

Histidine Decarboxylase Is Identified as a Potential Biomarker of Intestinal Mucosal Injury in Patients with Acute Intestinal Obstruction

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Various biomarkers currently used for the diagnosis of intestinal mucosal injury (IMI) in patients with acute intestinal obstruction have low sensitivity and specificity. In the present study, IMI, as indicated by the impaired expression of tight junction proteins, including zonula occludens-1, occludin and claudin-1, and inflammation were determined in colonic tissues of patients with 45 strangulated intestinal obstruction (STR-IO) and the adjacent "normal" colonic tissues of 35 patients with colon cancers by quantitative real-time polymerase chain reaction (QRT-PCR), Western blotting, immunohistochemistry and histological examination, respectively. Then, two-dimensional fluorescent difference gel electrophoresis coupled with linear trap quadrupole mass spectrometry was used to screen for potential biomarkers of IMI in the serum samples of 10 STR-IO, 10 simple intestinal obstruction (SIM-IO) and 10 normal healthy controls. A total of 35 protein spots were differentially expressed among the serum samples, and six of the proteins were identified as potential biomarkers. Among the six proteins, histidine decarboxylase (HDC) and ceruloplasmin (CP) were elevated significantly in patients with STR-IO, compared with patients with SIM-IO and healthy controls. Thus, HDC and CP were further validated by QRT-PCR, Western blotting, immunohistochemistry and enzyme-linked immunosorbent assay, respectively, in colonic tissues, serum and urine samples. Finally, the receiver operating characteristic curves were used to show the area under the curves of HDC, CP and several established biomarkers, followed by the determination of the appropriate cutoff values and their sensitivities and specificities. It was shown that for serum and urine, HDC levels achieved sensitivities and specificities compatible to or even greater than those of established biomarkers for the diagnosis of IMI in patients with acute intestinal obstruction, although further validation in a larger cohort is required.

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INTRODUCTION

The integrity of the intestinal mucosa, which is primarily maintained by the apical junctional complex consisting of tight junction proteins (TJPs) and adherens junction proteins, is essential for the function of the intestinal barrier (1). Intestinal mucosal injury (IMI) is a common clinical complication that may lead to dysfunction of the intestinal barrier. The most common cause of IMI includes acute intestinal obstruction (AIO), especially strangulated intestinal obstruction

(STR-IO), followed by chronic intestinal obstruction (2,3), severe trauma (4–6), intestinal ischemia (7–9) and acute pancreatitis (10–12). A cascade of intestinal events such as overproduction of intestinal cytokines (13) and increased intestinal permeability (14) and translocation of intestinal bacteria and endotoxins may be initiated in these diseases (15). These pathologic events not only influence the intestinal mucosa itself, but may also impair the remote tissues and organs, if not controlled, leading to systemic inflamma-

tory response syndrome, multiple organ dysfunction syndrome and/or even death (16). Therefore, prompt and accurate diagnosis of IMI is critical to prevent the patients from resultant systemic inflammatory response syndrome, multiple organ dysfunction syndrome and even death. However, the traditional diagnosis methods for diagnosing IMI in patients with the above-mentioned diseases, especially AIO, on the basis of fever, chills, peritonitis, tachycardia, leukocytosis and acidosis, are notoriously unreliable, even in the hands of experienced surgeons (17,18). Several serum biomarkers including D-lactate (19–21), α -glutathione S-transferase (α -GST) (22,23), intestinal fatty acid binding proteins (I-FABP) (24,25), D-dimer (26,27), cobalt-albumin binding assay (28), diamine oxidase (DAO) (29) and urine biomarkers, including I-FABP (24) and thromboxane B2

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(TXB2) (30) for IMI, were investigated; however, none of these biomarkers was proven clinically useful because of poor sensitivity and specificity. Therefore, identification of novel and effective biomarkers for the prognosis of IMI and the response to treatment in patients with AIO is urgently required.

Currently, the proteomic technique is considered to be a predominant tool for the global evaluation of protein expression and has been widely applied for the discovery of novel biomarkers for different diseases (31). This technique has been substantially advanced since it was first described in the mid-1970s (32). A fundamental improvement was the development of two-dimensional fluorescent difference gel electrophoresis (2-D DIGE). 2-D DIGE possesses some advantages over traditional two-dimensional polyacrylamide gel electrophoresis (2-DE PAGE); for instance, it adds a quantitative component, allowing for the direct comparison of protein abundance changes across multiple samples simultaneously (33–35), negates inter-gel variation by using an internal standard (35), offers more accurate quantitation than silver staining (36) and reduces the excessive time and labor costs encountered with standard 2-DE PAGE. Therefore, 2-D DIGE is a more accurate quantitative and qualitative method than 2-DE (37) and thus has been applied in proteomic studies of human diseases including cancer (38–40), autoimmune uveitis (41), chronic inflammatory demyelinating polyneuropathy (42) and psychiatric disease (43). In addition, 2-D DIGE has also been applied for purification of human monoclonal antibodies (44) and determination of drug resistance (45).

Blood constituents, notably serum proteins, reflect diverse physiological or pathological states in humans. Serological biomarkers can be analyzed relatively easily and economically; thus, they have the potential to be used for greatly enhancing screening acceptance (46). In our previous proteomic studies, different kinds of proteomic approaches and mass

spectrometry (including 2-DE, 2-D DIGE, matrix-assisted laser desorption/ionization–time of flight [MALDI-TOF] and MALDI–tandem time of flight [TOF/TOF]) have been used in the research field of colorectal cancer (38,47–50). Urine is a useful source of proteins for biomarker discovery and assessment. It can be obtained by noninvasive collection methods and thus is more easily collected than the tissue or blood, which enables the more convenient monitoring of a wide range of physiologic processes and diseases. Moreover, urine aliquots provide enough protein for multiple analyses (51,52).

An ideal test for the diagnosis of the IMI in patients with AIO would be one that is simple, accurate and noninvasive, and we believe that a blood or even urine test for a biomarker(s) would possess the potential to meet all the above requirements. Therefore, the present study was carried out to identify specific diagnostic biomarkers of IMI in patients with AIO, especially STR-IO, and to provide clues to further understand the molecular mechanisms involved in intestinal mucosal dysfunction.

MATERIALS AND METHODS

Subjects and Collection and Preparation of Colonic Tissues, Blood and Urine Samples

This prospective study was performed with 160 subjects, including 45 patients with STR-IO, 30 patients with simple intestinal obstruction (SIM-IO), 20 acute simple appendicitis (ASA) patients, and 35 patients with colon cancer recruited from The Sixth People's Hospital affiliated to Shanghai Jiao Tong University from June 2009 to February 2010. In addition, 30 healthy volunteers from the community of Shanghai were also recruited during the same time.

Colonic tissues, blood and urine samples were collected from these patients included the experiments described below. The study protocol was designed in accordance with human subject guidelines and approved by the Scientific and

Ethical Committee of The Sixth People's Hospital Affiliated to Shanghai Jiao Tong University, China. All subjects, including healthy controls, were able to read and understand the experiment and provided written informed consent. They all were selected on the basis of the strict criteria in the current study (Supplementary Table 1).

For the confirmation of IMI in colonic tissues of STR-IO patients, colonic tissues from 45 STR-IO patients and adjacent "normal" colonic tissues from 35 patients with colon cancer were collected (Table 1). For initial proteomic analysis, blood was obtained by venipuncture from 10 STR-IO patients before surgical resection, 10 SIM-IO patients and 10 healthy individuals (Table 1).

For the validation study, once the differentially expressed proteins were identified, colonic tissues from 45 STR-IO patients and adjacent "normal" colonic tissues from 35 patients with colon cancer were used for quantitative real-time polymerase chain reaction (QRT-PCR), Western blotting and immunohistochemistry (IHC) for the expression of the proteins in colonic mucosal tissues at mRNA and protein levels, respectively. Moreover, serum samples were collected from 35 of the STR-IO patients before surgical resection, 20 SIM-IO patients, 20 ASA patients before surgical resection and 20 healthy controls. In addition, urine samples were also collected from 25, 15, 15 and 15 of the above STR-IO, SIM-IO, ASA patients and healthy controls, respectively. The characteristics of these individuals for the validation study are summarized in Table 1. All of the colonic tissues were obtained from surgical resections of patients with STR-IO or patients with colon cancer, immediately frozen in liquid nitrogen and then frozen at -80°C until use. Serum and urine samples were collected from patients with STR-IO, SIM-IO or ASA upon hospital admission and healthy individuals in the fasting state and were subjected to centrifugation at $2,000g$ for 15 min. The supernatant was then frozen on liquid nitrogen within 30 min of collection.

Table 1. Clinical characteristics of patients and healthy controls included in the study.

Subject group	Clinical features	
	Mean age (range, years)	Sex (M/F)
Confirmation analysis in colonic tissues ^a		
STR-IO patients (n = 45) ^b	47.91 ± 13.08 (25–75)	25/20
Colon cancer patients (n = 35)	50.54 ± 13.63 (28–75)	19/16
Proteomic analysis in serum		
STR-IO patients (n = 10)	47.20 ± 13.68 (28–73)	6/4
SIM-IO patients (n = 10)	48.90 ± 11.28 (33–70)	5/5
Healthy controls (n = 10)	46.40 ± 12.58 (31–74)	4/6
Validation analysis ^c		
Serum samples		
STR-IO patients (n = 35)	48.11 ± 13.10 (25–75)	19/16
SIM-IO patients (n = 20)	51.40 ± 14.12 (29–73)	9/11
ASA patients (n = 20)	49.70 ± 11.57 (26–72)	11/9
Healthy controls (n = 20)	49.35 ± 11.80 (29–74)	8/12
Urine samples		
STR-IO patients (n = 25)	48.88 ± 13.97 (25–75)	13/12
SIM-IO patients (n = 15)	49.93 ± 14.34 (29–73)	8/7
ASA patients (n = 15)	52.13 ± 10.11 (37–72)	8/7
Healthy controls (n = 15)	47.87 ± 12.61 (29–74)	7/8

^aColonic tissues of 35 STR-IO patients and 20 colon cancer patients were included in the IHC for expression of TJPs.

^bSerum samples of 10 STR-IO patients were used for the proteomic analysis, and serum and urine samples of the other 35 STR-IO patients were used in the validation analysis.

^cThe subjects included in the validation analysis were from the same cohort that was unrelated to subjects in the proteomic analysis.

Removal of High-Abundance Proteins from Serum Samples in Initial Proteomic Analysis

The whole experimental design of proteomic analysis is shown in Figure 1. High-abundance proteins were removed from the serum samples as previously described (38,53). Briefly, a 4.6 × 100 mm² Agilent Multiple Affinity Removal Column (Agilent Technologies, Palo Alto, CA, USA) was attached to an EZChrome Elite HPLC (Hitachi High Technologies America, San Jose, CA, USA) to selectively remove albumin, immunoglobulin G (IgG), IgA, antitrypsin, transferrin and haptoglobin in serum samples. This column can process 20 µL human serum per sample run. Samples were processed according to the manufacturer's instructions. Approximately 90% of total serum proteins are removed by this method.

Fluorescence Labeling with CyDyes

A total of 50 µg serum proteins were minimally labeled with one of the three

CyDye DIGE Fluors (Amersham Biosciences, Piscataway, NJ, USA) (Table 2). Briefly, individual serum samples from three groups (STR-IO, SIM-IO and healthy control) were labeled with Cy2 [3-(4-carboxymethyl)phenylmethyl-3-ethylloxycarbocyanine halide N-hydroxysuccinimidyl ester], Cy3 [1-(5-carboxypentyl)-1-propylindocarbocyanine halide N-hydroxysuccinimidyl ester] and Cy5 [1-(5-carboxypentyl)-1-methylindocarbocyanine halide N-hydroxysuccinimidyl ester], respectively. CyDyes were reconstituted in anhydrous dimethyl formamide and combined with samples at a ratio of 400 pmol CyDye to 50 µg proteins. In addition, the pooled internal standard was labeled with Cy2. Labeling was performed on ice and in the dark for 30 min. The reaction was then quenched by incubating with 1.5 µL of 10 mmol/L lysine on ice in the dark for 10 min. Finally, four kinds of labeled samples (pooled standard, STR-IO, SIM-IO and control) were combined.

2-DE and Imaging

Unlabeled pooled internal standard sample (1 mg) was separately processed by 2-DE for purposes of protein identification. Proteins were focused on 13-cm, 3–10 immobilized pH gradient (IPG) strips (Amersham Biosciences). IPG strips were then equilibrated in equilibration buffer (50 mmol/L Tris-HCl, 6 mol/L urea, 30% glycerol, 2% sodium dodecylsulfate). Proteins were then separated on 10% Tris-glycine gels (ProtoGel, National Diagnostics, Atlanta, GA, USA) using an Ettan DALT II System (Amersham Biosciences). Samples were run in triplicate. The gels were scanned using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences). Excitation/emission wavelengths for Cy2, Cy3 and Cy5 were 488/520, 532/580 and 633/670 nm, respectively. The 2-D gel containing 1 mg unlabeled pooled internal standard sample was fixed in 30% methanol, 7.5% acetic acid, Coomassie stained (Colloidal Blue stain kit, Invitrogen, Carlsbad, CA, USA) and then scanned with the same imager, with an excitation wave length of 633 nm.

DIGE Analysis

Relative protein quantitation in the serum of 10 STR-IO patients, 10 SIM-IO patients and 10 healthy controls was performed using the DeCyder software (Version 6.5, Amersham Biosciences). The Differential In-gel Analysis module was used for pairwise comparisons of each gel and for the calculation of normalized spot volumes/protein abundance. The presence of a Cy2-labeled pooled internal standard on every gel allowed accurate relative quantitation of protein spot features across different gels. Protein spots that were differentially expressed among the three groups, or between any two of the groups, were identified. A one-way analysis of variance (ANOVA) test ($P < 0.05$) was performed for every matched spot-set, comparing the average and standard deviation (SD) of protein abundance for a given spot and calculating significant differences in relative abundances of protein spot features among three groups. Only protein spots that showed significantly increasing or de-

creasing trends among STR-IO, SIM-IO and healthy control serum samples were selected for further analysis to identify the potential markers. Pixel values from images of a small area of fluorescent stained gels were converted into 3-D representations to illustrate the differential quantification, using the Biological Variation Analysis module of the DeCyder software.

Tryptic Digestion and Protein Identification by Linear Trap Quadrupole Mass Spectrometry

Tryptic digestion was carried out as described in our published proteomic study (38). Separation and identification of the digested proteins were conducted by a Finnigan linear trap quadrupole mass spectrometer (ThermoQuest, San Jose, CA, USA) coupled with a Surveyor HPLC system (ThermoQuest). First, Microcore RP column (C18 0.15 × 120 mm²; Thermo-Hypersil, San Jose, CA, USA) was used to separate the protein digests. The gradient was held at 2% solvent B (0.1% v/v formic acid in 100% v/v acrylonitrile) for 3 min and increased linearly to 98% solvent B in 20 min. The peptides were eluted from the C18 microcapillary column at a flow rate of 150 µL/min and were then electrosprayed directly into an LCQ-Deca mass spectrometer with the application of the spray voltage of 3.2 kV and with the capillary temperature at 200°C. The full scan ranged from 400 to 2,000 *m/z*. Normalized collision energy was at 35.0. The number of ions stored in the ion trap was regulated by the automatic gain control. Voltages across the capillary and the quadrupole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. Protein identification using tandem mass spectrometry (MS/MS) raw data was performed with SEQUEST software (University of Washington, licensed to Thermo Finnigan) on the basis of the nonredundant International Protein Index (IPI) human protein database (version 3.26). The species was human. A relative molecular mass of 57 (57 D) was added to the average molecular mass of cysteines in MS/MS data searching. Both b ions and y ions were also included in the database

search. In addition, the searches were performed without constraining protein relative molecular mass (*Mr*) and isoelectric point (*pI*). Protein identification results were filtered with the *Xcorr* ≥ 1.9 with charge state 1+, *Xcorr* ≥ 2.2 with charge state 2+ or *Xcorr* ≥ 3.75 with charge state 3+. In addition, the delta *Cn* was ≥ 0.1.

QRT-PCR for mRNA Expression of Tight Junction Proteins and Histidine Decarboxylase and Ceruloplasmin in Colonic Tissues

In the confirmation study, the mRNA expression levels of TJPs (including occludin, zonula occludens-1 [ZO-1] and claudin-1) in 45 STR-IO colonic tissues and 35 adjacent “normal” colonic tissues were measured using QRT-PCR with SYBR Green I dye (Molecular Probes, Leiden, the Netherlands). Moreover, in the validation study, mRNA expression levels of histidine decarboxylase (HDC) and ceruloplasmin (CP) in 35 STR-IO colonic tissues and 20 adjacent “normal” colonic tissues were measured using the same technique. Briefly, total RNA was isolated from colonic tissues of STR-IO patients and adjacent “normal” colonic tissues of patients with colon cancer using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. QRT-PCR was performed with an ABI prism 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers were designed using the Primer Express[®] program (Applied Biosystems) (Supplementary Table 2). In a sterile RNase-free microcentrifuge tube, 1 µL of 20 µM oligo (dT) 15 primer was added to a total volume of 15 µL in water. The tube was heated to 70°C for 5 min to melt secondary structures within the template, and then the tube was cooled immediately on ice to prevent secondary structures from reforming. In addition, 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase, RNase H Minus (M-MLV RT RNase H-; Promega, Madison, WI, USA) were added to the reagent to obtain a 25 µL total reaction volume.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the house-

keeping gene control. Separate PCRs in a 25 µL mixture, consisting of 2.0 µL cDNA, 12.5 µL of 2 × SYBR Premix Ex Taq[™] (TaKaRa, Shiga, Japan) and 0.5 µL each of 10 µmol/L gene-specific forward and reverse primers, were conducted for each transcript. PCR conditions were optimized to 95°C (over 30 s), followed by 40 cycles (45 s each) of 10 s at 95°C, 5 s at 60°C and 30 s at 72°C. Five serial dilutions of cDNA were analyzed for each target gene and used to construct linear standard curves. The raw data for the expression of each target gene were divided by the quantity of GAPDH present to acquire the normalized value of the yield expressed in arbitrary units.

Western Blotting for Expression of TJPs, HDC or CP in Colonic Tissues

The protein expression levels of TJPs (including occludin, ZO-1 and claudin-1) in 45 STR-IO colonic tissues and 35 adjacent “normal” colonic tissues (in the confirmation study) and those of HDC and CP in 35 STR-IO colonic tissues and 20 adjacent “normal” colonic tissues (in the validation study) were measured using Western blotting. Briefly, the colonic tissues were ground in liquid nitrogen and lysed in radioimmunoprecipitation assay lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L NaF, 1 mmol/L Na₃V4 and 1 mmol/L phenylmethylsulfonyl fluoride). The extracted proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblotted using primary antibody against ZO-1 (Abcam), occludin (Abcam), claudin-1 (Santa Cruz), HDC (Santa Cruz) and CP (Abcam). The blots were visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). β-Actin was used as an internal control.

Histological Examination and IHC for the Expression of TJPs, HDC and CP in Colonic Tissues

For histological examination, 45 STR-IO colonic tissues and 35 adjacent “nor-

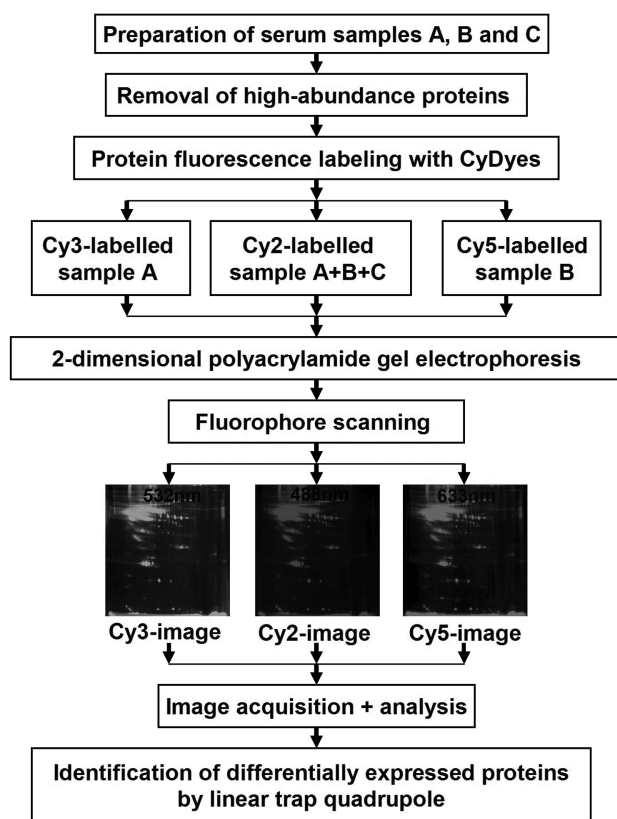


Figure 1. Flow chart illustrating the proteomic analysis for identifying serum biomarkers of IMI in patients with AIO by 2-D DIGE and linear trap quadrupole mass spectrometry. A, healthy controls; B, patients with SIM-IO; and C, patients with STR-IO.

mal" colonic tissues were fixed in 10% neutral phosphate-buffered formalin, routinely processed, sectioned at 5 μm and stained with hematoxylin and eosin for light microscopic examination. The colonic mucosal tissues were examined in a blind fashion by all four independent pathologists. The colonic tissues (cecum, ascending, transverse or descending colon) were assigned a histo-

logical score ranging from 0 to 4 on the basis of the criteria adapted from Berg *et al.* (54). Thus, the summation of these scores provided a total colonic inflammatory score ranging from 0 (no change in any colonic parts of the colon) to a maximum of 16 (four grades in all).

For IHC, 45 STR-IO colonic tissues and 35 adjacent "normal" colonic tissues were stained with anti-ZO-1 (Abcam),

occludin (Zymed) and claudin-1 (Santa Cruz), while among them, 35 STR-IO colonic tissues and 20 adjacent "normal" colonic tissues were stained with HDC and CP (Abcam) primary antibodies (Euro-Diagnostica AB) using the DAB substrate solution (Dako Cytomation, Hamburg, Germany) according to the manufacturer's instructions. For each section, a minimum of five representative fields with well colonic tissues were examined at 100 \times magnification, and 200 epithelial cells were counted for each field. The immunostaining was evaluated in our previously published study (47). Briefly, the immunostaining intensity was classified into four grades, as 0, lack of staining; 1, mild staining (yellow color); 2, moderate staining (brown color); and 3, strong staining (deep brown color). Epithelial cells with weak to strong staining (yellow to deep brown color) in the cytoplasm and/or nuclei were considered to be immunostaining-positive cells. Then, the proportion of immunostaining-positive cells over the five fields was calculated for each section and divided into four grades, as 0, $\leq 5\%$; 1, 6–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. The overall score for each section was measured as the proportion of immunostaining-positive cells multiplied by the overall immunostaining intensity and defined as negative (–), score 0; weakly positive (+), scores 1–3; moderately positive (++), scores 4–7; and strongly positive (+++), scores 8–12. Sections were examined separately by two independent pathologists who lacked any prior knowledge of patients' clinical information. Any discrepancies between these two investigators were resolved by reevaluation until agreement was reached.

Table 2. Experimental design of fluorescence labeling with CyDyes.

Gel	Cy2 standard	Cy3	Cy5
1	50 μg (16.67 μg each of A, B, C)	50 μg sample A	50 μg sample B
2	50 μg (16.67 μg each of A, B, C)	50 μg sample B	50 μg sample C
3	50 μg (16.67 μg each of A, B, C)	50 μg sample A	50 μg sample B
4	50 μg (16.67 μg each of A, B, C)	50 μg sample C	50 μg sample A
5	50 μg (16.67 μg each of A, B, C)	50 μg sample B	50 μg sample C

A, serum of healthy controls; B, serum of SIM-IO patients with SIM-IO; C, serum of patients with STR-IO.

Enzyme-Linked Immunosorbent Assay for HDC, CP, D-lactate, α -GST, DAO, I-FABP and TXB2 in Serum and Urine Samples of Patients with STR-IO, SIM-IO or ASA and Healthy Controls

In the validation study, serum HDC, CP, D-lactate, α -GST, DAO and I-FABP levels were measured in patients with

STR-IO (n = 35), SIM-IO (n = 20) or ASA (n = 20) and 20 healthy controls using commercial enzyme immunoassay kits (R&D Systems, United Kingdom) in accordance with the manufacturer's instructions. In addition, HDC, CP, I-FABP and TXB2 levels in the urine samples of patients with STR-IO (n = 25), SIM-IO (n = 15) or ASA (n = 15) and healthy controls (n = 15) were also measured using commercial enzyme immunoassay kits (R&D Systems) according to the manufacturer's instructions.

Evaluation of Diagnostic Performance of HDC, CP, D-lactate, α-GST, DAO, I-FABP and TXB2 in the Serum and Urine for IMI in Patients with AIO

In the present study, patients with IMI were defined as individuals with STR-IO in the validation analysis, since patients with SIM-IO did not require surgical treatment and thus the colonic tissues were not available from patients with SIM-IO. Then, receiver operating characteristic (ROC) curve and area under the curve (AUC) were used for determining the diagnostic performance of the different biomarkers in the serum or urine in discriminating patients with STR-IO from SIM-IO, ASA and healthy controls.

Statistical Analysis

Statistical calculations and analyses were performed by SPSS for Windows, release 13.0.0, statistical software (SPSS, USA). Numerical data were expressed as mean ± SD or standard error (SE), whereas categorical data were expressed as number (percentage). Comparisons of numerical data between the two groups were performed by the Student *t* test, Mann-Whitney test, independent sample *t* test or rank-sum test where appropriate. Comparisons of numerical data among multiple groups were performed by one-way ANOVA, Dunnett *t* test or least significant difference (LSD) *t* test, where appropriate. Comparisons of categorical data between or among groups were performed by the χ^2 test or Fisher exact test. The sensitivity and specificity were calculated according to the stan-

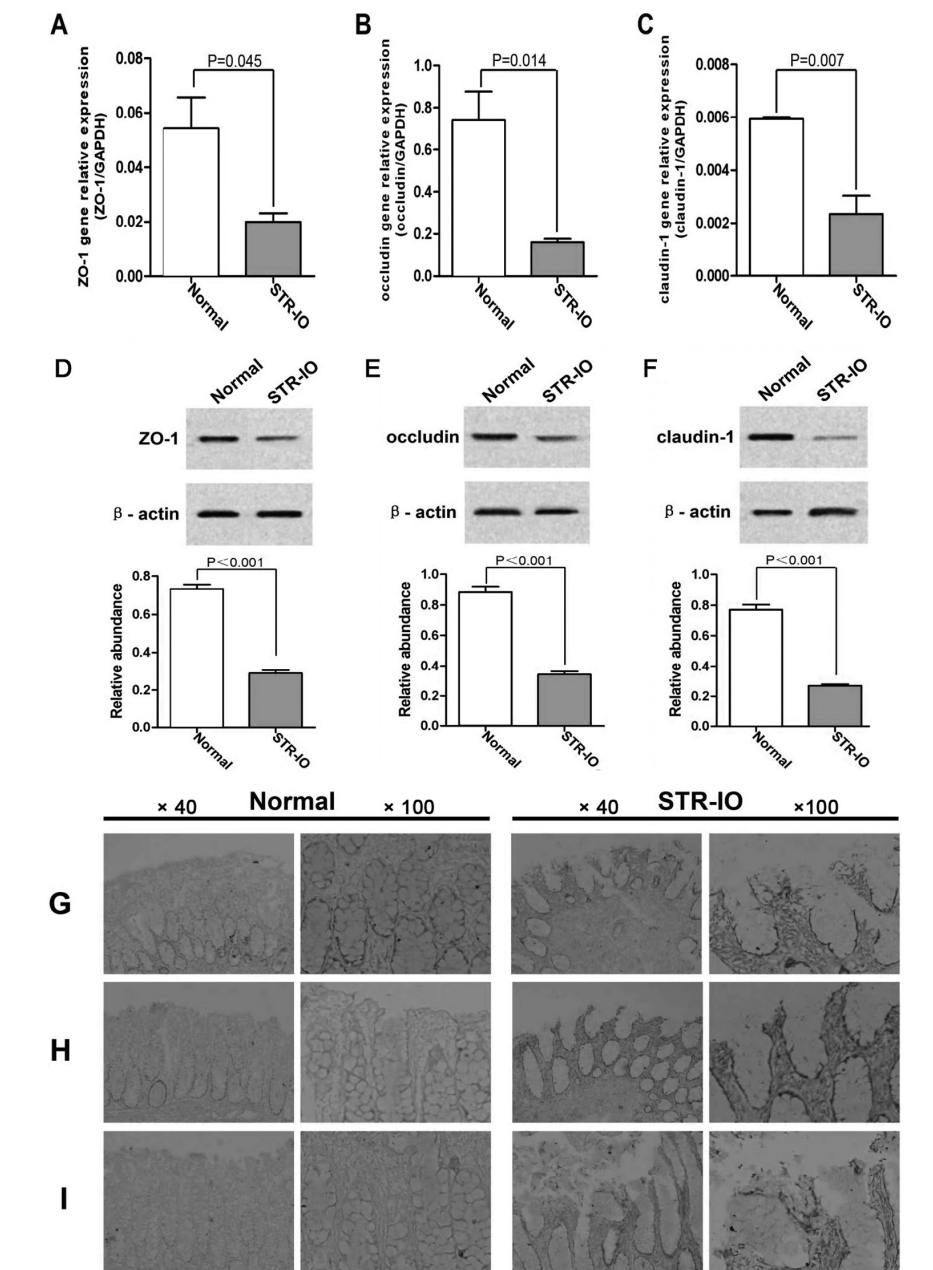


Figure 2. Confirmation of ZO-1, occludin and claudin-1 downregulation in colonic tissues of patients with STR-IO compared with the "normal" colonic tissues of patients with colon cancer. (A–C) QRT-PCR showing mRNA levels of ZO-1 (A), occludin (B) and claudin-1 (C) in STR-IO and "normal" colon tissues. (D–F) Western blotting showing representative images and protein levels of ZO-1 (D), occludin (E) and claudin-1 (F) in STR-IO and "normal" colon tissues. (G–I) Immunohistochemical analysis showing expression levels of ZO-1 (G), occludin (H) and claudin-1 (I) in STR-IO and "normal" colon tissues (40x and 100x magnification, respectively).

dard formulas. The ROC curve and AUC were estimated by the multivariate logistic regression model and used for discriminating between patients with and those without IMI. All *P* values were

two-sided, and a *P* value of <0.05 was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Individual Characteristics

There were no significant differences in age among patients with STR-IO (47.91 ± 13.08 years), patients with SIM-IO (50.57 ± 13.10 years), patients with ASA (49.70 ± 11.57 years), patients with colon cancer (50.54 ± 13.63 years) and healthy controls (48.37 ± 11.93 years) ($P = 0.856$, one-way ANOVA) (Table 1). The sex distribution (male:female) was 25:20 in the STR-IO group, 14:16 in the SIM-IO group, 11:9 in the ASA group, 19:16 in the patients with colon cancer group and 12:18 in the control group ($P = 0.677$, χ^2 test) (Table 1).

Decreased Expression of ZO-1, Occludin and Claudin-1 in STR-IO As Confirmed by QRT-PCR, Western Blotting and IHC

QRT-PCR analysis showed that mRNA expression levels of ZO-1, occludin and claudin-1 were significantly decreased in the STR-IO colonic tissues compared with the adjacent "normal" colonic tissues with colon cancer ($P < 0.001$, independent sample t test) (Figures 2A–C). Western blotting analysis also showed that the protein expression levels of ZO-1, occludin and claudin-1 were significantly decreased in the STR-IO colonic tissues (ZO-1 0.73 ± 0.02 ; occludin 0.94 ± 0.02 ; and claudin-1 0.77 ± 0.03) compared with the adjacent "normal" colonic tissues (ZO-1 0.29 ± 0.02 ; occludin 0.34 ± 0.02 ; and claudin-1 0.27 ± 0.01 ; all $P < 0.001$, independent sample t test) (Figures 2D–F).

In addition, IHC showed that ZO-1, occludin and claudin-1 were localized along the subapical region of the lateral plasma membrane representing the region of tight junctions in surface and crypt epithelial cells in the colonic mucosal epithelial cells (Figures 2G–I). As shown in Table 3, there was a significant difference in the staining intensity and positive cells for ZO-1, occludin and claudin-1 between "normal" colonic tissues and STR-IO colonic tissues (all $P < 0.01$, Mann-Whitney U test). The semiquantitative

Table 3. Immunostaining of tight junction proteins in "normal" colonic tissues of patients with colon cancer and colonic tissues of patients with STR-IO.

Index	Normal (n = 35)	STR-IO (n = 45)	P
ZO-1 (n (%))			<0.001
-	1 (2.9)	23 (51.1)	
+	0 (0.0)	17 (37.8)	
++	14 (40.0)	5 (11.1)	
+++	20 (57.1)	0 (0.0)	
Average score	7.46 ± 2.66	1.29 ± 1.77	<0.001
Occludin (n (%))			<0.001
-	0 (0.0)	18 (40.0)	
+	2 (5.7)	22 (48.9)	
++	11 (31.4)	5 (11.1)	
+++	22 (62.9)	0 (0.0)	
Average score	7.83 ± 2.90	1.44 ± 1.70	<0.001
Claudin-1 (n (%))			<0.001
-	0 (0.0)	23 (51.1)	
+	2 (5.7)	19 (42.2)	
++	10 (28.6)	3 (6.7)	
+++	23 (65.7)	0 (0.0)	
Average score	7.94 ± 2.68	1.00 ± 1.36	<0.001

overall scores of ZO-1, occludin and claudin-1 were significantly fewer in STR-IO colonic tissues than that in "normal" colonic tissues (1.29 ± 1.77 versus 7.46 ± 2.66 for ZO-1, 1.44 ± 1.70 versus 7.83 ± 2.90 for occluding and 1.00 ± 1.36 versus 7.94 ± 2.68 for claudin-1; all $P < 0.001$, one-way ANOVA). Thus, compared with the "normal" colonic mucosal epithelial cells of patients with colon cancer, the expression levels of these proteins were significantly

decreased in the colonic mucosal epithelial cells of patients with STR-IO.

Increased Inflammatory Scores in STR-IO as Confirmed by Histological Examination

Further histological examination showed that the inflammatory scores of STR-IO colonic mucosal tissues were significantly greater than those of the "normal" colonic mucosal tissues ($11.84 \pm$

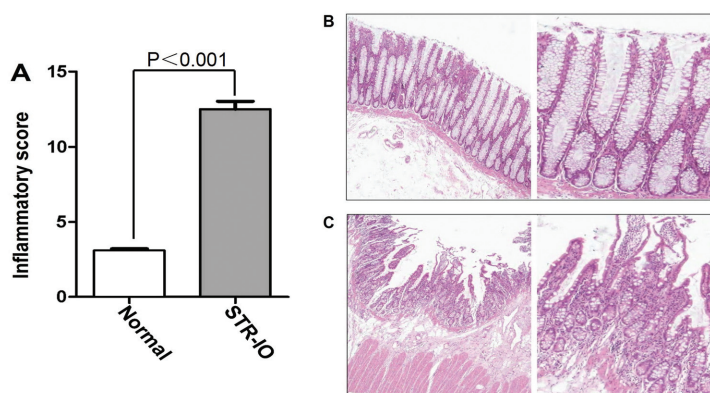


Figure 3. Confirmation of severe inflammation of colonic tissues of patients with STR-IO, compared with the "normal" colonic tissues of patients with colon cancer. (A) The inflammatory score of normal colonic tissues with adjacent colon tumor and STR-IO. (B,C) Representative histological images (hematoxylin and eosin staining) showing adjacent "normal" colonic mucosa of patients with colon cancer (B) and colonic mucosa of patients with STR-IO (C) (40x and 100x magnification, respectively).

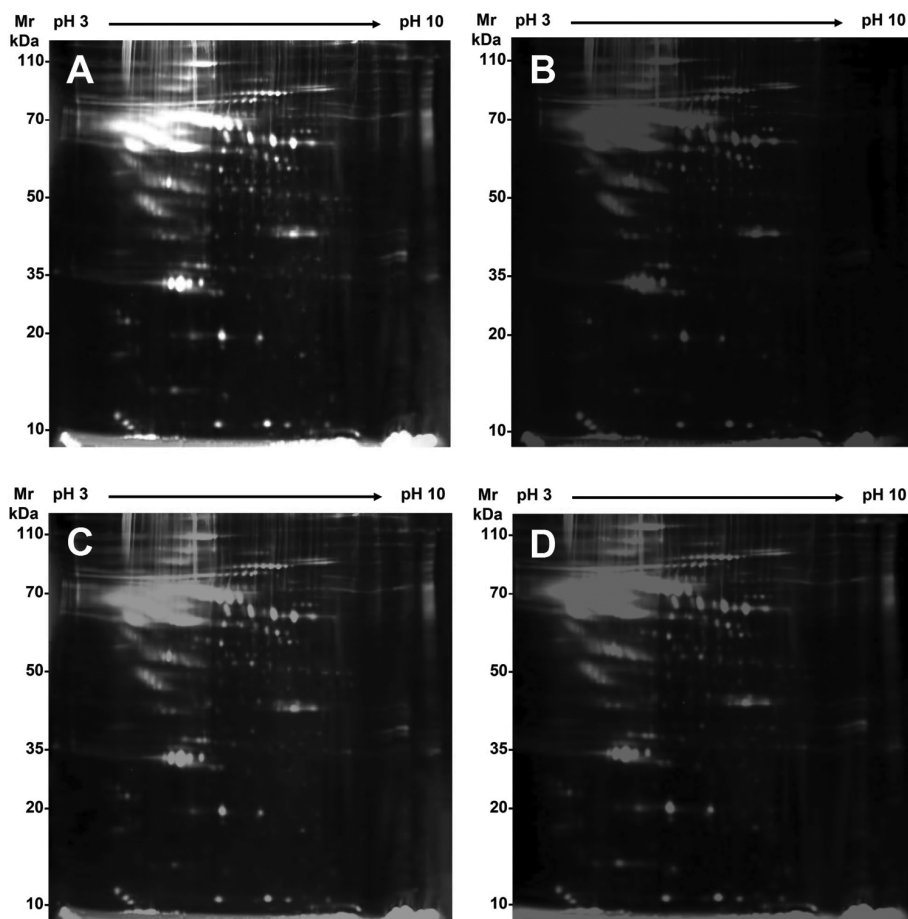


Figure 4. Representative example of analytical gels of 2-D DIGE of serum samples from patients with STR-IO, patients with SIM-IO and healthy controls. (A) Overlay of three individual channels with the following samples: STR-IO serum sample/Cy5/red, healthy control serum sample/Cy3/green and STR-IO + SIM-IO + healthy control serum pooled sample/Cy2/blue. (B) Cy2 image with equal amount of sample STR-IO + SIM-IO + healthy control sample. (C) Cy3 image with STR-IO sample. (D) Cy5 image with SIM-IO sample.

2.39 versus 3.06 ± 0.76 , $P < 0.001$, independent sample *t* test) (Figure 3A). Severe mucosal erosion, necrosis and a large number of lymphocyte and neutrophil infiltration in the lamina propria were present in STR-IO mucosal tissues (Figure 3B), whereas only mild inflammatory cell infiltration was present in the “normal” colonic mucosa (Figure 3C).

Differentially Expressed Proteins in the Serum among Patients with STR-IO, Patients with SIM-IO and Healthy Controls as Identified by Proteomic Analysis

Figure 4 shows the 2-D DIGE analytical gels. Overall, a total of 35 protein

spots were identified as being differentially expressed; 11 protein spots were differentially expressed among three groups, and 24 protein spots were differentially expressed between any two groups. Therefore, 11 differentially expressed proteins in the serum were further analyzed. Among them, only five differentially expressed proteins with an increasing trend and one protein with a decreasing trend, paralleling the increasing severity of colonic mucosal injury including erosion, necrosis and inflammation were identified (Figure 5) (all $P < 0.05$, one-way ANOVA). The mean molecular weight (MW)/pI and peptides of the identified six differen-

tially expressed proteins among STR-IO patients, SIM-IO patients and healthy controls were calculated and are summarized in Table 4, with the six proteins with increasing or decreasing trends being further analyzed.

The five proteins with an upregulated trend were identified as isoform 1 of sex hormone-binding globulin, CP, apolipoprotein E, serum amyloid P-component and HDC, and the protein with a downregulated trend was protein AMBP. Notably, the *P* values were the most significant for HDC ($P = 0.005$) and CP ($P = 0.014$) among six, as determined by one-way ANOVA (Figures 6A, B). Therefore, HDC and CP became the subsequent focus of this study from the results of mass spectrometry analysis.

Validation of HDC and CP from Colonic Tissues by QRT-PCR, Western Blotting and IHC

QRT-PCR analysis showed that mRNA expression of HDC and CP was significantly increased in the STR-IO colonic tissues compared with that in the “normal” colonic tissues ($P < 0.001$, independent sample *t* test) (Figures 7A, B). Western blotting analysis also showed significantly increased protein expression of HDC and CP in the STR-IO colonic tissues compared with that in the “normal” colonic tissues (0.73 ± 0.02 versus 0.29 ± 0.02 for HDC; 0.94 ± 0.02 versus 0.34 ± 0.02 for CP; both $P < 0.001$, independent sample *t* test) (Figures 7C, D).

In addition, IHC showed that HDC was mainly located in the membrane of STR-IO colonic tissue mucosal cells, whereas CP was located in the cytoplasm and the surrounding stroma of the STR-IO cells (Figures 7E, F). As shown in Table 5, there was a significant difference in the staining intensity and positive cells for HDC and CP between “normal” colonic tissues and STR-IO colonic tissues (all $P < 0.001$, Mann-Whitney *U* test). The semiquantitative overall scores of HDC and CP were significantly greater in STR-IO colonic tissues than in “normal” colonic

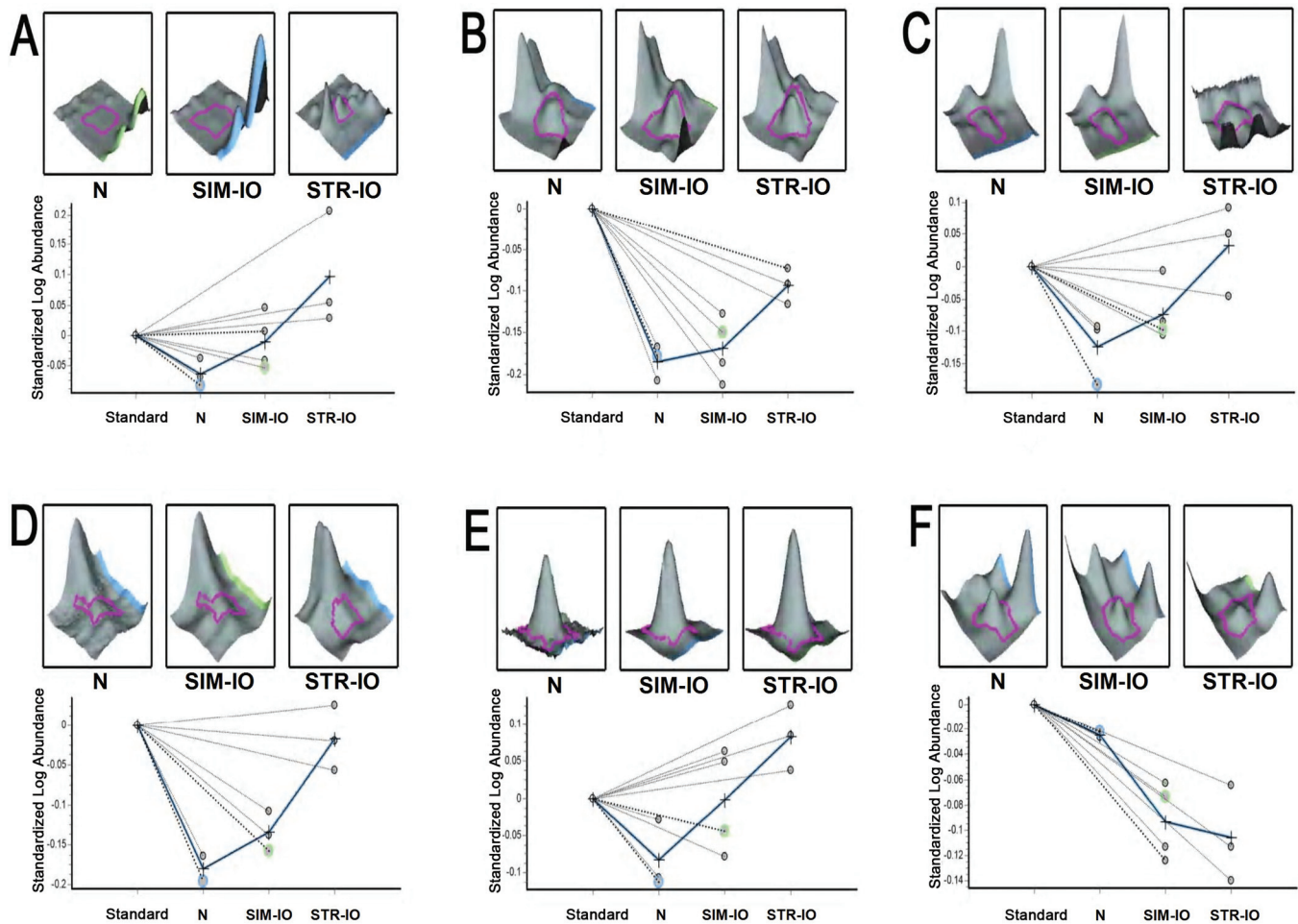


Figure 5. Cropped three-dimensional view of six identified differentially expressed proteins and their images of expression trends from the healthy controls to patients with SIM-IO and to patients with STR-IO. (A–E) Five proteins representing isoform 1 of sex hormone-binding globulin, CP, apolipoprotein E, serum amyloid P-component and HDC showing an increasing expression trend. (F) One protein representing protein AMBP showing a decreasing expression trend. One-way ANOVA was applied to compare the three groups using Decyder Biological Variation Analysis software.

tissues (7.46 ± 3.25 versus 2.05 ± 2.21 for HDC and 6.49 ± 3.48 versus 1.65 ± 1.53 for CP; both $P < 0.001$, independent sample t test).

Increased Expression Levels of HDC and CP in Serum and Urine in Patients with STR-IO As Determined by Enzyme-Linked Immunosorbent Assay

After confirmation of upregulation of HDC and CP in STR-IO colonic tissues, we further quantified the serum levels of HDC, CP and the established biomarkers (D-lactate, α -GST, I-FABP and DAO) by enzyme-linked immunosor-

bent assay (ELISA) analysis (Figure 8 and Supplementary Table 3). The serum levels of HDC, CP, D-lactate, α -GST, I-FABP and DAO in the STR-IO group were significantly greater than those in the SIM-IO group, ASA group and normal healthy controls ($P < 0.001$, one-way ANOVA). Meanwhile, HDC serum level in the SIM-IO group was greater than those in the ASA group and normal healthy controls (both $P < 0.001$, LSD t test), and I-FABP serum level in the SIM-IO group was higher than that in normal healthy controls ($P < 0.050$, LSD t test).

In addition, the urine levels of HDC, CP, I-FABP and TXB2 were also detected by ELISA analysis (Figures 9A–D and Supplementary Table 4). The urine levels of HDC, I-FABP and TXB2, but not CP, were significantly different among the STR-IO group, SIM-IO group, ASA group and normal healthy controls ($P < 0.01$, one-way ANOVA). However, the urine levels of CP in the STR-IO group were higher than those in the ASA group ($P < 0.05$, LSD t -test) and normal healthy controls ($P < 0.01$, LSD t test). Meanwhile, the HDC serum levels in the SIM-IO group were greater than those in the

Table 4. Six differentially expressed proteins in the serum among patients with STR-IO, patients with SIM-IO and healthy controls, as identified by linear trap quadrupole mass spectrometry after 2-D DIGE analysis.

Spot number	IPI accession number	Protein name	MW/pi (theoretical)	Sequence coverage (%)	Unique peptide matches	Peptides identified
844	IPI00023019.1	Isoform 1 of sex hormone-binding globulin	43779.28/6.22	3.98%	2	K.TSSSFEVR.T R.VVLSQGSK.M
859	IPI00017601.1	Ceruloplasmin	122205.24/5.44	0.94%	1	R.IYHSHIDAPK.D
1012	IPI00021842.1	Apolipoprotein E	36154.22/5.65	26.18%	7	K.VQASAVGTSASAPVPSDNH.- R.ASATVGS LAGQLQER.A R.AKLEEQAQQR.L R.LAVYQAGAR.E R.LGADMEDVCGR.L R.LGPLVEQGR.V R.LSKELQASAAQR.L
1256	IPI00022391.1	Serum amyloid P-component	25387.17/6.1	10.31%	3	K.IVLGQEQDSYGGK.F K.IVLGQEQDSYGGK.F R.QGYFVEAQQP.I
1059	IPI00290368.3	Histidine decarboxylase	74140.69/8.3	2.27%	1	R.LFLPATIQDKLIIR.F
1290	IPI00022426.1	Protein AMBP	38999.49/5.95	5.4%	2	K.GVCEETS GAYEKTDTDGK.F R.KGVCEETS GAYEK.T

ASA group and normal healthy controls ($P < 0.01$, LSD t test), and I-FABP and TXB2 urine levels in the SIM-IO group were greater than those in the ASA group and normal healthy controls (all $P < 0.01$, LSD t test).

Sensitivity and Specificity of Serum and Urine Biomarkers in Differentiating Patients with STR-IO from Patients with SIM-IO or ASA and Healthy Controls

The ROC curve was plotted for each of the biomarkers in the serum (Figure 10A)

and urine (Figure 10B) to determine the optimal cutoff value that would give the maximum sensitivity and specificity. The AUCs of serum HDC, CP, D-lactate, α -GST, DAO and I-FABP were 0.937 ± 0.023 , 0.770 ± 0.051 , 0.763 ± 0.054 , $0.849 \pm$

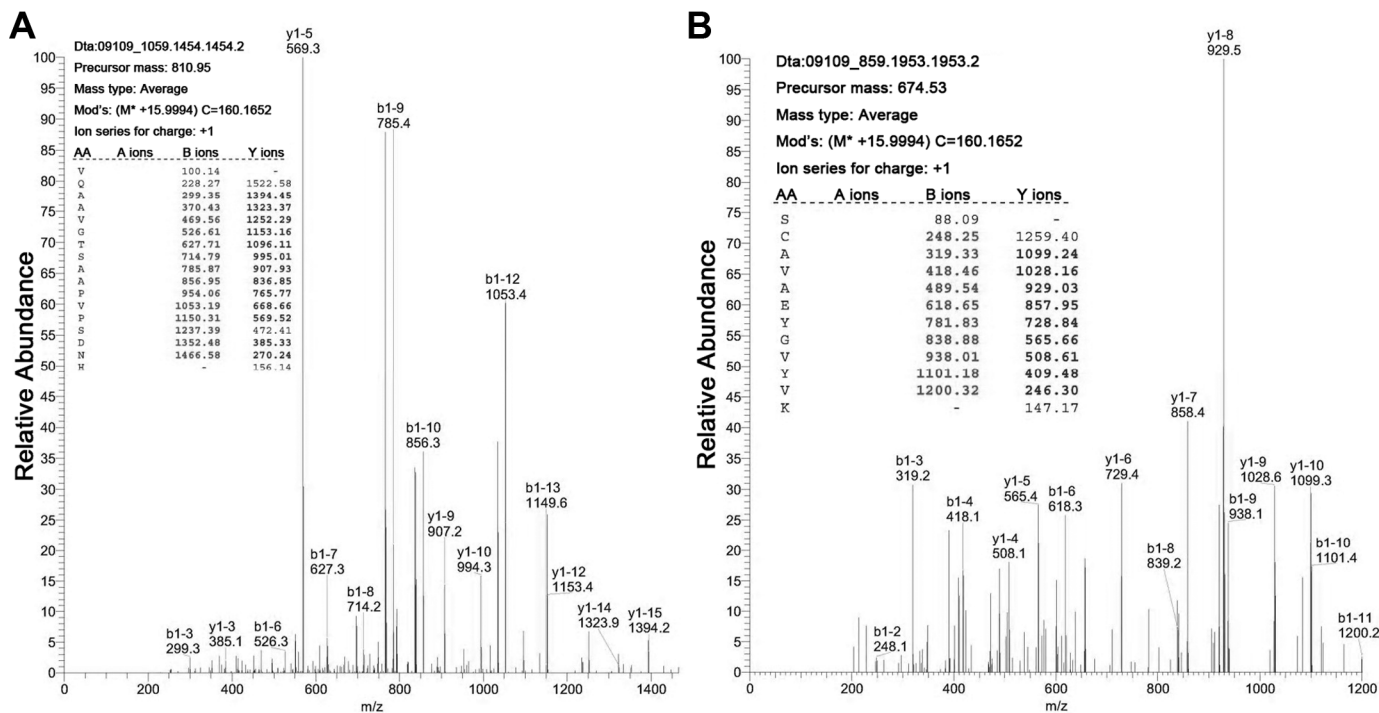


Figure 6. Maps of HDC (A) and CP (B) identification of the representative protein using linear trap quadrupole mass spectrometry.

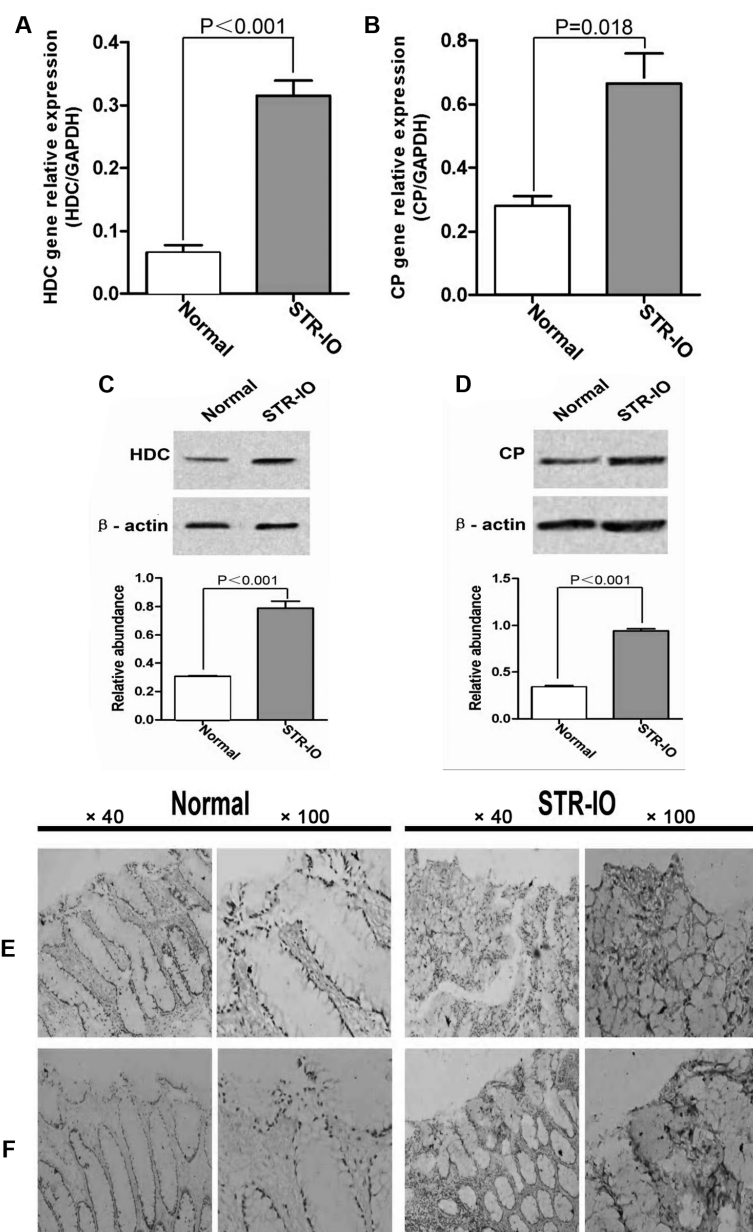


Figure 7. Confirmation of HDC and CP overexpression in STR-IO tissues. (A, B) QRT-PCR showing mRNA levels of HDC (A) and CP (B) in STR-IO and "normal" colonic tissues. (C, D) Western blotting analysis showing representative images and the protein levels of HDC (C) and CP (D) in STR-IO and "normal" colon tissues. (E, F) Immunohistochemical analysis showing expression levels of HDC (E) and CP (F) in STR-IO and "normal" colonic tissues.

0.040, 0.865 ± 0.043 and 0.915 ± 0.035 , respectively (Supplementary Table 5). In addition, the AUCs of urine HDC, CP, I-FABP and TXB2 were 0.916 ± 0.032 , 0.708 ± 0.064 , 0.884 ± 0.040 and 0.772 ± 0.060 , respectively (Supplementary Table 6). With the cutoff values of HDC in the serum and urine of 42.28 and

43.68 ng/mL, the sensitivity of 91.4% and 88.0% and specificity of 81.7% and 86.7% would be achieved, respectively. With cutoff values of CP in the serum and urine of 229.04 and 69.59 ng/mL, the sensitivity of 62.9% and 64.0% and specificity of 80.0% and 62.2% would be achieved. The cutoff values for other

Table 5. Immunostaining of HDC and CP in "normal" colonic tissues of patients with colon cancer and colonic tissues of patients with STR-IO.

Index	Normal (n = 20)	STR-IO (n = 35)	P
HDC (n (%))			<0.001
-	4 (20.0)	0 (0.0)	
+	13 (65.0)	3 (8.6)	
++	2 (10.0)	14 (42.8)	
+++	1 (5.0)	17 (48.6)	
Average score	2.17 ± 2.26	7.24 ± 3.28	<0.001
CP (n (%))			<0.001
-	3 (15.0)	0 (0.0)	
+	16 (80.0)	5 (14.3)	
++	1 (5.0)	13 (37.1)	
+++	0 (0.0)	17 (48.6)	
Average score	1.66 ± 1.33	6.49 ± 3.60	<0.001

biomarkers and the corresponding sensitivities and specificities are shown in Tables 6 and 7.

DISCUSSION

In the critical care setting, the development of IMI in patients with AIO is associated with a high mortality due to the lack of methods or biomarkers for diagnosis of IMI (55). Although several serum (such as D-lactate, α -GST, I-FABP and DAO) and urine biomarkers (such as I-FABP and TXB2) have been used for diagnosing IMI in patients with AIO, all of them lack sensitivity as well as specificity, and thus their diagnostic values in clinical practice are limited. Therefore, the identification of novel biomarkers of IMI with higher specificity and sensitivity for diagnosis, prognosis and treatment has potential benefits for improving the clinical strategy and outcome of IMI in patients with AIO, and a useful and unique serum or urine biomarker of IMI is urgently needed.

Recently, several studies have revealed that TJPs including ZO-1, occludin and claudin-1, which are the essential components of the intestinal mucosal barrier, reflect the changes of intestinal mucosal function (56–59), and thus the reduction of these proteins may indicate IMI. In the present study, the expression levels of

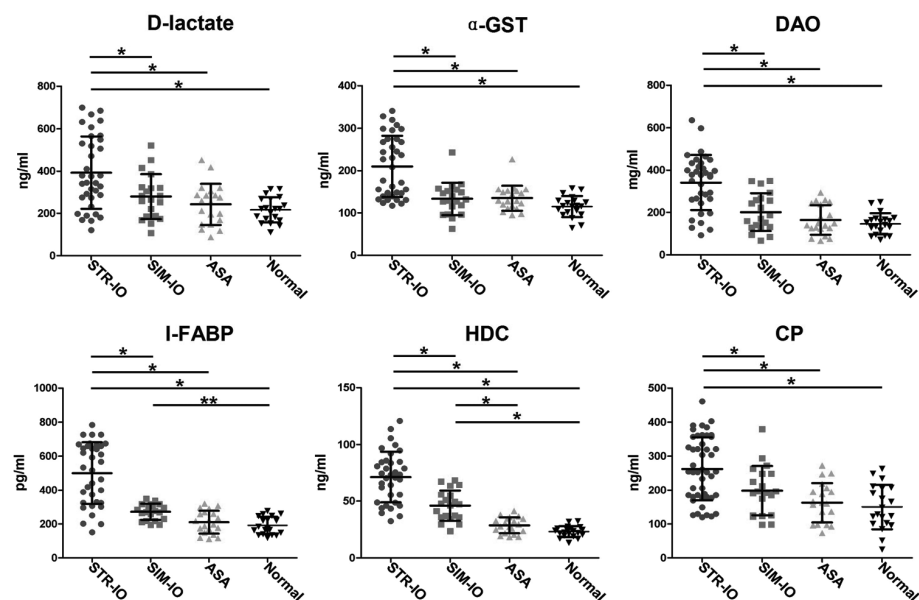


Figure 8. Serum concentrations of HDC, CP, D-lactate, α-GST, DAO and I-FABP in patients with STR-IO, SIM-IO or ASA and healthy subjects. *LSD *t* test, *P* < 0.01; **LSD *t* test, *P* < 0.05.

ZO-1, occludin and claudin-1 were tested at both mRNA and protein levels by QRT-PCR, Western blotting and IHC in colonic mucosal tissues of STR-IO patients and in the adjacent “normal” colonic tissues of patients with colon cancer, to confirm the existence of IMI in patients with AIO, mainly STR-IO. It was observed that the protein and mRNA expression of all three proteins was significantly decreased in STR-IO colonic tissues compared with that in “normal” colonic tissues. In addition, there were mucosal erosion, necrosis, infiltration of a large number of lymphocytes and neutrophils in the lamina propria of STR-IO mucosal tissues, and the overall inflammatory score of STR-IO mucosal tissues was significantly greater than that of the “normal” colonic mucosal tissues. All of these observations confirmed that there was IMI in STR-IO colonic tissues.

In the present study, we compared the global protein profiles among STR-IO, SIM-IO and healthy control serum using 2-D DIGE and linear trap quadrupole mass spectrometry. A total of six differentially expressed proteins with an increasing or a decreasing trend in parallel with increasing severity of colonic mucosal inflammation among the serum of patients with STR-IO, patients with SIM-IO and healthy controls were identified. These proteins, including isoform 1 of sex hormone-binding globulin, CP, apolipoprotein E, serum amyloid P-component, HDC and protein AMBP, were reported to be involved in various biological processes and thus may play important roles in IMI in patients with AIO.

Moreover, HDC and CP were further analyzed as potential biomarkers for IMI in patients with AIO. 2-D DIGE analysis revealed the expression levels of HDC and CP, with an increasing trend paralleling the increasing severity of colonic mucosal injury among normal healthy controls, patients with STR-IO and patients with SIM-IO, and their alterations were unambiguously confirmed by ELISA analysis. In addition, QRT-PCR, Western blotting and IHC showed that the mRNA and protein expression levels of HDC and CP were significantly increased in STR-IO colonic

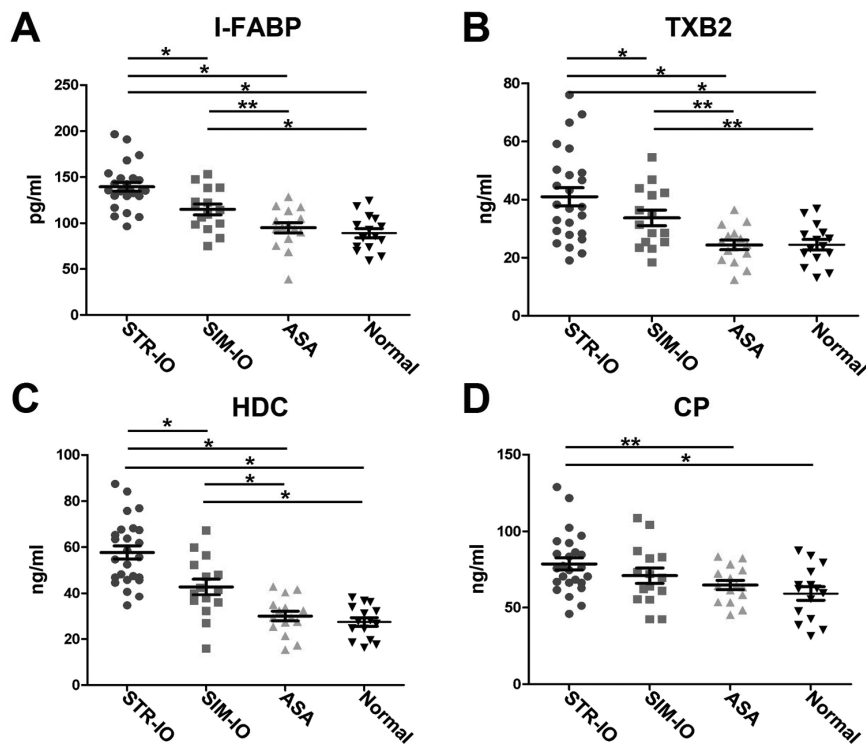


Figure 9. Urine concentrations of I-FABP (A), TXB2 (B), HDC (C) and CP (D) in patients with STR-IO, SIM-IO or ASA and healthy subjects. *LSD *t* test, *P* < 0.01; **LSD *t* test, *P* < 0.05.

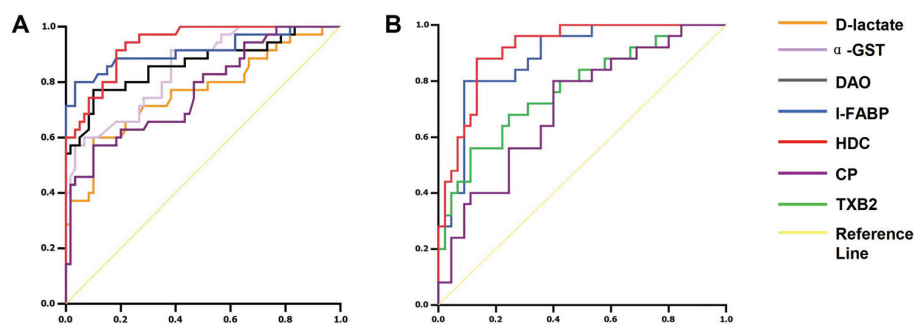


Figure 10. ROC curves of serum (A) and urine (B) biomarkers for differentiating patients with STR-IO from those with SIM-IO or ASA and healthy subjects.

tissues compared with the “normal” colonic tissues. IHC also showed that the overexpression of HDC, and CP in STR-IO colonic tissues, was associated with severity of the inflammation in the colonic mucosa, suggesting that the overexpression of these proteins might be attributable to the inflammatory response and may play an important role in IMI in patients with AIO, although more mechanistic studies are required to confirm this notion.

On the basis of the above observation that the expression of HDC and CP was significantly increased in STR-IO colonic tissues, we speculated that HDC and CP might be released from STR-IO colonic tissues to the circulation and then elimi-

nated through the urine, giving rise to elevated HDC and CP levels in the serum and urine. Therefore, we determined the levels of HDC and CP in the serum and urine of STR-IO patients, SIM-BO patients, ASA patients and healthy controls and confirmed that the serum and urine levels of HDC in STR-IO patients were indeed greater than those in SIM-BO patients, ASA patients and healthy controls, except the difference in the urine levels was not statistically significant between STR-IO and SIM-IO patients. In addition, other established serum and urine biomarkers (D-lactate, α -GST, I-FABP, DAO and TXB2) were also determined by ELISA. Similarly, the serum and urine lev-

els of these established biomarkers in STR-IO patients were greater than levels in SIM-BO patients, ASA patients and healthy controls.

ROC curve is a useful method for evaluating clinical usefulness of a biomarker and for comparing the effectiveness among different biomarkers (60,61). The potential clinical values of HDC and CP might be best discussed in the context of the established serum biomarkers (D-lactate, α -GST, I-FABP and DAO) and urine biomarkers (I-FABP and TXB2). Although these established biomarkers are not recommended for detection of IMI in patients with AIO because of the lack of sensitivity and specificity, they are still useful indicators for IMI in many current studies on IMI (19–25,29,30). The AUCs reached 0.937 and 0.770 for serum HDC and CP and 0.916 and 0.708 for urine HDC and CP, respectively, using ROC curve analysis. Moreover, the AUCs of HDC were the greatest among those of all other established biomarkers, both in serum and urine samples. The greater AUC represents the greater diagnostic ability (60,61), which means that HDC could be a potential, novel, sensitive and specific biomarker for diagnosis of IMI in patients with AIO. With the serum and urine cutoff values of 42.28 and 43.68 ng/mL, HDC would achieve a sensitivity of 91.4% and 88.0% and a specificity of 81.7% and 86.7%, respectively, in discriminating IMI in patients with AIO. However, with the serum and urine cutoff values of 229.04 and 69.59 ng/mL, CP would have a sensitivity of 62.9% and 64.0% and a specificity of 80.0% and 62.2%, respectively, which were lower than those achieved by serum and urine HDC levels. The sensitivities and specificities yielded by other established biomarkers (D-lactate, α -GST, I-FABP, DAO and TXB2) ranged from 60.0% to 88.6% and 73.3% to 85.0% for the serum levels and 72.0% to 80.0% and 68.9% to 84.4% for the urine levels, which were consistent with the findings reported by Evennett *et al.* (62). These observations indicate that HDC may achieve a better performance than the established biomarkers, both for the serum and urine lev-

Table 6. Sensitivity and specificity of serum biomarkers in differentiating patients with STR-IO from those with SIM-IO or ASA and healthy controls.

Biomarkers	Cutoff value	Sensitivity (%)	Specificity (%)
HDC (ng/mL)	42.28	91.4	81.7
CP (ng/mL)	229.04	62.9	80.0
D-Lactate (ng/mL)	320.04	60.0	85.0
α -GST (ng/mL)	169.93	68.6	73.3
I-FABP (pg/mL)	292.58	88.6	81.7
DAO (mg/mL)	251.61	77.1	83.3

Table 7. Sensitivity and specificity of urine biomarkers in differentiating patients with STR-IO from those with SIM-IO or ASA and healthy controls.

Biomarkers	Cutoff value	Sensitivity (%)	Specificity (%)
HDC (ng/mL)	43.68	88.0	86.7
CP (ng/mL)	69.59	64.0	62.2
I-FABP (pg/mL)	123.02	80.0	84.4
TXB2 (ng/mL)	28.91	72.0	68.9

els, and that the cutoff values of HDC in the serum and urine described above can be used to reliably identify IMI patients with AIO, although further investigation with a large sample size is required to confirm these preliminary observations.

HDC is the enzyme solely responsible for the production of histamine (63). Extensive studies have demonstrated that HDC activity is increased markedly in rapidly proliferating tissues, including fetal tissues of rats and mice, tissues undergoing repair and liver regeneration after hepatectomy (64). Fujimoto *et al.* (65) showed that HDC might also play a pivotal role in tissues repair and restoration of mucosal function observed after ischemia-reperfusion (I/R). In their study, where the superior mesenteric artery of the jejunal mucosa of male Sprague Dawley rats was occluded for 15 min, followed by reperfusion, they observed that histamine content and HDC activity were increased after I/R. The histamine output in the mesenteric lymph was also elevated after I/R. Their results suggest that histamine and HDC contribute to the restoration in the mucosal function observed at 48 h after I/R. In addition, the role of HDC in the repair of gastric mucosal injuries was also evaluated in another study, which demonstrated that the protein and mRNA levels of HDC rose gradually after the formation of the gastric-ulcer-of-rat model (66). The present study was the first to demonstrate HDC overexpression in IMI in patients with AIO; however, its precise role in the process of IMI in patients with AIO still needs further investigation.

There are a few limitations in the present study. First, the adjacent "normal" colonic tissues of patients with colon cancer were used compared with colonic tissues from patients with STR-IO. Ideally, the colonic tissues from healthy individuals should be used as normal controls. However, it is not ethical to collect colonic tissues from healthy individuals. Nevertheless, theoretically, the adjacent "normal" colon tissues should not suffer from IMI and thus would not be associated with changes in the expression of IMI-associated proteins. Second, the sample size in the validation

analysis appears small. Inclusion of a small sample size in the present study was mainly due to two reasons. The first reason is that our inclusion criteria and exclusion criteria were very strict. The second reason is that the collection of a large number of serum and urine samples from patients with STR-IO within a relatively short period of time in a single center was not possible. Obviously, the discriminating power of HDC identified and verified in the present study needs to be further confirmed in large cohort, multicenter, long-term and independent studies.

In conclusion, the expression of TJPs including ZO-1, occludin and claudin-1 was increased at both mRNA and protein levels in the colonic tissues of patients with STR-IO. Among six proteins that were differentially expressed in patients with STR-IO, HDC and CP were elevated significantly in patients compared with the "normal" healthy controls. Finally, serum and urine HDC levels achieved sensitivities and specialties compatible or even greater than those of established biomarkers for the detection of IMI in patients with AIO, although further validation in a larger cohort is required.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecu-*

lar Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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