. Data were presented as mean $\pm$ SD.

The sample size was determined by the number of female subjects from the previous study for whom urine or stool specimens were available. Ninety-three subjects were included in at least one of the analyses in this report; however, the sample size for specific analyses involving stool TFF3 varies from 72 to 89, depending on missing data for specific clinical or biomarker variables. Power for detecting a correlation of 0.30 thus varied from 75% to 82%, and power for detecting correlation of 0.35 varied from 87% to 92%. Power was lower for the analyses that were split by hormone contraception use.

Results

Characteristics of participants

Of the 93 women enrolled, the majority were White and had an education level of at least a bachelor's degree. The mean age was 40 ± 15 years, and the mean BMI was 27 ± 8 . Thirteen percent of the sample was IBS-constipation, 26% IBS-diarrhea, 58% IBS-mixed, and 3% unclassified stool pattern, based on Rome III research criteria. Twenty-eight women (32%) were currently using estrogen/progesterone contraception at the time of sample collection. Of these, 23 were on oral products and five had an estrogen/progesterone implant or intrauterine device. Fourteen percent of the participants used selective serotonin reuptake inhibitors on a regular basis.

Stool and urine TFF3

There were no significant differences in stool or urine TFF3 across predominant stool pattern groups (Table 1). As shown in Figure 1, stool and urine TFF3 levels differed by hormone contraception use. As a result, correlations of stool and urine TFF3 with other measures are presented separately for those using and not using hormone contraceptives.

Among women younger than 40, stool TFF3 was lower in those on hormone contraception ($P = 0.051$), while urine TFF3 was markedly higher in those on hormone contraception ($P < 0.001$). There was no correlation between stool and urine TFF3 levels ($r = -0.15$, $P > 0.20$).

GI symptoms

Higher stool TFF3 was significantly associated with more diarrhea, looser stools and more stools, and with less constipation (Table 2; Figure 2). Abdominal pain, urgency, and intestinal gas were not related to stool TFF3 levels. Among women on hormone contraception, the percent of days with very loose stools was highly correlated with stool TFF3 levels. These relationships persisted after controlling for stool protein content. Urine TFF3 levels were not significantly correlated with any of the GI symptom measures, with the exception of a negative correlation with diarrhea and very loose stools in those women on hormone contraceptives (Table 2).

Intestinal permeability

Gut permeability, as measured by the urinary L/M ratio based on the overnight urine collection, was inversely and significantly related to stool TFF3 based on total IBS sample value (Table 3; Figure 3). When examined based on hormone contraceptive use, this relationship was present only in those on hormone contraceptives. Urine TFF3 levels were not significantly correlated with L/M ratio.

Stool microbial diversity and abundance

Stool TFF3 levels were inversely related to the Shannon Diversity Index and the number of OTUs (Table 3). When the twenty most abundant bacterial families were correlated with stool TFF3 levels, two (*Mogibacteriaceae* and *Christensenellaceae*) were found to be inversely related. However, after controlling for multiple comparisons, only *Christensenellaceae* remained significant (Table 3; Supplement Table 1; Figure 4). The

lowest level of *Christensenellaceae* was found in those with more than 70% days with loose stools ($P < 0.02$). The genera within the 20 most abundant families were also analyzed, but excluding those present in less than 10% of families. Sixty-six genera associated with the twenty families were identified, six of which significantly correlated with stool TFF3 levels (Supplement Table 1). However, when corrected for multiple comparisons, no relationships between stool TFF3 and genera remained significant.

There was no hormone contraception effect on the relationship of stool TFF3 to microbial diversity. In addition, there was no significant relationship between urine TFF3 and stool microbial diversity.

Discussion

In this cross-sectional study of female IBS patients, stool TFF3 levels were positively correlated with diarrhea (very loose stools) and stool frequency (Figure 2). These relationships were stronger in those women on hormone contraception. Intestinal permeability decreased (i.e., lower L/M ratio) with increasing stool TFF3 levels (Table 3). Stool TFF3 levels were negatively associated with stool microbial diversity and with the abundance of one bacterial family - *Christensenellaceae* (Table 3). In contrast, there were no significant relationships between stool or urine TFF3 levels and abdominal pain, gas, or urgency symptoms (Table 2). Stool and urine TFF3 levels did not correlate.

Previous studies of TFF3 have been performed in mouse models of gut injury or inflammation, epithelial cell lines, or serum and tissue biopsy samples from patients with inflammatory bowel disease (Chaiyarit *et al.*, 2012; Fu *et al.*, 2015; Gronbaek *et al.*, 2006; Podolsky *et al.*, 2009; Srivastava *et al.*, 2015; Verey *et al.*, 2011). To our knowledge this is the first report of an association of stool TFF3 with loose and frequent stools in women with IBS. The increase in stool TFF3 in those with high percent of days with diarrhea-like symptoms was not a reflection of an overall increase in stool protein content as there were no statistically significant differences in protein content across IBS bowel pattern subgroups.

To gain further insight into the relationship of stool TFF3 with IBS pathophysiology, we evaluated intestinal permeability and pain-related symptoms. Stool TFF3 and permeability were inversely related, suggesting that TFF3 may be part of a response that protects the epithelium and reduces the likelihood of increased permeability. Increased gut permeability has been reported to be present in some children and adults with IBS, both diarrheal and constipation subtypes, and has been associated with visceral hypersensitivity (Camilleri *et al.*, 2012). In the current study, we found no association of TFF3 with pain-related IBS symptoms, including bloating and abdominal pain.

In the current study, we attempted to address the link between TFF3 and gut bacteria by using 16S rRNA gene sequencing to examine both diversity and composition. Stool TFF3 level was inversely related to diversity. Of the twenty most abundant bacterial families identified, five were found to be inversely related to stool TFF3. Of these, only the relationship of *Christensenellaceae* (Figure 4) remained statistically significant after controlling for multiple comparisons. *Christensenellaceae* is a family of gram-negative

anaerobic bacteria. It is in the order of Clostridiales and the phylum Firmicutes (Morotomi *et al.*, 2012). *Christensenellaceae* have been linked to better health (i.e., lower BMI) (Biagi *et al.*, 2016; Kasai *et al.*, 2015). However, little is known about its abundance in persons with IBS. An unclassified *Christensenellaceae* was recently described as one of four taxa that was enriched in controls and individuals with IBS-constipation, relative to individuals with IBS-diarrhea and IBS-mixed (Pozuelo *et al.*, 2015). This finding is consistent with a study of healthy Japanese adults whose stool frequency was inversely related to a network of bacterial families, including *Christensenellaceae* (Kasai *et al.*, 2015). Network analyses and deeper sequencing through the use of shotgun metagenomics may substantiate the role of these families—and specific taxa within these families—in eliciting host responses, such as decreased expression of TFF3.

In our prior study using a mass spectrometric approach with pooled urine samples, we discovered overexpression of TFF3 in the urine of women with IBS relative to healthy controls. This difference was confirmed by analyzing the individual samples with ELISA, and also confirmed in an independent sample of 55 IBS and 13 control women. However, in that previous study, as in the current study, we found no differences across IBS bowel pattern subgroups. In the prior study, we did not control for hormone contraceptive use.

In the current study, urine TFF3 values were remarkably higher in women on hormone contraception. In this subset, there was a significant positive relationship in urine TFF3 with age (Figure 1). At the same time, we found a positive correlation of stool TFF3 with very loose stools, as well as number of stools in those on hormone contraception (Table 1). This suggests that both urine and stool TFF3 are influenced by ovarian hormone levels (May and Westley, 2015). The lack of correlation between urine and stool TFF3 levels may represent the effect of hormone contraception on other tissues with resulting symptoms that overlap with IBS (May and Westley, 2015). For example, women with endometriosis also report abdominal pain, constipation, bloating and intestinal gas (Ek *et al.*, 2015). Henze *et al* (Henze *et al.*, 2016) investigated women with endometriosis and found that peritoneal fluid TFF3 levels were higher in these women when compared to those without endometriosis. Of note, regardless of endometriosis status, the researchers observed menstrual cycle phase differences in serum TFF3 levels (Henze *et al.*, 2016). The difference in the relationship of stool TFF3 and intestinal permeability between those on and off hormone contraception (Table 3) reinforces the importance of considering reproductive status and hormone therapy when testing biomarkers in this predominantly female patient group.

The current study has limitations. There was no healthy, non-IBS control group. Stool and urine samples were collected as part of a baseline assessment of IBS patients enrolling in a randomized clinical trial of behavioral therapy. We excluded women who had used antibiotics for two months prior to study entry. The Human Microbiome Project uses an antibiotic-free period of 6 months prior to microbiome testing (Aagaard *et al.*, 2013). Not all participants were able to complete the permeability testing due to missed urine collection. The focus on women limits the generalizability of the findings beyond women with IBS. No attempt was made to perform the biomarker assessments at a particular menstrual cycle phase. Finally, the composition of the GI microbiota may be influenced by diet and

probiotics which were not controlled for in this study. However, it is unlikely that those subjects with loose stools had a different diet than those with constipation.

Strengths of the study include the use of prospective measures of daily symptoms and stool characteristics in combination with microbiota and permeability measures. In addition, the relationship of TFF3 levels in urine and stool with consideration of hormone contraceptive use is novel to our study.

In summary, we report the novel finding that stool TFF3 levels are positively related to IBS symptoms of diarrhea, and inversely related to gut permeability, microbial diversity, and an abundance of *Christenellaceae*, in women with IBS. Our results underscore the importance of hormone contraception in evaluating biomarkers in IBS. We conjecture that stool TFF3 levels may reflect a defensive mucosal response in persons with IBS-diarrhea given its association with decreased intestinal permeability and reduced microbial diversity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement.

Supported by a grant from the National Institute of Nursing Research, National Institutes of Health R21NR014331.

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Figure 1:
Stool (A) and urine (B) trefoil factor 3 (TFF3) by age and hormone contraceptive use.

Figure 2:
Relationship of stool trefoil factor 3 (TFF3) with diarrhea and constipation. Percent of days with moderate/severe diarrhea (A) and constipation (B) were derived from a 28-day symptom diary.

Figure 3:
Relationship of stool trefoil factor 3 (TFF3) with permeability. Permeability is a ratio of urine lactulose to mannitol collected overnight following ingestion of a testing solution.

Figure 4:
Relationship of stool trefoil factor 3 (TFF3) with *Christensenellaceae*. Relative abundance of stool *Christensenellaceae* (logit transform) as determined by 16S rRNA.

Table 1.

Trefoil Factor 3 (TFF3) Levels in Stool and Urine of Females with Irritable Bowel Syndrome (IBS)

	IBS	IBS-Diarrhea n = 22	IBS-Constipation n = 12	IBS-Mixed n = 53
Stool protein (mg/g stool)	3.7 (0.2)	3.7 (0.7)	3.9 (0.9)	3.6 (0.7)
Stool TFF3 Total (ng/mg stool)	5.3 (8.8)	7.3 (13.1)	2.7 (2.2)	5.1 (7.6)
Stool TFF3/protein (ng/mg protein)	31.3 (53.2)	42.2 (77.8)	14.3 (11.0)	31.4 (46.3)
Urine, TFF3/creatinine (ng/mg creatinine)	1.1 (1.6)	1.4 (2.2)	0.9 (1.1)	1.1 (1.5)
L/M permeability	0.06 (0.04)	0.05 (0.04)	0.05 (0.03)	0.06 (0.04)

Note. Mean (SD); Stool protein, n = 89; Stool TFF3 Total, n = 89; Stool TFF3/protein, n = 86; Urine TFF3/creatinine, n = 73, L/M = lactulose/mannitol permeability, n = 81.

Relationship of Stool and Urine TFF3 to Gastrointestinal Symptoms and Stool Characteristics in Females with Irritable Bowel Syndrome

Table 2.

Symptoms ^a					Stool Characteristics ^b				
	Abdominal Pain	Constipation	Diarrhea	Intestinal Gas	Urgency	% Very Loose	% Loose	% Hard	Number of Stools
Log of Stool TFF3									
Simple correlation	0.05	-0.24 *	0.22 *	0.18	0.13	0.32 **	0.19	-0.06	0.22 *
Partial correlation ^c	0.03	-0.26 *	0.20	0.16	0.14	0.29 **	0.18	-0.09	0.15
Simple correlation, among participants not using hormone contraceptive	0.13	-0.22	0.17	0.19	0.18	0.22	0.16	-0.04	0.07
Simple correlation, among hormone contraceptive users	-0.26	-0.35	0.31	0.08	0.03	0.55 **	0.25	-0.28	0.39 *
Urine TFF3									
Simple correlation	-0.06	0.01	-0.09	-0.01	-0.08	-0.13	0.01	-0.05	-0.22
Partial correlation ^c	0.01	-0.05	-0.20	-0.02	-0.22	-0.22	-0.10	-0.01	-0.10
Simple correlation, among participants not using hormone contraceptive	-0.04	-0.08	-0.06	0.03	0.05	-0.03	0.02	-0.06	0.07
Simple correlation, among hormone contraceptive users	0.02	-0.06	-0.40 *	-0.03	-0.39	-0.41 *	-0.20	0.01	-0.20

Notes. TFF3 = Trefoil factor3. No Hormone Contraceptive (n = 45); Hormone Contraceptive (n = 24); Stool TFF3, n = 85.

^aSymptoms are measured as the % of days reported as moderate to severe on a 28-day diary.

^bStool characteristics are measured as percent of days with very loose stools, loose stools, etc.

^cPartial correlation controlling for hormone contraceptive. Pearson R was used.

* p < 0.05

** p < 0.01

Table 3.

Relationship of Stool and Urine Trefoil Factor 3 (TFF3) to Intestinal Permeability Lactulose/Mannitol (L/M), Stool Shannon Diversity, Number of OTUs and *Christensenellaceae* in Females with Irritable Bowel Syndrome

	L/M ratio, night	Shannon Diversity ^b	Number of OTUs ^b	<i>Christensenellaceae</i> ^b
Log of Stool TFF3				
Simple correlation	-0.23 *	-0.28 *	-0.34 **	-0.37 **
Partial correlation ^a	-0.24 *	-0.29	-0.36 **	-0.40 **
Simple correlation, among participants not using hormone contraceptive	-0.09	-0.27	-0.39 **	-0.34 *
Simple correlation, among participants using hormone contraceptive	-0.63 **	-0.30	-0.28	-0.61 **
Urine TFF3				
Simple correlation	0.00	0.06	0.03	0.10
Partial correlation ^a	0.05	0.14	0.13	0.19
Simple correlation, among participants not using hormone contraceptive	0.18	0.05	-0.06	-0.12
Simple correlation, among participants using hormone contraceptive users	0.07	0.30	0.34	0.46 *

Notes. TFF3 = Trefoil Factor 3; No Hormone Contraceptive (n = 36 to 46). Hormone Contraceptive (n = 19 to 24). For stool TFF3, n = 72 – 89.

^aPartial correlation controlling for hormone contraceptive. Pearson R was used.

^bCorrelations with microbiome measures are partial correlations controlling for total read count.

* p < 0.05

** p < 0.01