Serum Proteomics in African American Female Patients With Irritable Bowel Syndrome

An Exploratory Study

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Background: Sex and subtype differences within patients with irritable bowel syndrome (IBS) complicate the understanding of disorder pathogenesis and hinder the design of efficacious, therapeutic interventions.

Objectives: The aims of this study were to harness the power of shotgun proteomic analysis, identify circulating proteins that differentiate African American female patients with IBS from healthy controls (HC), and gain biological insight on symptomatology.

Methods: Serum proteome analysis was performed upon a cohort of overweight, African American female participants with constipation predominant IBS symptoms ($n = 5$) and HC ($n = 5$), matched on age, sex, years of education, body mass index, and 11 physiological markers. Tandem mass tags for multiplexed proteomic analysis were performed, incorporating reverse-phase liquid chromatography and liquid chromatography-tandem mass spectrometry.

Results: Participants with IBS did not differ from HC in demographics, clinical characteristics, or initial proteomic analysis. Nested case control analysis of six samples (IBS: $n = 3$, HC: $n = 3$), hierarchically clustered into two main groups, with 12 out of 1,317 proteins significantly different in levels of expression: TGFβ1, PF4V1, PF4, APP, MMP9, PPBP, CTGF, SRGN, THBS1, WRN, LTBP1 (Isoform 3), and IGLV5-48. Top associations of identified proteins in DAVID and STRING resources (upregulated in HC vs. IBS) involve platelet alpha granule lumen, platelet activation/degranulation, extracellular region, and secretion by cell.

Discussion: Differentially expressed proteins between participants with IBS and HC involving platelet-related associations prompt inquiry as to differences in serotonergic signaling, inflammatory or immunomodulatory mechanisms underlying IBS symptomatology. Although preliminary and requiring validation in larger cohorts, these findings bear relevance to understanding pathogenic processes of IBS and biological effects of the disorder.

Key Words: IBS-C • irritable bowel syndrome • platelet-associated proteins • proteomics • TGFB1

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Irritable bowel syndrome (IBS) is a gastrointestinal (GI) condition characterized by abdominal pain and disordered bowel habits (Lacy et al., 2016) that predominantly affects the female sex and carries a pooled prevalence rate of 8.1% in North America, Europe, Australia, and New Zealand (Sperber et al., 2017). Although not entirely understood, pathogenesis of IBS is acknowledged as multifactorial in nature, with contributors such as genetics, inflammation, dysbiosis, and intestinal permeability playing a role (Enck et al., 2016). Heterogeneity of the population of patients with IBS complicates the elucidation of mechanisms underlying symptomatology, as differences exist within patients with IBS by sex (Videlock et al., 2016) and bowel habit subtype (Wouters et al., 2014). Furthermore, the influence of race on IBS symptomatology has not been fully explored nor explained, as investigations of racial differences among patients with IBS in the United States is lacking (Iorio, Makipour, Palit, & Friedenberg, 2014). The purpose of this investigation was to explore circulating proteins that may differentiate African American female participants with IBS from matched healthy controls (HC), utilizing a shotgun proteomic approach. Identification of peripheral proteins associated with

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IBS symptomatology may help build a testable hypothesis for future studies and bear relevance to therapeutic interventions.

Treatment approaches for IBS are primarily symptom driven and include medications for pain, bowel disturbances, depression/anxiety, as well as psychological therapies, diet, and exercise recommendations (Chey, Kurlander, & Eswaran, 2015). Medical treatments for IBS are currently viewed as insufficient, however, and without a clear etiology or biomarker for IBS, the illness burden for patients is quite high (Ballou, Bedell, & Keefer, 2015). Biomarker development is challenging due to the variety of patient phenotypes and diversity of etiological mechanisms that potentially contribute to IBS (Jones et al., 2014). Mass spectrometry proteomics may enable improved diagnostics through the accurate quantification of biologically relevant proteins (Cifani & Kentsis, 2017). In a typical mass spectrometry proteomic "shotgun" bottom-up workflow, proteins are digested into peptides, separated, and measured and conclude with a database search (Li, Wang, & Chen, 2017). Therefore, proteomic analysis may offer insight as to physiological mechanisms that differentiate patients with IBS from HC and prove relevant to biomarker development and clinical interventions.

Proteome analysis has not been extensively utilized in IBS research, although representative studies have been performed in both animal models and clinical populations. Differential expression of proteins with roles in nerve regulation, inflammation, and intestinal tract immunity has been reported in the colonic tissue in a rat model of IBS (Ding et al., 2010). In female patients with IBS, a urinary proteomic investigation showed the protein gelsolin to be upregulated in pooled samples of patients with IBS compared with HC (Voss et al., 2011). A follow-up study identified proteins with roles including intestinal function homeostasis and inflammation, to be overexpressed in most IBS subgroups in comparison to HC, and detected proteomic differences among IBS subtypes (Goo et al., 2012). Given the fact that specific protein targets as identified by prior research have yet to become reliable biomarkers for diagnosing IBS, a discovery-driven research investigation was undertaken. Therefore, the purpose of this study was to conduct an exploratory serum proteome analysis of patients with IBS with well-matched HC, identify differential protein expression, and exert further efforts to understand physiological underpinnings of IBS.

METHODS

Recruitment

Participants of this investigation were part of a larger study evaluating chronic abdominal pain at the molecular level at the National Institutes of Health in Bethesda, Maryland (ClinicalTrials.gov NCT00824941; IRB Protocol 09-NR-0064). Participants were 13–45 years of age, male and female, both with and without chronic abdominal pain and of overweight and normal weight. Inclusion criteria required participants with abdominal pain to have experienced symptoms for more than 6 months prior to participation, and all female participants were required to have regular monthly menstrual cycles, with onset of menses at least 2 years prior to participation. Exclusion criteria included any evidence of organic GI disease, prior GI surgery, renal, cardiac, endocrine, neurologic, pulmonary or gynecological pathology, or psychiatric or comorbid pain condition. In addition, participants were excluded if taking medications daily for GI symptoms and medications that acted on catecholaminergic, serotonergic, or cortisol systems. Last, exclusion criteria included the consumption of more than 300 mg of caffeine in the evening or afternoon, more than two alcoholic beverages a day, or work during the night or late evening.

If deemed eligible after telephone screening, participants were instructed to fast overnight and present between 8 am and 11 am to the Clinical Research Center. After signed, written consent, participants' weight and height were measured in duplicate by trained personnel, and body mass index (BMI; kg/m^2) was calculated. A history and physical examination was performed with IBS diagnosis and subtype clinically determined per Rome Criteria. For this exploratory study, a cohort of participants with IBS and HC was selected from the overall study sample and included all female, overweight, African American participants with constipation predominant IBS (IBS-C; $n = 5$) or HC ($n = 5$), matched by age, years of education, BMI, and 11 physiological laboratory markers (see Table 1). Descriptive statistics and Mann–Whitney U tests were performed to assess for group differences on demographic and clinical characteristics, with level of significance set a priori as $<.05$. Statistics were run on SPSS Version 24 (IBM Corp. Released 2015; IBM SPSS Statistics for Windows, Version 24.0, IBM Corp., Armonk, NY). For proteome analysis, serum samples (previously aliquoted and frozen to −80°C) were brought to the National Heart, Lung, and Blood Institute Division of Intramural Research Proteomics Core.

Proteome Analysis

Tandem mass tags for multiplexed proteomic analysis were performed, with workflow as subsequently described. Briefly, approximately 150 μg of protein from each sample was broken down, with the cell lysate digested with trypsin. A coarse protein quantification check was performed, then lysates were immediately reduced with dithiothreitol and alkylated with iodoacetamide. Digestion and C18 desalting occurred, followed by tandem mass tag labeling of each sample (approximately 120 μg of peptides). A ratio check was performed to verify the major proteins and peptides were not changing between HC and IBS samples. The ratio check was confirmed, samples were combined, and then reverse-phase liquid chromatography separated the sample into 96 fractions. The sample was then concentrated into 24 final fractions for liquid chromatography-tandem mass spectrometry analysis performed on an Orbitrap

TABLE 1. Participant Characteristics

	Total (n = 10)		HC (n = 5)		IBS-C (n = 5)			Total (n = 6)		HC (n = 3)		IBS-C (n = 3)		
Variable	***M***	**(*SD*)**	***M***	**(*SD*)**	***M***	**(*SD*)**	***p***	***M***	**(*SD*)**	***M***	**(*SD*)**	***M***	**(*SD*)**	***p***
Age (years)	30.6	(7.1)	29.4	(9.3)	31.8	(4.9)	.42	31.3	(6. 9)	33.33	(9.7)	29.3	(3.5)	1.0
Formal education (years)[a]	14.6	(3.5)	14.4	(2.6)	14.8	(4.9)	.91	15.2	(4.2)	14.0	(2.0)	17.0	(7.1)	.80
BMI (kg/m^2)	30.5	(4.5)	31.1	(4.2)	29.9	(5.3)	.69	28.9	(4.9)	31.5	(5.6)	26.3	(2.8)	.20
ACTH (pg/ml)	17.2	(8.0)	15.2	(8.2)	19.1	(8.3)	.69	19.3	(8.6)	15.9	(9.8)	22.6	(7.5)	.70
ALT (U/L)	25.4	(11.3)	22.6	(8.4)	28.2	(14.1)	.42	28.3	(13.4)	27.3	(7.6)	29.3	(19.6)	.70
AlkPhos (U/L)	67.7	(14.0)	60.6	(7.1)	74.8	(16.3)	.22	64.5	(8.4)	64.3	(5.7)	64.7	(12.0)	1.0
AST (U/L)	18.0	(16.5)	15.6	(8.8)	20.4	(22.9)	1.0	23.0	(20.2)	20.3	(8.1)	25.7	(30.7)	.70
Cholesterol (mg/dl)	163.0	(23.9)	157.6	(26.6)	168.4	(22.6)	.42	155.2	(21.9)	156.0	(32.9)	154.3	(10.5)	.70
Cortisol (mcg/dl)	9.6	(3.3)	11.1	(3.3)	8.1	(2.9)	.22	10.1	(4.0)	11.4	(4.6)	8.9	(3.6)	.70
CRP (mg/L)	4.8	(5.0)	6.2	(6.6)	3.4	(2.9)	.55	5.3	(6.4)	8.0	(8.4)	2.6	(3.3)	.40
ESR (mm/h)	15.1	(6.5)	15.6	(8.5)	14.6	(4.7)	1.0	13.3	(4.1)	13.0	(3.0)	13.7	(5.7)	1.0
HCT (%)	37.2	(4.5)	37.2	(5.5)	37.2	(3.9)	1.0	37.2	(3.9)	38.5	(2.9)	35.8	(4.8)	1.0
HgbA1C (%)	5.5	(.36)	5.5	(.29)	5.4	(.45)	.84	5.3	(.35)	5.5	(.40)	5.1	(.20)	.40
Platelets (K/µl)	282.5	(61.6)	284.4	(82.3)	280.6	(42.1)	.55	272.2	(56.2)	275.7	(69.9)	268.7	(54.6)	.70

Note. ACTH = adrenocorticotropin hormone; ALT = alanine aminotransferase; Alk Phos = alkaline phosphatase; AST = aspartate aminotransferase; BMI = body mass index; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; HC = healthy controls; HCT = hematocrit; HgbA1C = hemoglobin A1C (glycated hemoglobin); IBS-C = constipation predominant irritable bowel syndrome; *M* = mean; *SD* = standard deviation. [a]Years of formal education: n = 9.

Fusion Tribrid Mass Spectrometer (ThermoFisher Scientific). Peptides were separated using a gradient of 2%–28% Solvent B for 120 minutes and 28%–70% Solvent B for 40 minutes (Solvent A: 0.1% formic acid, Solvent B: 100% acetonitrile in 0.1% formic acid). Data analysis for peptide identification and quantification was performed with Proteome Discoverer Software 2.2 (ThermoFisher Scientific). Normalized $\log_2$ intensity of proteins was used to evaluate correlations among replicates. *t* Tests were performed between groups to evaluate for protein differences, with false discovery rate set at 0.05, fold cutoff of 2, and level of significance value set a priori as <.05.

RESULTS

Participants with IBS-C were adequately matched with HC, as no differences were noted in age, years of education, BMI, or 11 laboratory markers: ACTH, AST, ALT, AlkPhos, cholesterol, cortisol, CRP, ESR, HCT, HgbA1C, and platelets (see Table 1). For proteomic data, proteins with a reporter intensity value of less than 1,000 were removed, yielding a total of 1,317 proteins for analysis. The normalized $\log_2$ intensity of proteins hierarchically formed two main groups from unsupervised clustering analysis, HC and IBS, although variation of intensity between samples was noted. *t* Tests comparing groups did not reveal significant differences in protein composition. Nested case control analysis was completed to exclude two samples (one from IBS and one from HC) that contained a lipid-type layer within the sample and two additional samples (one from IBS and one from HC) that deviated from others within their group on heat map visualization. In unsupervised clustering analysis, the six samples hierarchically formed two main groups, HC and IBS, as noted on heat map illustration (see Figure 1). Heat maps can be used to visualize proteomic data with columns corresponding to the samples, rows to the proteins, columns/rows with similar profiles displayed closer to one another, and data displayed as a color to view patterns graphically (Key, 2012). *t* Tests indicated there was differential expression of 12 proteins between HC and IBS groups, with 11 upregulated in HC and 1 upregulated in IBS, respectively: TGFβ1, PF4V1, PF4, APP, MMP9, PPBP, CTGF, SRGN, THBS1, WRN, LTBP1 (Isoform 3), and IGLV5-48 (see Table 2).

Differentially expressed proteins were uploaded into DAVID Bioinformatics Resources 6.8 (National Institute of Allergy and Infectious Diseases, National Institutes of Health) to identify annotation clusters (Huang da, Shermin, & Lempicki, 2009b) and to help extract biological meaning of the associated gene list (Huang da, Shermin, & Lempicki, 2009a). The protein list was also uploaded into the Search Tool for the Retrieval of Interacting Genes/Proteins or STRING v10.0 to explore functional associations between proteins likely to influence a mutual biological purpose (Szklarczyk et al., 2015). Of the 11 genes recognized for analysis in DAVID, Annotation Cluster 1, with an Enrichment Score of 8.5 (default setting of medium stringency), included the following: platelet alpha granule lumen (six genes), platelet degranulation (six genes), and extracellular region (eight genes). Functional annotation clustering in DAVID groups functionally similar terms associated with the submitted gene list, with the enrichment or importance score, ranking the groups (score of 1.3 equal to 0.05, non-log scale; Huang da et al., 2009b). Similarly, in STRING, the functional protein association network (composed of 10 proteins) included the following biological processes: platelet degranulation (six counts in gene set), platelet activation (six), and secretion by cell (seven). In STRING, a visual network is

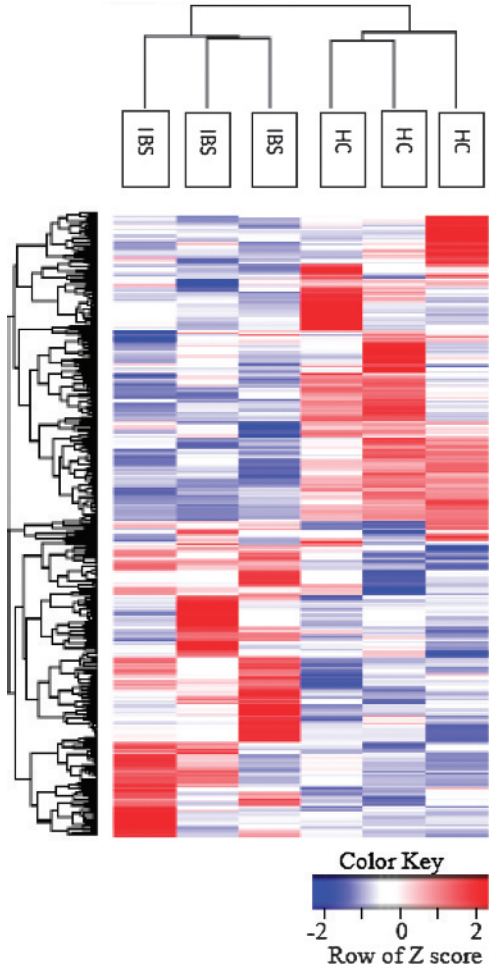

FIGURE 1. Heat map illustration. Columns correspond to samples; rows correspond to proteins.

composed of proteins depicted as nodes and protein–protein predicted associations by edges or lines. In this analysis, only known interactions were used for network construction, with pink lines representing experimentally determined interactions and blue lines representing interactions from curated databases (see Figure 2). Proteins IGLV5-48 and LTBP1 (Isoform 3) did not contribute to these analyses, either because of their nonfunctional nature or they are not recognizable.

DISCUSSION

The purpose of this study was to harness the power of shotgun proteomic analysis to detect biological underpinnings of IBS symptomatology. As sex and subtype differences within patients with IBS complicate our understanding of disorder pathogenesis, this study evaluated a cohort of IBS-C, overweight female participants, matched with HC by age, race, and clinical profile. Limitations of this study include its small sample size; but as this analysis was exploratory in nature, findings naturally require validation in larger cohorts. A strength of this investigation is the inclusion of phenotypically similar participants, as such efforts increase the likelihood of detecting pertinent findings for patients' sex and IBS subtype. Second, by analyzing protein expression within a racial/ethnic minority group, this study will foster understanding as to how the disorder affects African Americans.

Of the 1,317 proteins quantified in this analysis, 12 were found to significantly differ between participants with IBS and HC and were uploaded for functional analysis. Primary top networks upregulated in HC versus participants with IBS from 10 proteins, as previously reviewed, involve platelet alpha granule lumen, platelet activation/degranulation, extracellular region, and secretion by cell. The predominance of these

TABLE 2. Differentially Expressed Proteins Identified Between HC and Patients With IBS

UniProt no.	Protein name	Gene	$-\log_{10}$ (*p* value)	$\log_2$ (HC/IBS)	HC/IBS ratio
Q14191	Werner syndrome ATP-dependent helicase	*WRN*	3.26	2.49	+5.63
P02776	Platelet factor 4	*P4*	3.40	1.76	+3.39
P07996	Thrombospondin-1	*THBS1*	2.76	1.59	+3.00
P10720	Platelet factor 4 variant	*PF4V1*	2.90	1.49	+2.80
P05067	Amyloid beta A4 protein	*APP*	3.12	1.47	+2.76
P02775	Platelet basic protein	*PPBP*	3.34	1.42	+2.68
P10124	Serglycin	*SRGN*	3.02	1.40	+2.64
P14780	Matrix metalloproteinase-9	*MMP9*	2.84	1.40	+2.63
P29279	Connective tissue growth factor	*CTGF*	3.74	1.34	+2.53
P01137	Transforming growth factor beta-1	*TGFB1*	3.46	1.27	+2.41
Q14766-3	Isoform 3 of latent-transforming growth factor beta-binding protein 1	*LTBP1 (Isoform 3)*	4.13	1.11	+2.16
A0A075B6I7	Immunoglobulin lambda variable 5–48 (nonfunctional)	*IGLV5-48*	2.82	–1.45	–2.70

Note. HC = healthy controls; IBS = irritable bowel syndrome.

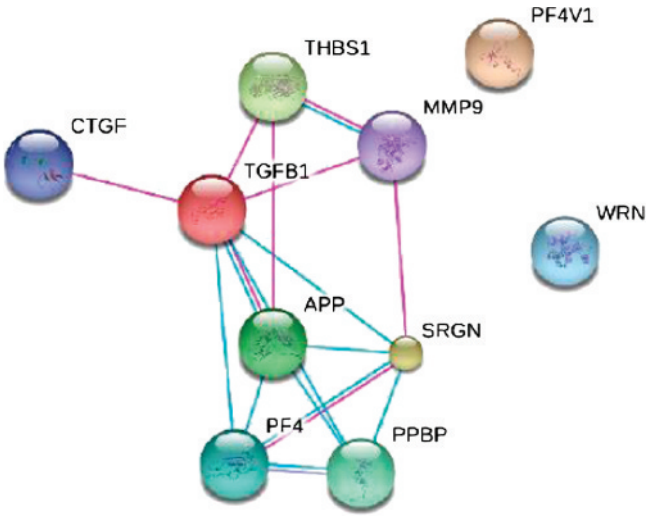

FIGURE 2. STRING network. Proteins with known interactions. Pink lines represent experimentally determined interactions; blue lines represent interactions from curated databases.

proteins involving platelet-related associations prompts inquiry as to serotonergic signaling and IBS symptomatology. It is well understood that platelets take up serotonin, store it in granules, and release it upon activation (Offermanns, 2006); therefore, differential expression of platelet-related proteins may have implications upon serotonin signaling. In peripheral blood cells, platelets have been found as the major contributors to serotonin reuptake transporter (SERT) function (Beikmann, Tomlinson, Rosenthal, & Andrews, 2013), with serotonin (5-HT) signaling and SERT potentially involved in the pathogenesis of IBS (Jin et al., 2016). Differential 5-HT signaling in patients with IBS has been found by investigations, as well as differences in 5-HT signaling by IBS subtype: Patients with IBS-C were suggested to have reduced enterochromaffin cell release of 5-HT through the evaluation of 5-HT and its metabolite 5-hydroxyindoleacetic acid (Atkinson, Lockhart, Whorwell, Keevil, & Houghton, 2006). Recent investigations have reported similar findings of decreased 5-hydroxyindoleacetic acid levels in patients with IBS-C compared with HC, possibly relating to a diminished uptake of 5-HT by SERT or a decrease in 5-HT metabolism (Thijssen et al., 2016). Therefore, a downregulation of platelet-associated proteins in patients with IBS, who do not significantly differ from HC in overall platelet count, may play a role in altered release/metabolism of 5-HT and bear relevance to IBS-C symptomatology.

In addition to its significance in the gut, 5-HT from peripheral blood platelets has been used as a proxy for neuronal 5-HT, with platelet 5-HT concentrations noted as diminished, for instance, in patients with schizophrenia with depressive symptoms in comparison to those without (Peitl et al., 2016). Moreover, bioprofiling of platelets has been performed in patients with major depressive disorder, noting a downregulation of serotonin, platelet factor 4 (PF4), and interleukin-1β in comparison with HC, with these bioactive compounds mediating the immunomodulatory and inflammatory mechanisms of platelets (Hüfner et al., 2015). Platelet activation leads to the release of inflammatory mediators such as chemokines (e.g., PF4; Bhatnagar et al., 2012) and the release of growth factors such as TGFβ1 (Meyer et al., 2012) and CTGF (Cicha, Garlichs, Daniel, & Goppelt-Struebe, 2004). These three proteins, found to be downregulated in our cohort of participants with IBS compared with HC, suggest an inflammatory or immune component to underlie group differences that may prove relevant to IBS-C symptomatology. Furthermore, SRGN, possibly involved in regulating the inflammatory response and experimentally demonstrated to bind PF4 (Kolset, Mann, Uhlin-Hansen, Winberg, & Ruoslahti, 1996), was noted along with PF4 to be differentially expressed in our IBS cohort.

Inflammatory and cytokine profiles have been extensively investigated in the population of patients with IBS, and although not fully understood, research suggests a disturbance in the balance of pro-/anti-inflammatory cytokines in patients with IBS (Bashashati et al., 2012). In addition to immune markers in the peripheral circulation and in the intestinal mucosa, the microbiota may play a role in the immune response of patients with IBS (Martin-Viñas & Quigley, 2016). Interestingly, a study where rats were inoculated with human fecal samples showed the microbiota of patients with IBS-C to exhibit anti-inflammatory properties, in that it protected animals from dextran sulfate sodium salt-induced colitis to a greater extent than in comparison to rats inoculated with the microbiota of healthy subjects (Gobert et al., 2016). Therefore, the underexpression of such proteins/chemokines as PF4, PF4V1, and PPBP in participants with IBS-C compared with HC may relate to the intersection of inflammatory, immune, and microbiota processes along the gut–brain axis and bear relevance upon symptom manifestation in IBS.

In conclusion, this exploratory study incorporated serum proteomic analysis to facilitate biological insight in African American, overweight, female participants with IBS-C in comparison with HC. As recently noted, there are limited IBS investigations that incorporate serum/plasma proteome analysis to identify protein biomarkers for IBS (Tsigaridas et al., 2017). Our preliminary results suggest this cohort of participants with IBS have a downregulation of proteins related to platelet-associated processes, which may have bearing upon inflammatory and immune signaling and possible links to 5-HT. As previously acknowledged, these preliminary results will require validation in a larger cohort as well as evaluation in patients with IBS of different races, sex, and subtypes to determine sensitivity and specificity of findings. Nevertheless, these results are encouraging in their ability to differentiate participants with IBS from HC, may prove relevant to understanding biological processes of the disorder, offer insight for biomarker development and therapeutic applications, and illustrate the potential for proteome analysis to advance nursing research.

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The authors declare no conflicts of interest.

Ethical Conduct of Research: This investigation was performed as a substudy of a larger investigation, which has received institutional review board approval from the National Institutes of Health (Protocol 09-NR-0064).

Clinical Trial Registration: This study was a substudy of a larger investigation, which registered on ClinicalTrial.gov (NCT00824941). The first participant was enrolled in February, 2009.

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