Mass spectrometry-based metabolomics for irritable bowel syndrome biomarkers

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Abstract

Background: Irritable bowel syndrome (IBS) is a common gastrointestinal disorder without obvious structural abnormalities or consistent associated biomarkers, making its diagnosis difficult. In the present study, we used a urine-based metabolomics approach to identify IBS biomarkers.

Methods: We used an ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) on urine samples from patients suffering from IBS and healthy controls. Data were coupled for multivariate statistical analysis methods.

Results: We selected 30 differential metabolites associated with IBS and found steroid hormone biosynthesis and histidine metabolism alterations in patients with IBS that may be involved in the pathogenesis of the disease. In addition, we identified a panel of five metabolite markers composed of cortisone, citric acid, tiglylcarnitine, N6,-N6,-N6-trimethyl-L-lysine and L-histidine that could be used to discriminate between patients and healthy controls and may be appropriate as IBS diagnosis biomarkers.

Conclusion: Our findings indicate that metabolomics combined with pattern recognition can be useful to identify disease diagnostic IBS markers.

Clinical trial registration: ChiCTR1800020072

Keywords: biomarkers, irritable bowel syndrome, metabolomics

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Introduction

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by chronic or recurrent abdominal pain, abdominal bloating and discomfort in association with bowel habit changes.[1,2] Studies suggest that altered gastrointestinal motility, visceral hyperalgesia, increased intestinal permeability, chronic low-grade mucosal inflammation, immune activation, altered microbiota, and disturbances in brain–gut function[3,4] may be involved in the development of IBS, but the exact pathogenesis remains unclear. The lack of obvious structural abnormality or reliable biomarkers associated with IBS[5] means the diagnosis is one of exclusion based on symptoms (the Rome criteria and the Manning criteria).[6]

The prevalence of IBS varies by region and diagnostic criteria,[7] but the global prevalence of IBS reported by a systematic review was 11.2% [95% confidence interval (CI) 9.8–12.8%].[5] In China, studies applying the Rome criteria have reported a prevalence ranging from 5 to 10%.[8] Among patients with IBS, 13–88% seek care.[9] Patients experiencing IBS symptoms for more than 6 months can be diagnosed with IBS. These symptoms can then be classified into four subtypes using the Bristol stool form scale (BSFS) of bowel movement, such as IBS constipation type (IBS-C), diarrhea type (IBS-D), mixed type (IBS-M), and non-classifiable type (IBS-U).[10–12] In Japan, a study examining 30,000 adults 20–78 years of age found a prevalence rate of IBS of 16.5% (15.5%

male and 17.4% female). Among those with IBS, IBS-M was most frequent at about 8.3%, while for IBS-D 4.5% and IBS-C 2.8% of people who met the subtype criteria.[4,13] IBS treatment is based mainly on symptomatic therapy aimed at alleviating pain and discomfort and does not address the poorly understood pathology.[14]

Recently, small molecular products found downstream of biomolecular processes close to the biological phenotypes[15] have been identified. As we know, the human gut contains trillions of microorganisms, including bacteria, fungi, viruses, eukaryotes, and archaea, which are involved in maintaining gastrointestinal homeostasis.[16,17] Previous work has confirmed more than 2000 bacterial species that reside in the gut from main phyla: Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria.[18–20] Recently, several omics-based classifications of IBS using biomarker identification-based technologies, including Trefoil factor 3 (TFF-3), dimethyl sulfide, benzene, gelsolin (GSN), histamine, volatile organic metabolites (VOMs), and short-chain fatty acid cyclohexanecarboxylic acid in humans using 2-DE and HPLC-MS/MS, 2-DE and MALDI-TOF/MS, and GC-MS have been completed.[21] Most studies focus on proteome. A study using urine samples demonstrated that clinical subtypes of IBS, which are specifically altered in the urinary proteome, assessed through mass spectrometry (MS) and enzyme-linked immunosorbent assay (ELISA) were quantitatively detected. This work highlights the importance of research investigating the proteome in diseased conditions.[22] Recent work using a proteomic approach to examine the differentially expressed proteins in IBS patients compared to healthy human serum samples ($n = 10$/group) from each IBS classification (IBS-D, IBS-C and IBS-M) showed eight significantly expressed proteins that may contribute to a better understanding of IBS cases.[23] Interestingly, the light chain immunoglobulin isotypes such as kappa (IGKC) and lambda (LAC3) have been reported in many inflammatory and autoimmune diseases, including asthma, multiple sclerosis and rheumatoid arthritis; however, their contribution to IBS pathogenesis is unknown.[24–26]

Indeed, metabolomics are powerful and sensitive tools to describe perturbations in metabolic networks and have been used to identify novel biomarkers for a variety of functional and organic diseases.[27] Compared with radiological and endoscopic examinations for the clinical diagnosis of IBS, urinary metabolomics provide a noninvasive approach to objectively measure biochemical changes caused by IBS. In this study, we tested a nontargeted metabolomics approach based on the ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) technology combined with multivariate statistical analysis. We profiled 60 patients with IBS and 63 healthy individuals to identify potential metabolite biomarkers for IBS diagnosis and to help elucidate its mechanisms.

Materials and methods

Patients

Ethics approval was given by the Shanghai Changhai hospital Ethics Committee of the Naval Medical University (ID: SCHEC2013-183). We obtained signed and informed consent from each participant, and this study was conducted according to the Declaration of Helsinki principles.

Based on the Rome III diagnosis criteria, we recruited 60 patients with IBS and diarrhea and 63 gender- and age-matched healthy volunteers from Changhai Hospital of Naval Medical University in Shanghai, China. The volunteers in this study were selected from the physical examination centers of the hospital and corresponded to healthy male and female volunteers of the same age group ($p > 0.05$). These patients were not selected in a double-blind manner. Data were collected from January 1, 2014 to December 31, 2015; patients were between 18 and 60 years old. Exclusion criteria: (1) age less than 18 years old; (2) refused to participate in the study. There were initially 63 individuals in each of the control group (25 male and 38 female volunteers) and inpatient group (25 male and 38 female volunteers). However, three individuals in the patient group refused to participate in the study. Therefore, there were 60 individuals in the patient group (24 male and 36 female volunteers). Neither the IBS patients with diarrhea or the healthy volunteers had taken any medications or supplements before the urine sample collections.

Chemicals and reagents

We purchased LC/MS grade (1) methanol, (2) acetonitrile, and (3) water from Thermo Fisher

(Geel, Belgium). LC/MS grade (1) formic acid and spectroscopic grade (2) leucine enkephalin were purchased from FLUKA (Sigma-Aldrich Company, St. Louis, MO, USA). All the chemicals used are listed here: (1) N6,N6,N6-trimethyl-L-lysine, (2) aspartylglycosamine, (3) pipecolic acid, (4) L-histidine, (5) N1-methyl-4-pyridone-3-carboxamide, (6) creatinine, (7) citric acid, (8) ciliatine, (9) aminoadipic acid, (10) glutarylcarnitine, (11) tiglylcarnitine C, (12) dodecanoic acid, (13) dodecanedioic acid, (14) androstenedione, (15) 17-hydroxyprogesterone, (16) cortisone, (17) myristic acid, (18) dehydroepiandrosterone 3-glucuronide, decanoylcarnitine, (19) palmitic acid, (20) 9,10-DiHODE, (21) 5a-androst-3-en-17-one, (22) TXB2, (23) gamma-2-solamarine, (24) myristoleic acid, (25) 2,6,10-trimethylundecanoic acid, (26) monoethylhexyl phthalic acid, (27) sorbitan stearate, and (28) chenodeoxyglycocholic acid. Standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample collection and preparation

In the morning, we drew 2 ml urine samples from each overnight-fasting individual. We centrifuged the samples using a Thermo Heraeus Fresco 17 machine (Thermo Fisher Scientific, Waltham, MA, USA) at 12,000 rpm for 10 min at 4°C immediately to remove any solid debris. We then aliquoted the supernatants into 100 µl samples and stored them at –80°C. Before analysis, we thawed each sample at 4°C for 30 min. We added 300 µl of methanol to each tube and mixed well for 30 s on a vortex. We then centrifuged each sample for 15 min (14,000 rpm, 4°C) and transferred the supernatant to autosampler vials and stored them at –80°C for LC-MS analysis. We then pooled 20 µl samples to generate a pooled quality control (QC) sample and extracted 100 µl aliquots from this pooled sample by the same method.

UPLC-Q-TOF-MS assay

Chromatographic conditions. We performed the metabolomics analysis on an ACQUITY™ UPLC system (Waters, Milford, MA, USA) coupled to a Synapt G2-Si Q-TOF MS system (Waters) equipped with an electrospray ionization source. We used a Waters ACQUITY™ UPLC BEH C18 a 1.7 µm, 2.1 × 100 mm ACQUITY UPLC® BEH C18 column with the temperature maintained at 35°C. The mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The injection volume was 3 µl. The gradient duration program included 0–0.5 min, 5% B; 0.5–6 min, 5–80% B; 6–8 min, 80–100% B; 8–12 min 100% B; and 12.1–16 min 95% B (post-time). The flow rate was 400 µl/min.

MS conditions. The mass detection was operated in a positive mode with parameters set as follows: capillary voltage, 3 kV; cone gas flow, 50 L/h; desolvation gas, 500 L/h; sampling cone 50; source temperature, 120°C; desolvation temperature, 550°C; nebulizer pressure, 6.5 bar; and lock spray capillary voltage, 2.0 kV. A full scan mass range of 50–1200 m/z was acquired. Leucine enkephalin was used as the lock mass (LE, m/z 556.2771). We used the MSe mode of Q-TOF MS for rapid and comprehensive analysis of metabolites with a collision energy ranging from 10 to 40V. The raw LC-MS data were preprocessed using the Progenesis QI Version 2.0 software (Nonlinear Dynamics, Newcastle, UK).

Sequence analysis

The pooled QC samples were analyzed at the beginning, at the end and randomly throughout the analytical run to monitor the stability of the sequence analysis. The typical batch sequence of urine samples consisted of the consecutive analysis of one QC sample (at the beginning of the study) followed by six unknown urine samples, then one QC urine sample before running another six unknown urine samples, and so on. The samples were analyzed in a random order for normal good practice. We repeated an identical sequence to complete the total set of injections ($n = 6$, including QCs) analyzed in less than 1 day per mode as described in previous studies.

Data processing

The peak deconvolution, identification and autointegrator were carried out using the Progenesis QI (Waters Technologies, UK). The resulting three-dimensional matrix involving peak index (RT–m/z pair), sample names (observations) and ion intensity were introduced into the EZinfo 3.0 software (Umetrics, Umeå, Sweden) for chemometric analysis. The unsupervised principal components analysis (PCA) and supervised orthogonal partial least square-discriminant analysis (OPLS-DA) were both applied to show the separation between

diarrhea IBS profiles and healthy controls. The significant ions were extracted from the variable importance in the projection (VIP) matrix in OPLS-DA based on their contribution to the classification in the dataset. These ions were further filtered using nonparametric tests of t test and fold change calculation. Ions satisfying the criteria of three filters [VIP >1.5, p values <0.05, fold changes >1.2 (or <0.83)] were regarded as significant. The selected ions were finally identified and interpreted as follows: we searched for their accurate masses in the metabolite databases of METLIN (www.metlin.scripps.edu), HMDB (www.hmdb.ca) and KEGG (www.genome.jp/kegg). Then, we confirmed the isotopic distribution, retention time and fragments of commercial standards with those metabolites of interest. p values < 0.05 were considered as statistically significant.

Biomarkers identification and pathways analysis

The receiver operating characteristic (ROC) curve is an important statistical technique created by plotting the sensitivity against the false-positive rate at various threshold settings. This technique depends on cumulative distribution functions under the probability distribution from discrimination threshold. The area under the ROC curve (AUC) is equal to the probability that reflects the overall diagnostic accuracy of a certain index in the diagnosis of diseases. We performed multivariate ROC curve exploration and metabolic pathway analysis using the MetaboAnalyst (http://www.metaboanalyst.ca) software to help identify multiple biomarkers and to reveal metabolic disturbances.

Results and discussion

Diarrhea-predominant IBS (IBS-D) is a major subtype of IBS, and the pathogenesis of IBS-D remained unknown until recently. A single factor cannot fully explain the pathogenesis and symptoms of IBS-D. These factors may have complex interactions and their positions may vary in the pathogenesis of the disorder in the individual IBS-D patient, resulting in heterogeneity of IBS-D.[28] Several causes, including diarrhea,[29,30] for IBS have been reported. The complexity of environmental, epigenetic or genetic factors play an important role in the pathogenesis of IBS. As such, reports on current techniques used are required to inform future epigenetic research to address the approaches to the complex disorder for future health benefits.[31] Therefore, we recruited healthy persons as the control group in the present study.

Analysis of metabolic pattern

We demonstrated the LC-MS system stability for large-scale sample analysis by testing the pooled QC samples. The PCA score plot shows that the QC samples are tightly clustered (Figure 1), indicating that the large-scale sample analysis had hardly any effects on the reliability of the data. We evaluated the urine metabolic profiles between normal controls and patients with IBS using an unsupervised PCA model. As shown in Figure 1, the PCA score plot with the first two principal components showed clear separation trends between the samples from healthy individuals and those from patients with IBS, suggesting that IBS results in alteration of the normal metabolism.

Identification and selection of different metabolites

Metabolite profiling demonstrated a clear difference between the urine of controls and individuals with IBS. To further investigate the potential biomarkers that account for IBS, we performed supervised OPLS-DA. As indicated in Figure 2(a), the patients with IBS were separated completely from the healthy controls in the score plot of the OPLS-DA model. The corresponding S-plot (Figure 2(b)) shows the distribution of potential biomarkers. A total of 758 out of 12,280 ions were different between patients with IBS and healthy controls. We identified 30 different metabolites by searching the Biofluid Metabolites Database (http://metlin.scripps.edu) and Human Metabolome Database (http://www.hmdb.ca) listed in Table 1. Among these, 20 metabolites had high levels in the IBS group (Figure 2(c)).

Metabolic pathway and function analysis

Changes occurring at critical positions within a biologic pathway or network are thought to always have a more severe impact than changes at marginal or relatively isolated positions. In order to identify biologically meaningful patterns that are significantly enriched in metabolomic data, we

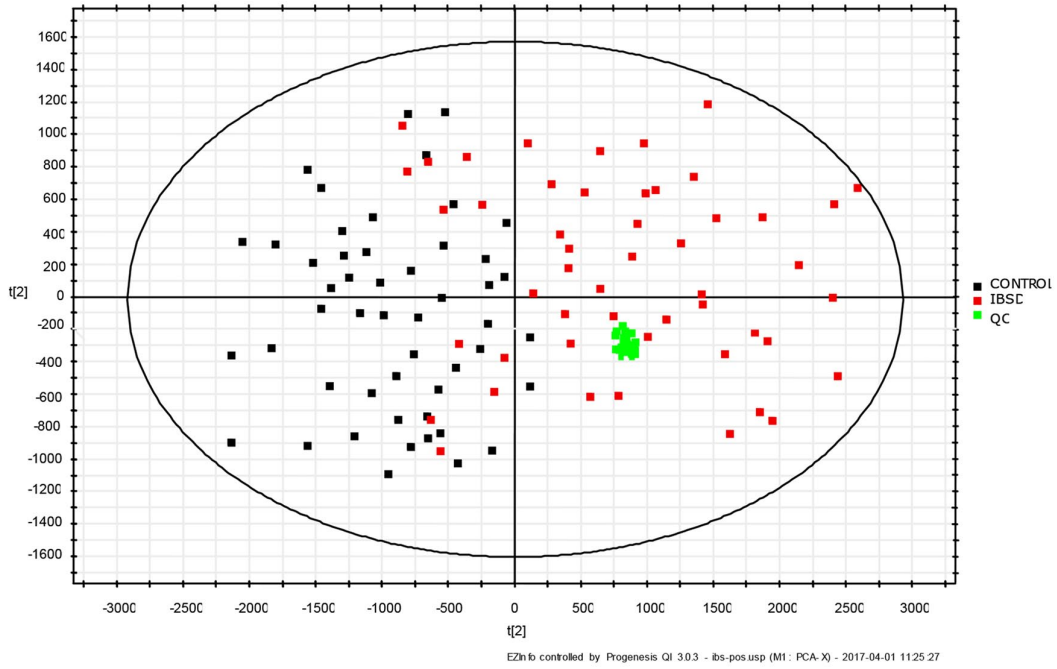

Figure 1. Principal component analysis score plot for samples from healthy controls and patients with irritable bowel syndrome.

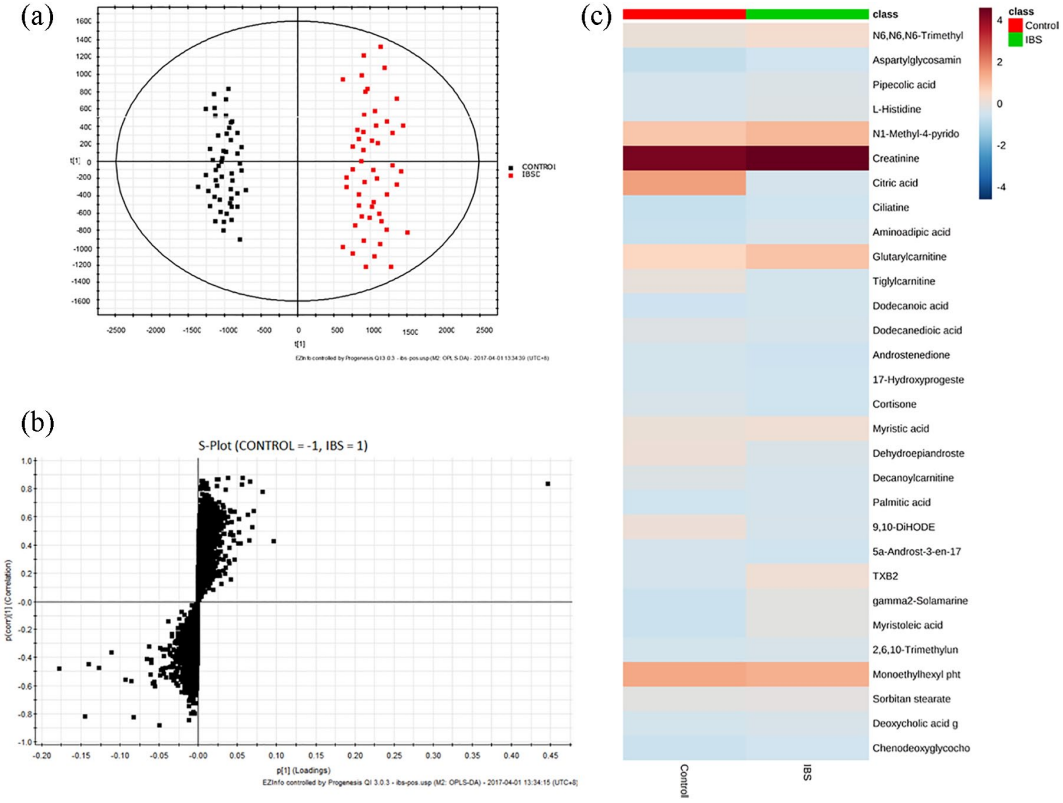

Figure 2. Orthogonal partial least square-discriminant analysis (OPLS-DA) analysis of healthy controls and patients with IBS. (a) OPLS-DA score plot for controls and patients with IBS; (b) S-plot of the OPLS-DA model; (c) heat map of the different metabolites.

Table 1. Thirty different metabolites of IBS detected by UPLC-QTOF-MS.

No.	Retention time (min)	m/z	Compound	Formula	Fold change
1	0.6	189.1602	N6,N6,N6-trimethyl-L-lysine	C9H20N2O2	1.76
2	0.62	358.1231	Aspartylglycosamine	C12H21N3O8	2.53
3	0.62	130.0868	Pipecolic acid	C6H11NO2	1.67
4	0.62	156.0774	L-histidine	C6H9N3O2	1.92
5	0.62	170.093	N1-methyl-4-pyridone-3-carboxamide	C7H8N2O2	1.7
6	0.67	114.0669	Creatinine	C4H7N3O	1.65
7	1.08	215.0165	Citric acid	C6H8O7	0.17
8	1.38	126.0314	Ciliatine	C2H8NO3P	1.71
9	1.39	184.0578	Aminoadipic acid	C6H11NO4	2.39
10	1.41	276.1442	Glutarylcarnitine	C12H21NO6	1.94
11	2.51	266.1393	Tiglylcarnitine	C12H21NO4	0.44
12	3.56	218.2119	Dodecanoic acid	C12H24O2	1.61
13	4.16	253.1444	Dodecanedioic acid	C12H22O4	0.78
14	4.37	287.2005	Androstenedione	C19H26O2	0.57
15	4.39	331.2264	17-hydroxyprogesterone	C21H30O3	0.67
16	4.41	361.2014	Cortisone	C21H28O5	0.58
17	4.44	246.2432	Myristic acid	C14H28O2	1.55
18	4.46	465.2482	Dehydroepiandrosterone 3-glucuronide	C25H36O8	0.63
19	4.59	316.2482	Decanoylcarnitine	C17H33NO4	0.71
20	4.86	274.2745	Palmitic acid	C16H32O2	1.47
21	5.08	330.2643	9,10-DiHODE	C18H32O4	0.54
22	5.15	273.2217	5a-androst-3-en-17-one	C19H28O	0.67
23	7.03	741.4795	TXB2	C20H34O6	3.62
24	7.03	722.4449	Gamma 2-solamarine	C39H63NO11	3.98
25	7.05	227.2012	Myristoleic acid	C14H26O2	3.89
26	7.76	246.2433	2,6,10-trimethylundecanoic acid	C14H28O2	1.43
27	8.09	301.1417	Monoethylhexyl phthalic acid	C16H22O4	1.43
28	8.58	453.322	Sorbitan stearate	C24H46O6	1.45
29	8.75	450.3195	Deoxycholic acid glycine conjugate	C26H43NO5	1.46
30	8.95	450.3196	Chenodeoxyglycocholic acid	C26H43NO5	1.49

Fold change was calculated by comparing metabolites differing between patients with IBS and healthy controls.

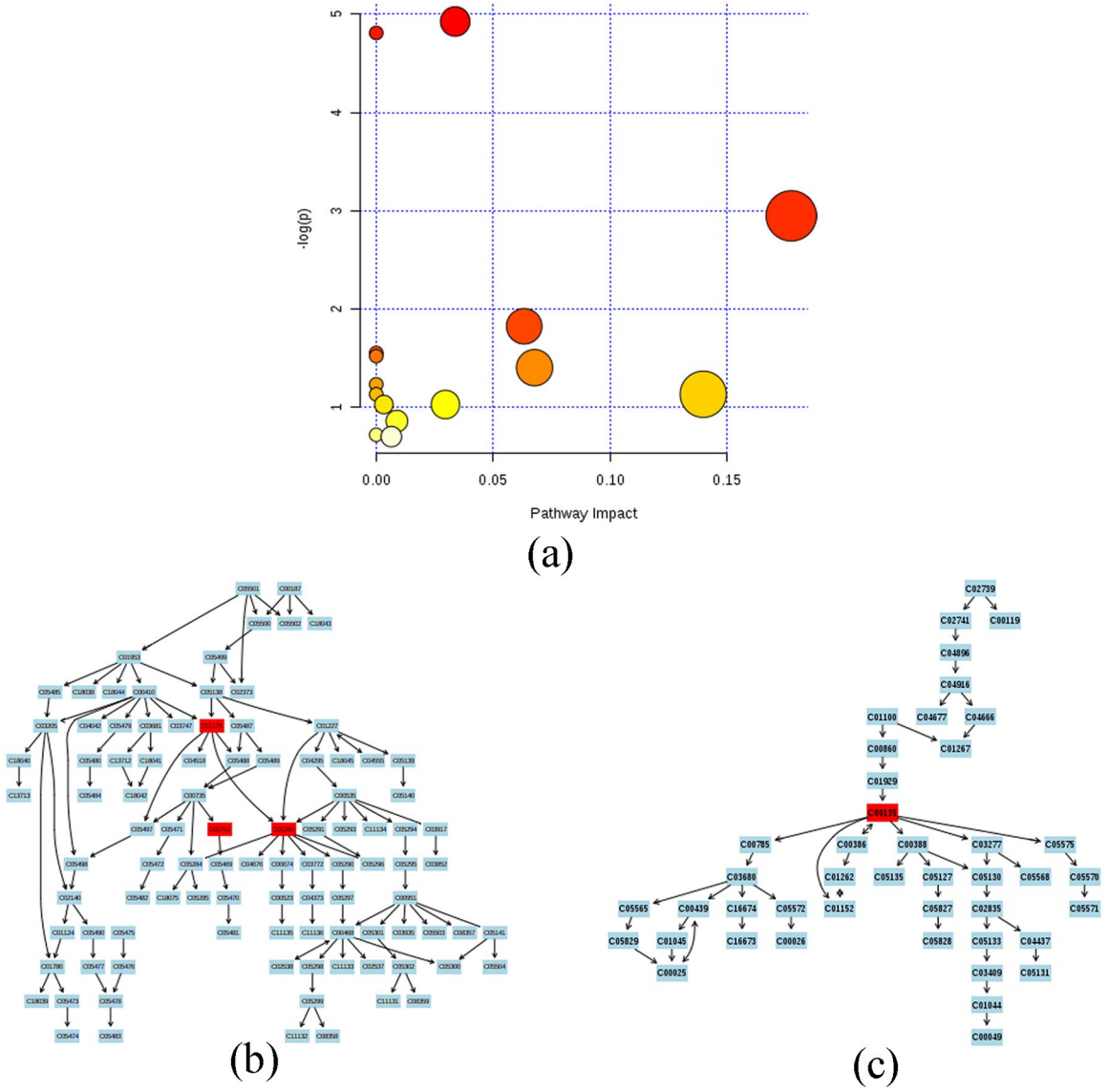

Figure 3. Pathway analysis of metabolic biomarkers in urine of patients with irritable bowel syndrome with MetaboAnalyst. (a) Summary of pathway analysis visualized by bubble plots; (b) steroid hormone biosynthesis metabolism; (c) Histidine metabolism.

performed a pathway analysis using Metabo Analyst. Figure 3(a) depicts a summary of the pathway analysis; the pathway impact value was calculated from pathway topology analyses. Here, the impact-value threshold was set to 0.1; values above the threshold were filtered out as potential pathway targets. In this study, we found two metabolic pathways of steroid hormone biosynthesis and histidine metabolism (Figure 3(b,c)) to be disturbed in patients with IBS. These biochemical changes may help understand key features of the disease and may provide useful clues for future IBS mechanism exploration.

The steroid hormone biosynthesis pathway in our study shows marked perturbations on the IBS-based pathway analysis. There are reports on the effects of hormonal status changes on the colonic motor response to stress.[32] Stress is implicated as an important factor in the development and exacerbation of various disorders, from depression to IBS.[33] Stress results in the activation of the hypothalamic–pituitary–adrenal (HPA) axis, which releases corticotropic-releasing hormone (CRH). Cortisone released from the adrenal cortex into the blood as a stress hormone affects metabolic actions, immune responses, inflammation,

locomotion, sexual behavior and learning in patients with IBS.[34] In addition, clinical and experimental studies indicate that declining or low sex hormone levels may contribute to the occurrence or exacerbation of bowel symptoms.[35] During pregnancy, ovarian hormone levels and progestogen are elevated, and many chronic pain syndromes frequently associated with IBS, such as migraine headaches, are amplified during pregnancy.[36] In our study, 17-hydroxyprogesterone and androstenedione in the urine of IBS patients were reduced compared with the levels in controls, and they may have affected the HPA axis[37] and intestinal movements[38] in the patients with IBS.

Histidine is an alpha-amino acid that exists in free form circulating in the blood, available for use in tissues. The histidine metabolism is closely related to those of oxidative stress and inflammation.[39] Studies have shown that people with oxidative stress or inflammatory disease (such as chronic kidney disease or rheumatoid arthritis[40]), tend to have low plasma histidine levels.[41] In addition, histidine is a precursor for the biosynthesis of physiologically active substances like histamine and carnosine,[42] and the serum concentration is considered a biomarker for the diagnosis of different diseases.[43] Inflammation has been found to play a critical role in IBS,[44] and histamine has been implicated in the pathogenesis of IBS.[45] Thus, histidine may mediate the development of IBS and its symptoms by affecting inflammation and adjusting the histamine concentration.

Acquisition of specific biomarkers by ROC analysis

Our multivariate ROC curve exploration was used to identify multiple biomarkers and evaluate their performances. Figure 4(a) shows the ROC curves for all models created by MetaboAnalyst. Model 3 (with just five features) displays relatively good performance. Figure 4(b) shows the metabolites ranked by their selection importance for the five-feature panel of model 3. We selected cortisone, citric acid, tiglylcarnitine, N6,-N6,-N6-trimethyl-L-lysine and L-histidine as the most significant differential metabolites for the differentiation of the patients with IBS from the healthy controls. Figure 5 shows the levels of the five metabolites in urine; all of the different metabolites had an AUC value within the range 0.78–0.85. The ROC curve analysis of the combined metabolic biomarkers yielded an AUC of 0.951 (95% CI 0.908–0.991) (Figure 4(c)), with good performance for the diagnosis of IBS. Further studies are needed to confirm the potential utility of these initial findings.

Study limitations

We are aware of the limitations of our study. Mainly our control group was composed only of volunteer healthy patients. Indeed, we only screened the samples of IBS patients with diarrhea for biomarkers and did not compare them with those of patients with other IBS symptoms, or those with diarrhea but no IBS. While this was beyond the scope of this study, future research could examine these relationships to further our understanding of the development and possible treatment of IBS.

We did not detect metabolic changes in patients with normal diarrhea, and also found it technically challenging to group a sufficient number of patients of the same gender and age presenting with normal diarrhea within this study. As such, we anticipated that the biomarkers observed resulted from IBS but not from diarrhea alone. Nevertheless, we agree that further studies comparing healthy patients to patients with normal diarrhea should be conducted in the presence of a confirmatory cohort to strengthen the potential clinical value of the current study.

Conclusion

In the present study, an UPLC-QTOF-MS-based urine metabolomics approach coupled with multivariate statistical methods provides a powerful approach to clearly differentiate patients with IBS from matched, healthy controls. In total, we selected 30 differential metabolites associated with IBS by analyzing global changes in an individual's metabolic profile. Moreover, we found disturbances in steroid hormone biosynthesis and histidine metabolism in patients with IBS, which may be involved in the pathogenesis of IBS. More importantly, a panel of five metabolite markers composed of cortisone, citric acid, tiglylcarnitine, N6,-N6,-N6-trimethyl-L-lysine and L-histidine, which showed good discrimination between patients and healthy controls, may

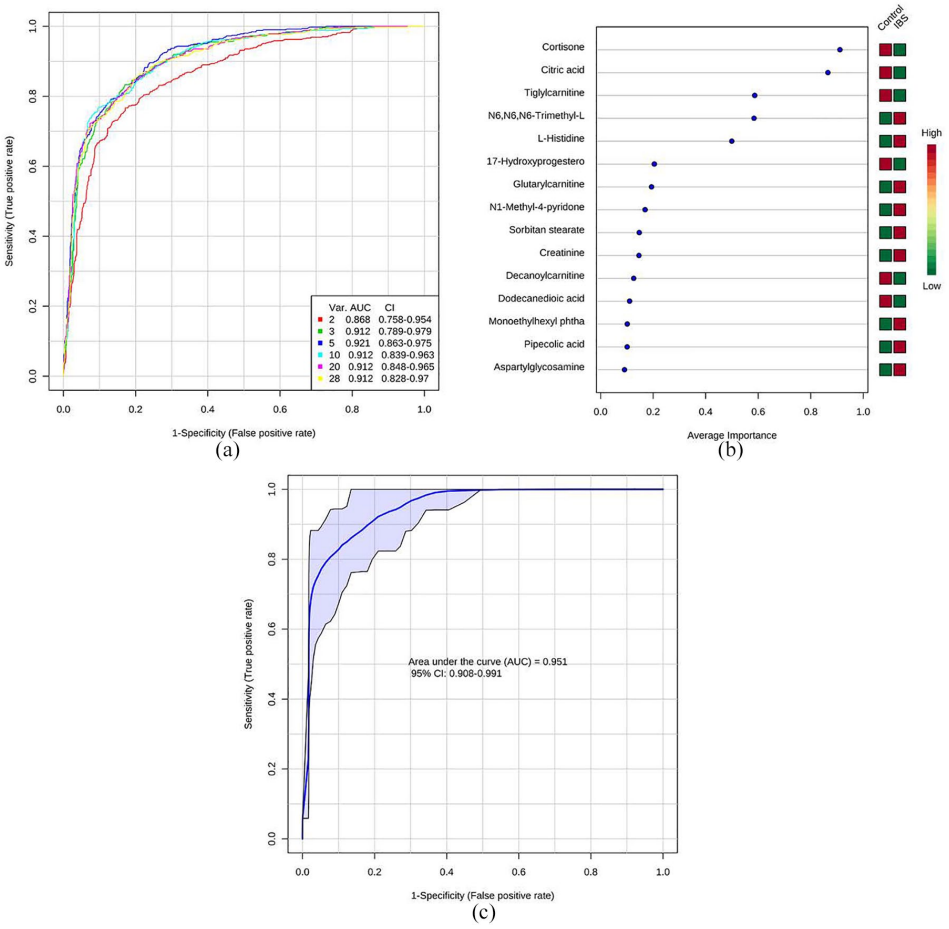

Figure 4. Multivariate receiver operating characteristic (ROC) curve exploration. (a) ROC curves for all models created by MetaboAnalyst; (b) metabolites ranked by their selection importance in the five-feature panel of model 3; (c) ROC curve analysis of the five metabolic biomarkers.

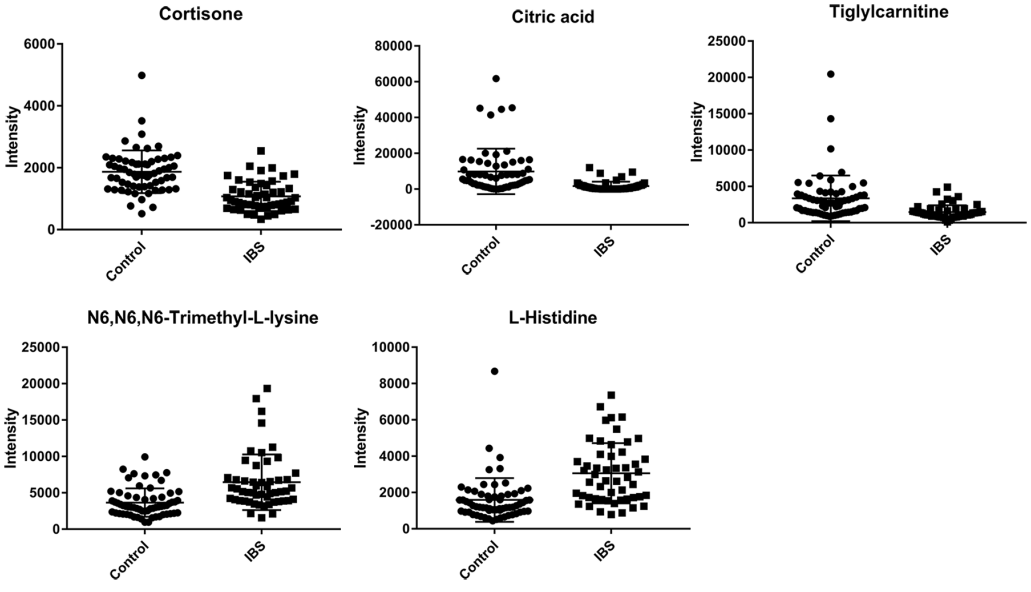

Figure 5. Scatter plots of five potential biomarkers in urine.

be potential biomarkers for IBS diagnosis. In conclusion, metabolomics combined with pattern recognition is an attractive strategy to identify disease diagnostic markers.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethical statement

The work is approved by the Shanghai Changhai hospital Ethics Committee of the Naval Medical University (No: SCHEC2013-183). All patients and healthy participants provided informed consent and the reported samples belong to healthy volunteers and IBS patients of 18 years of age or older. The study ended after diagnosis and collection of the urine samples.

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