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ORIGINAL ARTICLE

Serum and urine metabolomic fingerprinting in diagnostics of inflammatory bowel diseases

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Abstract

AIM: To evaluate the utility of serum and urine metabolomic analysis in diagnosing and monitoring of inflammatory bowel diseases (IBD).

METHODS: Serum and urine samples were collected from 24 patients with ulcerative colitis (UC), 19 patients with the Crohn's disease (CD) and 17 healthy controls. The activity of UC was assessed with the Simple Clinical Colitis Activity Index, while the activity of CD was determined using the Harvey-Bradshaw Index. The analysis of serum and urine samples was performed using proton nuclear magnetic resonance (NMR) spectroscopy. All spectra were exported to Matlab for preprocessing which resulted in two data matrixes for serum and urine. Prior to the chemometric analysis, both data sets were unit variance scaled. The differences in metabolite fingerprints were assessed using partial least-squares-discriminant analysis (PLS-DA). Receiver operating characteristic curves and area under curves were used to evaluate the quality and prediction performance of the obtained PLS-DA models. Metabolites responsible for separation in models were tested using STATISTICA 10 with the Mann-Whitney-Wilcoxon test and the Student's *t* test ($\alpha = 0.05$).

RESULTS: The comparison between the group of patients with active IBD and the group with IBD in remission provided good PLS-DA models (*P* value 0.002 for serum and 0.003 for urine). The metabolites that

allowed to distinguish these groups were: *N*-acetylated compounds and phenylalanine (up-regulated in serum), low-density lipoproteins and very low-density lipoproteins (decreased in serum) as well as glycine (increased in urine) and acetoacetate (decreased in urine). The significant differences in metabolomic profiles were also found between the group of patients with active IBD and healthy control subjects providing the PLS-DA models with a very good separation (P value < 0.001 for serum and 0.003 for urine). The metabolites that were found to be the strongest biomarkers included in this case: leucine, isoleucine, 3-hydroxybutyric acid, *N*-acetylated compounds, acetoacetate, glycine, phenylalanine and lactate (increased in serum), creatine, dimethyl sulfone, histidine, choline and its derivatives (decreased in serum), as well as citrate, hippurate, trigonelline, taurine, succinate and 2-hydroxyisobutyrate (decreased in urine). No clear separation in PLS-DA models was found between CD and UC patients based on the analysis of serum and urine samples, although one metabolite (formate) in univariate statistical analysis was significantly lower in serum of patients with active CD, and two metabolites (alanine and *N*-acetylated compounds) were significantly higher in serum of patients with CD when comparing jointly patients in the remission and active phase of the diseases. Contrary to the results obtained from the serum samples, the analysis of urine samples allowed to distinguish patients with IBD in remission from healthy control subjects. The metabolites of importance included in this case up-regulated acetoacetate and down-regulated citrate, hippurate, taurine, succinate, glycine, alanine and formate.

CONCLUSION: NMR-based metabolomic fingerprinting of serum and urine has the potential to be a useful tool in distinguishing patients with active IBD from those in remission.

Key words: Metabolomics; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Proton nuclear magnetic resonance spectroscopy; Urine; Serum; Partial least-squares-discriminant analysis

Core tip: This study demonstrates that proton nuclear magnetic resonance -based metabolic fingerprinting of human serum and urine combined with multivariate data analysis could be a useful tool for differentiation between the active and remission phase of the disease that is of great importance in inflammatory bowel diseases (IBD) monitoring. At the same time, the results indicate that this diagnostic method has rather a weak potential in the differential diagnosis of IBD.

Dawiskiba T, Deja S, Mulak A, Ząbek A, Jawień E, Pawełka D, Banasik M, Mastalerz-Migas A, Balcerzak W, Kaliszewski K, Skóra J, Barć P, Korta K, Pormańczuk K, Szyber P, Litarski A, Młynarz P. Serum and urine metabolomic fingerprinting in diagnostics of inflammatory bowel diseases. *World J Gastroenterol* 2014; 20(1): 163-174 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i1/163.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i1.163

INTRODUCTION

Ulcerative colitis (UC) and the Crohn's disease (CD) are the main well-characterized forms of inflammatory bowel diseases (IBD). Although the etiology of IBD remains not fully explained, it is widely accepted that this multifactorial condition is associated with an inadequate immune response to environmental factors in genetically predisposed individuals[1,2]. The diagnosis of IBD is based on the combination of clinical, endoscopic, histopathological, serological and radiological features but the precise distinction between UC and CD still remains a challenge in 5%-20% cases[3-6]. The diagnosis may be difficult due to the clinical overlapping between these two conditions, an incomplete expression of IBD or deficiency in histological response of the intestinal mucosa[4]. Moreover, the serological markers like anti-*Saccharomyces cerevisiae* antibodies and perinuclear antineutrophilic cytoplasmic antibodies recently implemented in IBD diagnostics have been found to have a high specificity in some studies, but a relatively low sensitivity[5,7]. The differential diagnosis between UC and CD is also essential regarding distinct approaches with respect to surgical treatment, moreover the diseases differ from each other in terms of prognosis, a tendency for recurrence and risks of cancer[3,4]. This situation as well as the limited number of IBD monitoring tools (the commonly used erythrocyte sedimentation rate and C-reactive protein do not always correlate with disease activity and are not specific to IBD inflammation) force researchers to look for new approaches in IBD diagnostics[8].

The use of metabolomics in medical diagnostics has recently become a very promising idea that is extensively studied in oncological conditions, diabetes, cardiovascular diseases, rheumatoid arthritis or multiple sclerosis[9-17]. Unlike the genomic and proteomic studies that examine genes and more or less complex proteins, metabolomic analysis allows to assess the simplest low molecular weight metabolites that are involved in disease processes (the basic forms and various derivatives of amino acids, ketones, fatty acids, amines, organic acids, nucleosides, aromatic compounds, sterols, saccharides *etc.*)[3,18-20]. There are several thousands of such metabolites in tissues and fluids of living organisms like blood, urine, cerebrospinal fluid or saliva, and most of those metabolites have a well-defined function. At the same time, the highly informative metabolic profiles as a potential source of biomarkers can be used not only for prognostic purposes, but also as an input for discriminant analysis, even when there are only premorphological biochemical changes that are not evident on histopathological examination[4,21].

So far there are only a few research studies that evalu-

Table 1 Demographic data and clinical profile of patients

	Ulcerative colitis	Crohn's disease	Healthy controls
Number of patients	24	19	17
Sex (male/female)	15/9	11/8	8/9
Average age (mean/range)	40.7 (18-70)	35.8 (18-61)	44.7 (24-62)
Mean activity score SCCAI/HBI (range)	6.1 (0-13)	6.7 (1-18)	-
Number of patients in active/remission phase of disease	12/12	16/3	-
Average disease duration (yr)	8.2 (0.1-32)	5.5 (0.3-21)	-

SCCAI: Simple clinical colitis activity index; HBI: Harvey-bradshaw index.

ate the usefulness of metabolomics in IBD diagnostics in humans. Fecal extracts[22], intestinal tissue biopsies[1,3,4,23], urine[1,9,24,25] and blood[21,24,26,27] were assessed as the source of potential biomarkers. It should be noted that the results of some investigations were contradictory, at the same time, to date there has been no study in which proton nuclear magnetic resonance (^{1}H NMR)-based combined serum and urine metabolomic analysis had been used to differentiate patients in the active and remission phase of IBD. The aim of our study was to answer the question if the ^{1}H NMR spectroscopy-based metabolic fingerprinting of human serum and urine combined with multivariate data analysis could be useful diagnostic tool in diagnosing and monitoring of IBD.

MATERIALS AND METHODS

Patient recruitment

Samples were collected from patients who were hospitalized at the three cooperating clinics of Wroclaw Medical University: the Department of Gastroenterology and Hepatology, the First Department and Clinic of General, Gastroenterological and Endocrinological Surgery and the Department of Vascular, General and Transplantation Surgery. The protocol of this study was approved by the Commission of Bioethics at Wroclaw Medical University (Approval no. KB-597/2011) and written informed consents were obtained from all the patients before enrolment in the study.

Serum and urine samples were collected from adult individuals with established diagnosis of UC (n = 24) and CD (n = 19). Reference samples were taken from healthy control individuals (n = 17). No patients with indeterminate colitis were included. The activity of UC was assessed with the Simple Clinical Colitis Activity Index (SCCAI)[28], while the activity of CD was determined using the Harvey-Bradshaw Index (HBI)[29]. Based on the individual SCCAI/HBI scores the patients were classified into the subgroup with the active or remission phase of the disease (remission was defined as SCCAI < 5 for patients with UC and HBI < 5 for patients with CD).

Patients aged over 75 years or below 18 years, with severe mental illness, infectious diseases, structural abnormalities of the gastrointestinal tract, diabetes mellitus, renal failure, hepatic dysfunction, evidence of malignancy and other severe diseases were excluded from our study. To avoid the dietary influence on the metabolic fingerprints of serum and urine the samples were taken after overnight fasting. Signals related to medications (5-aminosalicylate, azathioprine and acetaminophen) were eliminated from the statistical and chemometric evaluation. The characteristics of patients and healthy control subjects are shown in Table 1.

Sample handling and NMR spectroscopy

Serum samples were thawed in room temperature and mixed using a vortex mixer. According to a well-established protocol[30], aliquots of 200 μL of serum were transferred into a polypropylene Eppendorf tube and 400 μL of 0.9% saline solution in 15% D_2O, containing 3 mmol/L 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionate sodium salt TSP-d4 (TSP) were added. The samples were centrifuged for 10 min at 12000 × g and 550 μL of each sample supernatant was subsequently moved into a 5 mm NMR tube. ^{1}H NMR experiments were performed with the following parameters: relaxation delay, 3.5 s; acquisition time, 2.73 s; number of transients (scans), 128; number of points, 65536; pulse program, cpmgpr1d (Bruker notation); number of loops, 80; spin echo delay, 400 ms; spectral width, 20 ppm; line-broadening factor, 0.3 Hz. The spectra were manually corrected for phase and baseline distortions and were referenced to α-glucose signal (δ = 5.225 ppm).

Urine exhibits high pH and ionic strength variability which can dramatically affect NMR spectra quality by introducing an unwanted variance in the chemical shifts of resonances. In order to overcome this issue we used a modified method of Jiang *et al*[31] utilizing the additional step of precipitation of the most abundant urine salts: Mg^{2+} and Ca^{2+}. All urine samples were thawed in room temperature and mixed using a vortex mixer. Aliquots of 570 μL of urine were transferred into polypropylene Eppendorf tubes and 30 μL of KF solution was added, and samples were mixed again creating insoluble in water MgF_2 and CaF_2 salts. The samples were centrifuged for 10 min at 12000 × g and 540 μL of supernatant was transferred into a new Eppendorf tube. Next, the samples were mixed with 60 μL of 2.5 mol/L phosphate buffer solution with a pH of 7.00 in 99.8% D_2O containing TSP as an internal standard. The final concentration of TSP in the sample was 1 mmol/L. The samples were mixed again and finally 550 μL was moved into a 5 mm NMR tube. ^{1}H NMR experiments were performed with the following parameters: relaxation delay, 3.5 s; acquisition time, 1.36 s; number of transients, 64; number of points, 32768; pulse program, noesypr1d (Bruker notation); spectral width, 20 ppm, line-broadening factor, 0.3 Hz. The spectra were manually corrected for phase and baseline distortions and were referenced to the TSP resonance at δ = 0.0 ppm.

All NMR spectra were recorded at 300 K using a Bruker Avance II 600 spectrometer (Bruker GmBH,

Germany) operating at proton frequency of 600.58 MHz.

Data processing and multivariate statistical data analysis

All spectra were exported to Matlab (Matlab v. 8.1, Mathwork Inc.) for preprocessing which resulted in two data matrixes for serum and urine, respectively. Regions affected by solvent suppression were excluded (4.645-5.100 ppm for serum and 4.660-5.055 ppm for urine) and alignment procedures involving correlation optimized warping (COW)[32] and interval correlation shifting (icoshift) algorithms were applied[33].

Spectra of urine were aligned in two steps: first a global correction was performed using COW algorithm, while in the second step a custom interval approach was applied to adjust peak positions in baseline separated regions defined by the researcher. Urine spectra consisted of 24609 data points and were normalized using the Probabilistic Quotient method (PQ) to overcome the issue of dilution[34].

Serum samples are much more stable compared with urine and usually demonstrate only small variation in resonance chemical shift. However, alignment may be required especially for the fingerprinting approach where even small errors could affect the multivariate analysis and bilinear data are required. For that reason correction of peak shifts was done using only the icoshift algorithm. Serum spectra consisted of 24446 data points and were normalized using the PQ to overcome the issue of dilution[34].

Prior to the chemometric analysis, both data sets were unit variance (UV) scaled. The differences in metabolite fingerprints were assessed using partial least-squares-discriminant analysis (PLS-DA). A default 7-fold cross validation (CV) was applied to each PLS-DA model (1/7 of the samples being excluded from calculations in each round).

Receiver Operating Characteristic (ROC) Curves and Area Under Curves (AUC) were used to evaluate the prediction performance of the obtained PLS-DA models. A performance curve algorithm from the Matlab statistics toolbox (Matlab v. 8.1, Mathwork Inc.) was utilized for calculations. Specificity and sensitivity were determined based on sample class prediction: predicted values of the fitted Y for observations (in SIMCA-P+ software: Y-predcv computed based on the 7-fold cross validation procedure).

Metabolites responsible for separation in models were tested using STATISTICA 10 with the Mann-Whitney-Wilcoxon test (MWW) and the Student's *t* test. A 0.05 level of probability was used as the criterion for statistical significance.

RESULTS

Metabolic fingerprinting of serum samples from IBD patients

Median spectra of serum from IBD patients and healthy control subjects are shown in Figure 1, while the parameters of PLS-DA models obtained from ^{1}H NMR analysis of serum samples are shown in Table 2.

In total, 43 low molecular weight compounds were assigned in serum spectra. The comparison between the group of patients with active IBD and the group with IBD in remission provided a good PLS-DA model. The metabolites that allowed to distinguish these groups were down-regulated low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) as well as up-regulated *N*-acetylated compounds, phenylalanine and the unknown metabolite with signal at δ 1.39 ppm. The significant differences were also found between the group of patients with active IBD and healthy control subjects providing the PLS-DA model with a very good separation that passed validation test (CV-ANOVA, $P < 0.05$). In this case the following metabolites were increased in IBD patients: leucine, isoleucine, 3-hydroxybutyric acid, *N*-acetylated compounds, acetoacetate, glycine, phenylalanine, lactate and one unknown (Unk) metabolite with signal at δ 1.39 ppm mentioned above. Simultaneously lower levels of creatine, dimethyl sulfone, choline and its derivatives glycerophosphocholine (GPC) and phosphocholine (PC) as well as histidine were observed (Table 3).

No clear separation in PLS-DA models was found between CD and UC patients (all the patients as well as only those in the active phase of the disease). Only one metabolite (formate) in univariate statistical analysis was significantly lower in patients with active CD, and two metabolites (alanine and *N*-acetylated compounds) were significantly higher in patients with CD when comparing jointly patients in the remission and active phase of the diseases. It was also not possible to distinguish IBD patients in remission from healthy control subjects, although there were two statistically relevant differences in serum metabolic fingerprints between those cohorts of patients. The IBD patients in remission were characterized by a significantly higher concentration of dimethylamine and a lower level of dimethyl sulfone.

Metabolic fingerprinting of urine samples from IBD patients

Median spectra of urine from IBD patients and healthy control subjects are shown in Figure 2, while the parameters of PLS-DA models obtained from urine samples based on ^{1}H NMR analysis are shown in Table 4.

As in the case of the serum samples, the most significant differences in urine were found between the group of patients with active IBD and the group with IBD in remission, and between the group of patients with active IBD and healthy control subjects. In the first case a lower concentration of acetoacetate and a higher concentration of glycine were detected, while in the second case citrate, hippurate, trigonelline, taurine, succinate, 2-hydroxyisobutyrate (down-regulated) and an unknown metabolite with 4-hydroxyphenyl group and signal at δ 6.85 ppm (up-regulated) were found to be the strongest biomarker candidates (Table 5).

Contrary to the results obtained from the serum sam-

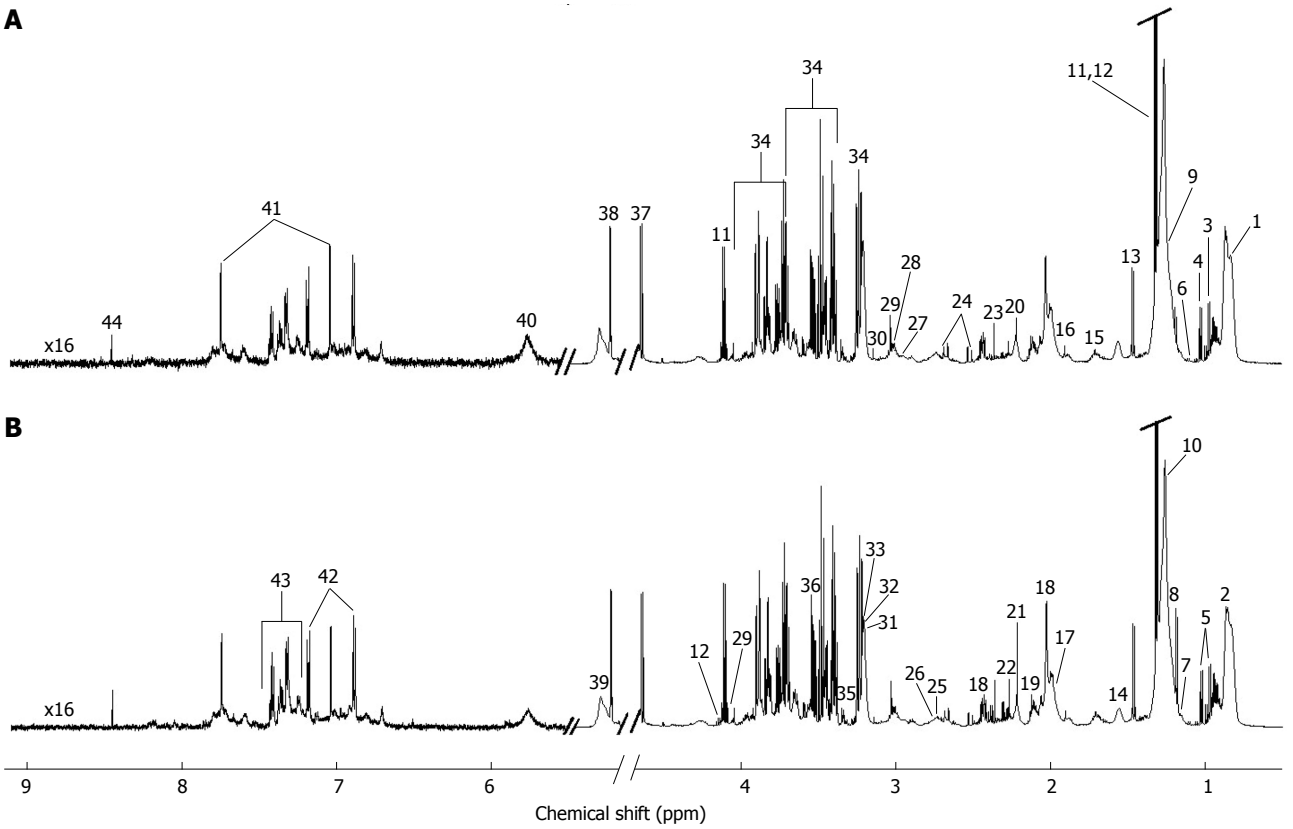

Figure 1 Median 600 MHz proton nuclear magnetic resonance noesy spectra of serum samples. A: Healthy control subjects; B: Patients with inflammatory bowel diseases. 1: Lipids LDL CH_3-$(CH_2)_n$-; 2: Lipids VLDL CH_3-$(CH_2)_n$-; 3: Leucine; 4: Isoleucine; 5: Valine; 6: 3-Methyl-2-oxovalerate; 7: Isobutyrate; 8: 3-Hydroxybutyrate; 9: Lipids LDL CH_3-$(CH_2)_n$-; 10: Lipids VLDL CH_3-$(CH_2)_n$-; 11: Lactate; 12: Threonine; 13: Alanine; 14: Lipids VLDL CH_2-CH_2-C=O; 15: Lysine; 16: Acetate; 17: Lipids -CH_2-CH=CH-; 18: N-acetylated compounds; 19: Glutamine; 20: Lipids -CH_2-C=O; 21: Acetone; 22: Acetoacetate; 23: Pyruvate; 24: Citrate; 25: Dimethylamine; 26: Lipids =CH-CH_2-CH=; 27: Trimethylamine; 28: Creatine; 29: Creatinine; 30: Dimethyl sulfone; 31: Choline; 32: Phosphocholine; 33: Glycerophosphocholine; 34: α and β-Glucose; 35: Methanol; 36: Glycine; 37: β-Glucose; 38: α-Glucose; 39: Lipids -CH=CH-; 40: Urea; 41: Tyrosine; 42: 1-Methylhistidine; 43: Phenylalanine; 44: Formate.

Table 2 The partial least-squares-discriminant analysis models obtained from proton nuclear magnetic resonance-based analysis of serum samples

Comparison	Model	*P* value	Q^2 (cum)	Number of latent variables
CD *vs* UC	PLS-DA	0.069	0.43	2
CD active *vs* UC active	PLS-DA	0.798	0.46	2
IBD active *vs* Control	PLS-DA	< 0.001	0.79	4
IBD remission *vs* Control	PLS-DA	0.372	0.26	2
IBD active *vs* IBD remission	PLS-DA	0.002	0.76	2

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel diseases; PLS-DA: Partial least-squares-discriminant analysis.

ples, the analysis of urine samples allowed to distinguish patients with IBD in remission from healthy control subjects. The following metabolites were down-regulated in IBD patients in remission: citrate, hippurate, taurine, succinate, glycine, alanine and formate, and only acetoacetate was up-regulated (Table 5). It is worth noticing that the first four metabolites listed above (citrate, hippurate, taurine, succinate) significantly changed in the same direction in patients in both the active and remission phase of IBD.

As in the case of serum samples, the PLS-DA models between CD and UC patients did not pass the validation test (all the patients as well as only those in the active phase of the disease). There were no statistically relevant differences in urine metabolic fingerprints between those two cohorts of patients.

DISCUSSION

In the present study we used a ^{1}H NMR-based metabolomic approach to demonstrate metabolic differences between patients with active IBD and those in remission. Additionally, we investigated the differences between CD and UC patients as well as IBD subjects and healthy controls. The obtained results confirm the potential of this method in IBD monitoring, simultaneously it may provide the understanding of pathogenic mechanisms of the diseases. Notably, to date there are only few research studies that evaluated the metabolomic based diagnostic tests of serum[21,24,26,27] and urine[1,9,24,25] in patients with IBD. The earlier studies in animals (most often using a model of acute colitis in interleukin-10 gene deficient mouses or dextran sulfate sodium-induced colitis) provided only the comparison of the disease state *vs* healthy

Table 3 Significantly changed serum metabolites

Metabolite	Percentage difference					Relative Standard Deviation (%)						
	CD *vs* UC	CD active *vs* UC active	IBD active *vs* control	IBD remission *vs* control	IBD active *vs* IBD remission	CD	CD active	UC	UC active	IBD active	IBD remission	Control
Lipids LDL CH_3-$(CH_2)_n$-	-7.2	-4.0	-11.6	0.4	-11.9[a]	20.7	20.5	20.4	18.1	19.2	20.9	24.8
Lipids VLDL CH_3-$(CH_2)_n$-	-6.4	-6.0	-12.9	-2.2	-10.9[c]	18.3	18.9	20.9	26.0	22.2	13.7	24.0
Leucine	1.2	0.7	15.3[a]	11.6	3.3	25.1	25.9	19.9	21.2	23.6	19.3	10.8
Isoleucine	6.2	6.5	23.6[a,c]	12.4	9.9	22.2	20.5	22.5	24.8	22.1	22.0	13.9
3-Hydroxybutyrate	-6.5	3.8	52.6[a,c]	45.9	4.6	50.2	48.1	76.8	53.9	49.6	94.4	34.1
Unk1 δ 1.39 ppm	4.9	1.8	9.3[a,c]	-3.0	12.7[a,c]	11.3	10.4	10.4	7.5	9.2	10.0	10.8
Alanine	12.2[c]	13.7	8.5	6.0	2.4	22.1	24.1	24.3	27.5	25.8	19.8	16.2
N-acetylated compounds	8.9[a,c]	3.2	16.1[a,c]	1.0	15.0[a,c]	11.1	11.3	14.1	13.1	12.0	11.1	8.4
Acetoacetate	-2.8	4.4	77.3[a]	50.7	17.7	79.8	76.7	97.9	79.8	76.6	118.6	49.1
Citrate	2.6	2.5	-3.9	-10.9	7.9	20.5	20.2	23.9	26.1	22.4	21.5	27.5
Dimethylamine	-4.5	- 6.0	14.5	16.1[a,c]	-1.5	23.4	25.4	16.3	16.9	21.8	15.0	22.7
Creatine	5.5	-4.1	-14.7[c]	-13.7	-1.1	24.9	22.6	31.4	31.0	26.2	32.9	28.4
Dimethyl sulfone	-0.9	4.0	-31.0[a,c]	-22.9[a]	-10.5	31.2	33.1	30.1	25.6	29.8	30.4	36.1
Choline + PC + GPC	-5.0	-0.6	-15.0[a]	-2.2	-13.0	21.5	21.9	24.0	21.2	21.2	23.3	27.9
Glycine	6.8	0.8	26.6[a,c]	13.7	11.3	20.3	21.2	24.6	26.8	23.3	19.9	21.2
Phenylalanine	6.8	0.1	19.8[a,c]	-0.6	20.5[a,c]	25.9	26.4	22.3	20.7	23.7	18.8	18.4
Histidine	2.6	7.8	-16.4[a,c]	-6.7	-10.4	33.2	36.3	22.6	26.4	32.5	16.6	19.0
Formate	-10.1	-22.7[c]	27.3	32.4	-3.8	48.5	51.1	32.5	30.6	42.7	34.2	45.6
Lactate	4.1	10.3	17.8[c]	13.2	4.1	20.7	19.9	28.1	29.4	24.0	26.8	36.5

Percentage difference was calculated based on the mean values of relative signal integrals in each group. The calculations were made from left to right, *e.g.*, a positive value in IBD active *vs* Control means that a particular metabolite exhibits higher concentration in patients with active IBD compared to the Control group, [a]$P < 0.05$ using the Student's *t* test, [c]$P < 0.05$ using the Mann-Whitney-Wilcoxon test. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel diseases; LDL: Low-density lipoproteins; VLDL: Very low-density lipoproteins; PC: Phosphocholine; GPC: Glycerophosphocholine; Unk1: Unknown metabolite.

Table 4 The partial least-squares-discriminant analysis models obtained from proton nuclear magnetic resonance-based analysis of urine samples

Comparison	Model	*P* value	Q^2 (cum)	Number of latent variables
CD *vs* UC	PLS-DA	0.388	0.1	2
CD active *vs* UC active	PLS-DA	1.000	-0.2	2
IBD active *vs* control	PLS-DA	0.003	0.6	4
IBD remission *vs* control	PLS-DA	0.037	0.31	2
IBD active *vs* IBD remission	PLS-DA	0.003	0.38	3

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel diseases; PLS-DA: Partial least-squares-discriminant analysis.

controls and did not allow to evaluate CD *vs* UC or to compare exacerbation *vs* remission[35-42].

To the best of our knowledge this is the first study in which ^{1}H NMR-based combined serum and urine metabolomic analysis is used to differentiate patients in the active and remission phase of IBD. The obtained results allowed to create very good cross-validated PLS-DA models with a high predictability for both biofluids (Figures 3 and 4). The metabolites that were found to be the strongest candidates for biomarkers were: phenylalanine and *N*-acetylated compounds in serum as well as glycine in urine (higher concentration), and LDL and VLDL in serum as well as acetoacetate in urine (lower concentration). The postulated mechanism of serum LDL and VLDL down-regulation demonstrated in our study is the systemic activation of the inflammatory response with an increase in secretory phospholipase A2 activity that promotes hydrolysis of phospholipids in LDL and VLDL[43]. Moreover, the effects of the proinflammatory cytokines like tumor necrosis factor-α, interleukin-1 and interferon-c, which affect the expression of lipoprotein lipase that hydrolyzes triglycerides in lipoproteins, are also considered[44,45]. In addition, it has been shown that lipoproteins in IBD patients are synthesized with abnormal apolipoprotein structure[46,47]. The up-regulation of serum *N*-acetylated compounds that was demonstrated in the active phase of the disease could be mainly associated with α1-acid glycoprotein that is an acute-phase protein[26,48,49], while the increase in the concentration of serum phenylalanine is most likely caused by the impairment of phenylalanine-4-hydroxylase activity (a hepatic enzyme that converts phenylalanine into tyrosine) associated with immune activation and inflammation[50]. The increased glycine concentration in urine in patients in the active phase of IBD observed in our study seems to be related with its anti-inflammatory and cytoprotective effects including the inhibition of glycine-gated chloride channels, direct effects on target cells and/or inhibition of neutrophils and macrophages activation[51,52]. At the same time, the decrease in urine acetoacetate concentration was rather unexpected. Acetoacetate as a ketone body reflects the demand of the body for energy, so it is naturally increased in the serum of patients with inflammatory disorders (just as in our study when comparing the group

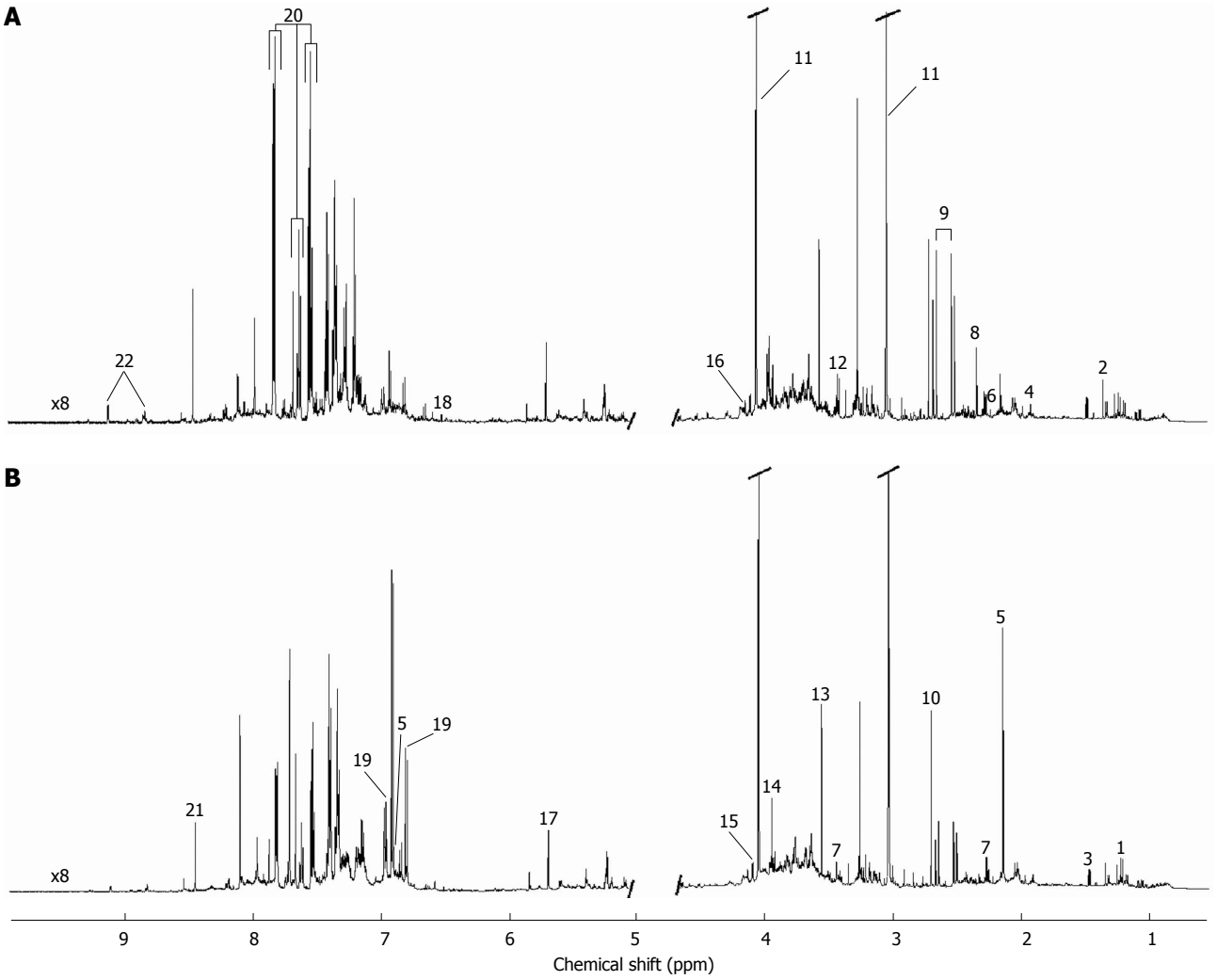

Figure 2 Median 600 MHz proton nuclear magnetic resonance noesy spectra of urine samples. A: Healthy control subjects; B: Patients with inflammatory bowel diseases. 1: 3-hydroxyisovalerate; 2: 2-hydroxyisobutyrate; 3: Alanine; 4: Acetate; 5: Acetaminophen; 6: Acetone; 7: Acetoacetate; 8: Succinate; 9: Citrate; 10: Dimethylamine; 11: Creatinine; 12: Taurine; 13: Glycine; 14: Creatine; 15: Lactate; 16: Threonine; 17: Cis-aconitate; 18: Fumarate; 19: 5-aminosialicylate; 20: Hippurate; 21: Formate; 22: Trigonelline.

of patients with active IBD and healthy controls). In only two found in literature research studies that compared the metabolomic profiles of patients in the active and remission phase of IBD no significant differences were found in one case (only urine evaluation of UC patients)[1], while in the other one based on the chromatography data system and generation of a multivariate index using the "AminoIndex™ technology" (MIAI) a very good separation was achieved with a decrease in the concentration of tryptophan and histidine (only serum analysis)[21].

Our results demonstrate that there are many metabolites that can be considered as potential biomarkers of IBD. At the same time, it is worth noticing that no comparison between IBD patients in the remission phase of the disease and healthy control subjects has been made so far. The metabolomic serum analysis in IBD patients in the active phase of the disease compared to healthy controls revealed an increase in the concentration of leucine, isoleucine, 3-hydroxybutyric acid, *N*-acetylated compounds, acetoacetate, glycine, phenylalanine, lactate and a simultaneous decrease in the concentration of creatine, dimethyl sulfone, choline compounds, and histidine. At the same time, we found only two statistically relevant differences in serum metabolomic profiles between IBD patients with quiescent disease and healthy control subjects (a higher level of dimethylamine and a lower level of dimethyl sulfone) and in this case it was not possible to distinguish the cohorts by multivariate modeling. In contrast to the serum analysis, urine metabolomic tests proved that it is possible to distinguish IBD patients from healthy controls both in the active and remission phase of the disease. The active phase of IBD was associated with a higher concentration of an unknown metabolite with 4-hydroxyphenyl group and a lower concentration of citrate, hippurate, trigonelline, taurine, succinate, 2-hydroxyisobutyrate. Remission was characterized by a higher concentration of acetoacetate and a lower concentration of citrate, hippurate, taurine, succinate, glycine, alanine and formate. The obtained results reflect changes in cellular energy metabolism that occur in patients with

Table 5 Significantly changed urine metabolites

Metabolite	Percentage difference					Relative Standard Deviation (%)						
	CD *vs* UC	CD active *vs* UC active	IBD active *vs* control	IBD remission *vs* control	IBD active *vs* IBD remission	CD	CD active	UC	UC active	IBD active	IBD remission	Control
Citrate	6.9	-4.0	-32.7[c]	-47.6[a,c]	28.3	73.2	72.8	91.4	111.7	90.4	45.1	57.8
Hippurate	7.2	29.7	-53.3[a,c]	-35.1[c]	-28.1	66.6	61.3	123.5	70.7	64.9	122.9	78.4
Glycine	23.3	11.4	12.6	-29.8[a]	60.3[a,c]	43.6	40.1	47.9	45.9	42.0	36.3	50.3
Alanine	-0.2	-12.2	-9.1	-30.3[a,c]	30.3	36.8	36.0	47.7	49.4	42.8	34.9	51.7
Formate	2.9	1.5	-17.0	-30.6[a,c]	19.5	47.1	42.1	37.4	37.2	39.4	45.3	38.8
Trigonelline	-10.2	139.6	-73.5[a,c]	-51.7	-45.1	148.3	144.8	137.4	64.3	150.8	121.9	133.7
Acetoacetate	-6.2	82.2	7.8	85.1[c]	-41.7[c]	125.9	133.8	122.5	58.0	129.3	108.4	92.3
Taurine	7.0	21.7	-28.1[a,c]	-31.8[a,c]	5.5	66.5	66.1	35.1	35.0	57.8	38.3	41.4
Succinate	2.3	-11.4	-63.7[a,c]	-51.8[a,c]	-24.7	128.9	120.5	93.6	95.4	107.4	111.1	74.1
2-hydroxyisobutyrate	17.3	30.2	-68.1[c]	-66.8	-3.9	41.5	44.9	38.4	31.4	42.9	37.2	256.5
Unk2 with 4-hydroxyphenyl group δ 6.85 ppm	0.8	-5.0	78.5[a,c]	33.1	34.1	63.8	62.2	73.0	81.9	70.4	53.3	45.3

Percentage difference was calculated based on the mean values of relative signal integrals in each group. The calculations were made from left to right, *e.g.*, a positive value in IBD active *vs* Control means that a particular metabolite exhibits higher concentration in patients with active IBD compared to the Control group, [a]$P < 0.05$ using the Student's *t* test, [c]$P < 0.05$ using the Mann-Whitney-Wilcoxon test. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel diseases; Unk2: Unknown metabolite.

IBD. The increase in serum concentration of ketone bodies (like acetoacetate and 3-hydroxybutyric acid) used as an alternative source of energy when glucose level is insufficient[27,53], as well as the decrease in the concentration of Krebs cycle intermediates (such as citrate and succinate) and creatine (that serves as an energy reservoir[54]) indicate a high demand of the organism for energy and rapid utilization of metabolites[9,24]. Similarly, the increased concentration of lactate that is probably secreted from inflamed colonic mucosa cells[36] indicates the up-regulation of glycolysis, which occurs in case of higher cellular energy need and/or lack of energy sources under inflammatory conditions[42,55]. The decreased level of membrane metabolites like choline and its derivatives (PC and GPC) that was demonstrated in the study is most probably caused by the increased use of choline in IBD patients[26] associated with a change in mucosal membrane integrity[3,56], extensive apoptosis and turnover of intestinal cell membranes under inflammatory conditions[26]. Additionally, choline is indirectly involved in the synthesis of the platelet-activating factor, which is a major inflammatory mediator[54,57]. Finally, a lower urine concentration of gut microbial-host cometabolites like hippurate (its level positively correlates with the presence of *Clostridia* spp.[25,58]) and formate (formed in the colonic lumen by intestinal bacteria such as *Enterobacteriaceae*, in the process of unabsorbed carbohydrates fermentation[3,24,59]) may be associated with changes in gut microbiome that occur in IBD patients[60].

Our observations concerning the discrimination of IBD patients from healthy controls correlate well with most of previously published results[9,21,24-27], although, as it was mentioned before, the comparison between IBD patients in the remission phase of the disease and healthy control subjects was never made before. In the serum of IBD patients as most prominently increased there were reported: *N*-acetylated glycoprotein, lactate[26], methanol, mannose, formate, 3-methyl-2-oxovalerate, 2-hydroxybutyrate, lysine, creatine[24], 3-hydroxybutyrate, phenylalanine, α and β-glucose[27] while those decreased included: lipids[26,27], urea, τ-methylhistidine, valine, tyrosine, citrate, choline, creatinine, dimethyl sulfone[24], histidine, tryptophan[21] and alanine[26]. Concomitantly, as most prominently increased metabolites in urine were characterized: mannitol, allantoin, xylose, carnitine, lactate, threonine[24] and formate[25], whereas decreased hippurate[9,24,25], succinate, citrate, 1-methylhistidine, methanol, formate, trigonelline, urea, taurine[9,24], asparagine, lysine, histidine, acetate, methylamine, creatine, trans-aconitate[9], betaine[24] and 4-cresol sulfate[25]. It should be noted that an increase in the concentration of leucine and acetoacetate in serum reported in our study was earlier described only in animal models[35,36] while a decrease in the concentration of 2-hydroxyisobutyrate in urine has never been demonstrated before. The elevated concentration of isoleucine in serum (conflicting results in the literature[24,26]) as well as a decrease in the concentration of creatine (once reported as elevated[24]) should be also pointed out. There is only one study (applies only to the urine of UC patients) in which the obtained results did not allow to distinguish IBD patients from healthy individuals[1].

The utility of metabolomics in the differential diagnosis of IBD remains controversial. Our results indicate that no chemometric model for distinction between CD and UC could be constructed both for serum and urine samples, although there are statisticaly significant differences between some metabolites in these diseases. Comparing the combined group of CD patients with active disease and remission to UC patients in the active and remission phase of the disease we found a significantly increased serum concentration of alanine and *N*-acetylated compounds. Comparing the patients with active CD and active UC we observed a significantly lower serum level of formate in the first group (not reported before). There were no

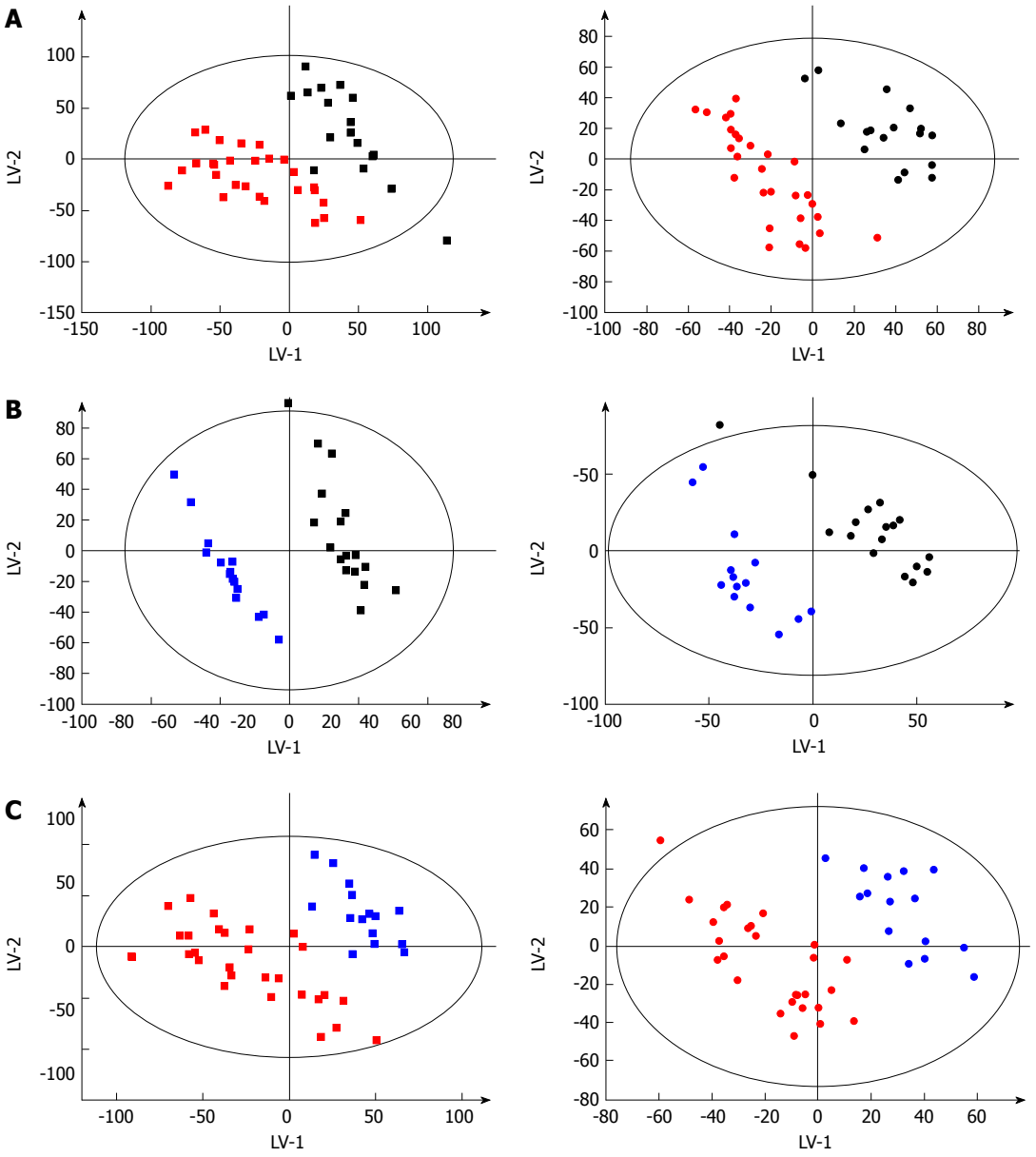

Figure 3 The partial least-squares-discriminant analysis score plots based on the metabolic fingerprints. A: IBD active *vs* control; B: IBD remission *vs* control; C: IBD active *vs* IBD remission. Boxes: Serum samples; Circles: Urine samples; Red: IBD active; Blue: IBD remission; Black: Control; IBD: Inflammatory bowel diseases.

statistically relevant differences in urine metabolic fingerprints between the cohorts of CD and UC patients. Our findings are substantially consistent with the results of Schicho *et al*[24] and differ from the results of Williams *et al*[25,26]. The first study revealed poorly pronounced differences between serum of the CD and UC cohorts, that allowed to create only a weak predictive chemometric model (AUROC 0.655 with Q^2Y 0.024 for serum, and AUROC 0.7325 with Q^2Y 0.115 for plasma), and no possibilities to create such model based on urine metabolomic analysis. Regarding to blood analysis, increased concentrations of arginine, 3-methyl-2-oxovalerate, τ-methylhistidine, lysine, leucine, glycerol in CD and elevated glutamine, citrate, tyrosine, glucose, mannose, carnitine, 2-hydroxybutyrate, betaine, creatynine, isoleucine in UC patients were reported[24]. The results of Williams *et al*[26] revealed that on the basis of OSC-PLS-DA serum analysis it is possible to distinguish CD and UC cohorts with a significant predictive accuracy. The metabolites of importance included up-regulated *N*-acetylglycoprotein in CD patients, and down-regulated LDL and HDL cholesterol, unsaturated lipids, choline, isoleucine and alanine. Similarly, it has been shown that the differential diagnosis of IBD is possible on the basis of the urinary metabolic profile and the statistically significant differences concerned: hippurate, 4-cresol sulfate and citrate (decreased in CD) as well as formate (increased in CD)[25]. Two other research groups estimated the usefulness of metabolomics in the differential diagnosis of IBD, but in these cases only one biofluid was evaluated. Stephens *et al*[9] observed that CD could be differentiated from UC using the urine metabolomic profile but only when IBD

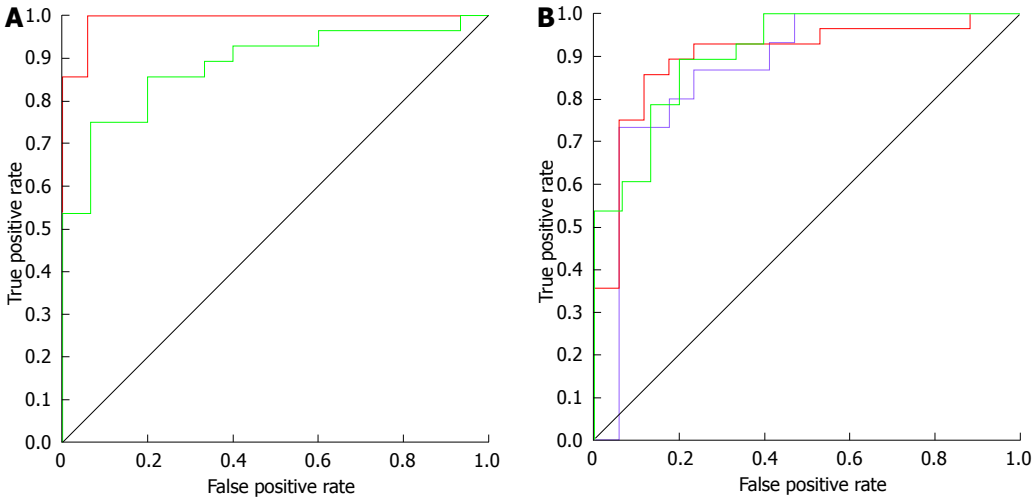

Figure 4 Receiver operating characteristic curves based on partial least-squares-discriminant analysis models for nuclear magnetic resonance spectra. A: Serum samples; B: Urine samples. Serum: Red - IBD active *vs* control (AUC = 0.992); Green - IBD active *vs* IBD remission (AUC = 0.883). Urine: Red - IBD active *vs* control (AUC = 0.900); Green - IBD active *vs* IBD remission (AUC = 0.909); Violet - IBD remission *vs* Control (AUC = 0.871). IBD: Inflammatory bowel diseases; AUC: Area under curves.

patients with intestinal resection were included in the cohorts (no differences were seen if the urine sample data from individuals with intestinal resection were removed from the analysis). Hisamatsu *et al*[21] using MIAI technology, demonstrated a potential for discrimination between CD and UC patients with AUROC = 0.879 in the active phase of the disease and 0.744 in remission. The authors underlined the fact that although the alterations in amino acid metabolism in IBD patients could be found, it is difficult to establish a single biomarker for a clinical use.

The results of our study demonstrate that ^{1}H NMR-based metabolic fingerprinting of human serum and urine combined with multivariate data analysis could be a useful tool in distinguishing patients with active IBD from those in remission that is of great importance in IBD monitoring. At the same time, the results indicate that this diagnostic method has rather a weak potential in the differential diagnosis of IBD.

COMMENTS

Background

Difficulties in the differential diagnosis between ulcerative colitis and the Crohn's disease, as well as the limited number of monitoring tools in inflammatory bowel diseases (IBD) force searching for new approaches in IBD diagnostics.

Research frontiers

The use of metabolomics in medical diagnostics has recently become a very promising idea that is extensively studied in oncological conditions, diabetes, cardiovascular diseases, rheumatoid arthritis or multiple sclerosis. Unlike the genomic and proteomic studies that examine genes and more or less complex proteins, metabolomic analysis allows to assess the simplest low molecular weight metabolites that are involved in disease processes. The highly informative metabolic profiles as a potential source of biomarkers can be used for both diagnostic and prognostic purposes. So far the usefulness of metabolomics in IBD diagnostics has been evaluated only in a few studies.

Innovations and breakthroughs

In the present study the utility of serum and urine metabolomic analysis in diagnosing and monitoring of IBD has been valuated using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The significant differences in metabolomic profiles were found between the group of patients with active IBD and the group with IBD in remission providing a good partial least-squares-discriminant analysis (PLS-DA) model.

Applications

NMR-based metabolomic fingerprinting of serum and urine has the potential to be a useful tool for differentiation between the active and remission phase of the disease that is of great importance in IBD monitoring.

Terminology

Metabolomics is the systematic study allowing the simultaneous and relative quantification of low molecular weight metabolites which form unique chemical fingerprints associated with the specific condition of the living system. PLS-DA was used to assess the differences in metabolite fingerprints. ^{1}H-NMR denotes proton nuclear magnetic resonance spectroscopy which allows to quantify the concentration and determine the structure of chemical compounds.

Peer review

The study is well conducted from the methodological point of view and presents interesting data concerning serum and urine metabolomic fingerprinting in the diagnostics of IBD. A new finding that metabolomics may be also helpful in differentiating between the active and remission phase of the disease is of particular importance in IBD monitoring.

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