

Quantitative Metabolomic Profiling of Serum, Plasma, and Urine by ^1H NMR Spectroscopy Discriminates between Patients with Inflammatory Bowel Disease and Healthy Individuals

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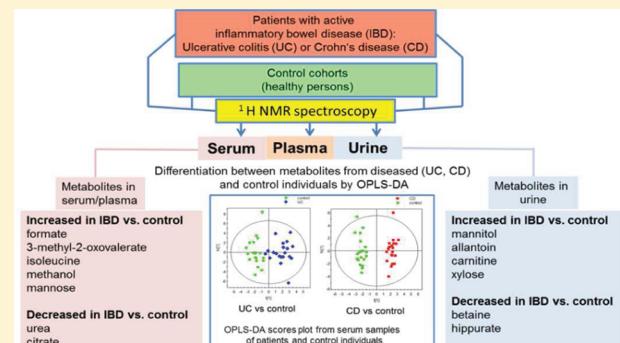
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Supporting Information

ABSTRACT: Serologic biomarkers for inflammatory bowel disease (IBD) have yielded variable differentiating ability. Quantitative analysis of a large number of metabolites is a promising method to detect IBD biomarkers. Human subjects with active Crohn's disease (CD) and active ulcerative colitis (UC) were identified, and serum, plasma, and urine specimens were obtained. We characterized 44 serum, 37 plasma, and 71 urine metabolites by use of ^1H NMR spectroscopy and "targeted analysis" to differentiate between diseased and non-diseased individuals, as well as between the CD and UC cohorts. We used multiblock principal component analysis and hierarchical OPLS-DA for comparing several blocks derived from the same "objects" (e.g., subject) to examine differences in metabolites. In serum and plasma of IBD patients, methanol, mannose, formate, 3-methyl-2-oxovalerate, and amino acids such as isoleucine were the metabolites most prominently increased, whereas in urine, maximal increases were observed for mannitol, allantoin, xylose, and carnitine. Both serum and plasma of UC and CD patients showed significant decreases in urea and citrate, whereas in urine, decreases were observed, among others, for betaine and hippurate. Quantitative metabolomic profiling of serum, plasma, and urine discriminates between healthy and IBD subjects. However, our results show that the metabolic differences between the CD and UC cohorts are less pronounced.

KEYWORDS: *inflammatory bowel disease, OPLS, metabolomics, chemometrics*



INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two major subtypes of chronic inflammatory bowel disease (IBD), cause significant morbidity in affected individuals. The prevalence ranges from 37 to 249 cases per 100,000 people for UC and from 26 to 319 cases per 100,000 people for CD in the North American population, with similar incidences in other developed countries.^{1,2} While the pathophysiology of IBD is not fully understood, it has been widely accepted that multiple components, including genetic, environmental, and microbiological factors, contribute to the occurrence and perpetuation of the disease.^{3,4}

Current therapeutic options consist of anti-inflammatory medications as well as corticosteroids and immunosuppressive and novel biological agents. However, some individuals fail to respond to these therapies, and these agents are associated with significant side effects. In addition, CD and UC, while sharing several similar pathologic and clinical features, do have distinct

differences in prognosis and management. Therefore, to minimize side effects in IBD therapy, appropriate therapeutic decision-making through accurate diagnosis and regular surveillance is crucial. Currently, diagnosis relies upon clinical, endoscopic, histologic, and radiologic techniques that can be time-consuming and costly. Endoscopy is a technique with risks,⁵ including a 1 in 1000 risk of bowel perforation.⁶ Furthermore, differentiating between the two subtypes of disease endoscopically and even histologically may be challenging in certain situations. Less invasive methods for diagnosis, such as determination of biomarkers from urine, serum, or feces, however, would be of significant advantage and useful for primary diagnosis, surveillance, and early detection of relapses. Additionally, non-invasive biomarkers could be used by gastroenterologists to triage referral for patients with

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symptoms such as abdominal pain and diarrhea. Several investigators have addressed this issue by performing non-targeted analysis of metabolites in animal models of colitis using mass spectroscopy^{7–9} as well as ¹H NMR spectroscopy.¹⁰ The latter method has also been applied to IBD patients to characterize metabolites in urine,¹¹ fecal extracts,¹² and biopsy samples.¹³ A recent study employing ion cyclotron resonance-Fourier transform mass spectrometry to discriminate between 1000 metabolites revealed differences in fecal samples collected from identical twin pairs, including healthy individuals and CD patients.¹⁴ Another study clearly distinguished between IBD patients and healthy individuals by use of multivariate indexes established from plasma aminograms.¹⁵ Analysis of metabolites is therefore rapidly emerging as a powerful method for characterizing IBD in experimental animal models and humans.

Though various markers or marker panels have been tested in clinical trials, there is no ideal marker that is able to diagnose or predict IBD.¹⁶ Most useful markers in clinical practice include acute phase proteins such as C-reactive protein, the fecal markers calprotectin and lactoferrin, and serologic markers such as the DNase-sensitive antineutrophil cytoplasmic antibody p-ANCA that is present in sera of 60% of UC and 20% of CD patients.¹⁷ However, a recent study concluded that the predictive values of the latest serological panel for pediatric IBD screening (Prometheus Inflammatory Bowel Disease Serology 7 [IBD7] panel) were less useful than the routine laboratory tests,¹⁸ suggesting the use of genomic or metabolomic biomarkers for diagnosis and prediction of IBD.

“Quantitative” analysis of metabolites may significantly improve the discovery of disease-related biomarkers. This approach involves analyzing a large group of compounds whose characteristics (e.g., NMR spectra) are known and stored in a database library. The complex mixture of individual metabolite spectra in the biofluid can be analyzed by overlaying spectra taken from a database to identify and quantify the targeted metabolites. This approach quantifies a large number of metabolites in a single specimen in a high-throughput manner and is highly suitable for studies of polar metabolites.¹⁹

Metabolite analysis of serum and plasma in IBD subjects has not been reported before, and so far only one study has investigated metabolite profiles in urine of patients with active UC²⁰ but not in active CD. Another previous urinary metabolomic study did not restrict enrolment to individuals with active disease.¹¹ In the current study, we therefore aimed to investigate the correlation of intestinal inflammation with the presence or absence of specific metabolites that could potentially be used as biomarkers in serum, plasma, and urine of IBD patients. To determine the metabolites, we used ¹H NMR spectroscopy and performed quantitative analysis of metabolites of the three biofluids from human subjects identified clinically, endoscopically, and histologically to have active CD or UC. We were specifically interested to examine all three biofluids to determine whether all of them could separate IBD from healthy subjects because it is known that urinary metabolomic profiles are more affected by environmental factors^{21,22} than blood metabolites and because differences in metabolite profiles between plasma and serum have been reported.^{23,24}

MATERIALS AND METHODS

Experimental Design

Subjects. The study was approved by the Conjoint Health Research Ethics Board of the University of Calgary (Protocol no. 18142), and all participants were provided written, informed consent. Serum, plasma, and urine samples were collected from adult individuals with confirmed CD ($n = 20$) and UC ($n = 20$) and from healthy control ($n = 40$) subjects. All three biofluids were taken from each individual, i.e., serum and plasma were from the same subject. For collection of serum, SST vacutainer tubes and for plasma, K2 EDTA vacutainer tubes from BD Biosciences (Franklin Lakes, NJ, USA) were used, processed according to the manufacturer’s instructions and frozen at -80°C . Urine was collected in sterile urine containers, pipetted into transport tubes, and also frozen at -80°C . Patients included in the study were recruited from the Foothills Medical Centre, the major tertiary care specialist center in Calgary, Alberta, (population 1.2 million) and had been diagnosed by experienced gastroenterologists according to rigorous criteria on the basis of endoscopic, histologic, and radiological findings. In addition, details of disease activity scores, based on the Harvey-Bradshaw Index for CD²⁵ and the Simple Clinical Colitis Activity Index for UC,²⁶ were calculated for later subgroup analysis. Patients in whom there was diagnostic uncertainty (e.g., those with IBD type unclassified) were excluded from the study. To avoid influence of aging and gender on metabolomic profiles, subjects in the healthy control cohort were matched each to corresponding subjects in disease cohorts by gender and age (age matched within 5 years).

Addressing Potential Confounding Factors of Patients and Metabolites. Several recent studies have elucidated the degree of variation in NMR spectroscopic profiles from urine samples of healthy subjects.^{21,22,27} Analysis of serum metabolites, however, demonstrated minimal variability between subjects and study days. Because different diets have been shown to influence urinary metabolic profiles,²⁶ we collected fasting samples. To replicate normal circumstances, no dietary exclusions were imposed. Details of chemical contraceptive usage and reproductive status were obtained in females, to ensure that the groups were similar. Metabolites related to medication (e.g., acetaminophen, acetamide) were eliminated from the statistical evaluation. Subjects with significant comorbidities and individuals with an intercurrent illness, who were pregnant, or who were taking antibiotics, biologics, prebiotics, or probiotics were excluded from the study.

¹H NMR Spectroscopy of Serum, Plasma, and Urine Samples

Metabolite Sample Preparation. Serum, plasma, and urine samples were thawed on ice. Of each sample, 400 μL was applied to 3-kDa Nanosep microcentrifuge filters for filtration to remove proteins and insoluble impurities. D₂O (100 μL) was added during filtration of serum and plasma for filter washing. The final volume of filtrate ranged from 100 to 400 μL . Samples were brought to 650 μL by addition of D₂O, 130 μL of sodium phosphate buffer (final concentration 0.1 M) containing dimethyl-silapentane-sulfonate (final concentration 0.5 mM) for NMR chemical shift reference and concentration calibration, and 10 μL of 1 M sodium azide to prevent growth of bacteria. The final sample pH was adjusted to 7 \pm 0.01. Samples from the CD, UC, and healthy control cohorts were analyzed in a blinded, randomized manner.

Table 1. Information on Patients and Healthy Persons That Participated in the Study

	ulcerative colitis ^a	Crohn's disease ^a	healthy controls
number (male/female)	20 (13/7)	20 (18/2)	40 (31/9)
mean age (range) in years	46.2 (21–85)	39.6 (20–57)	53.7
mean weight (range) in kg	81.8 (54–140)	73.9 (50–110)	79.3 (49–100)
height (range) in cm	171 (147–188)	175 (160–191)	174.5 (152–198)
current smoker	1	7	
mean activity score - physician (range)	9.7 (6–13)	9.9 (6–19)	
mean disease activity index - patient (range)	11.0 (4–22)	8.1 (1–17)	
mean disease duration (range) in years	5.5 (0.5–19)	10.6 (0.3–30)	
mean age at diagnosis (range) in years	39.6 (16–83)	27.5 (2–56)	
earlier bowel resection	1	9	
family history of IBD	4	5	
disease location	A1:1 A2:10 A3:9	B1:5 B2:6 B3:9	
disease complications	C1:0 C2:0 C3:0 C4:0 C5:0	C1:7 C2:3 C3:4 C4:3 C5:4	
extraintestinal manifestations of IBD	4	6	
medications within prior 4 weeks	M1:15 M2:15 M3:10 M4:5	M1:3 M2:14 M3:6 M4:7	
prior use of immunosuppresives	6	10	

^aA1, proctitis; A2, disease limit to distal splenic flexure; A3, disease proximal to splenic flexure; B1, ileal disease; B2, colonic disease; B3, ileocolonic disease; C1, stricturing; C2, penetrating; C3, fistula; C4, stenoses; C5, abscess; M1, 5-ASA; M2, Oral Steroids; M3, IV Steroids; M4, 6-MP/AZA.

NMR Spectra Acquisition. NMR spectra were acquired using an automated NMR Case sample changer on a Bruker Avance 600 spectrometer (Bruker Biospin) operating at 600.22 MHz and equipped with a 5-mm TXI probe at 298 K. Regular one-dimensional proton NMR spectra were obtained using a standard pulse sequence (Bruker pulse program prnoesy1d) that has good water suppression characteristics and is commonly used for metabolite profiling of serum or plasma samples.^{19,28} It utilizes the following pulse sequence RD-90°-t1-90°-tm-90°-acquire FID; where RD is a relaxation delay of 1 s, during which the water resonance is selectively irradiated; t1 is set to 4 μ s, and tm has a value of 100 ms, during which the water resonance was again selectively irradiated. Initial samples for each batch were shimmed to ensure half-height line width of <1.1 Hz for the dimethyl-silapentane-sulfonate peak, calibrated to 0.0 ppm. Spectra were acquired with 1024 scans, then zero filled and Fourier transformed to 128 k data points. For proper quantitative fitting of the NMR spectra, it is important that the spectra are collected under the same conditions as the metabolite standard spectra in the *Chenomx* database. Additional 2-dimensional NMR experiments were performed for the purpose of confirming chemical shift assignments, including homonuclear total correlation spectroscopy (2D ^1H - ^1H TOCSY) and heteronuclear single quantum coherence spectroscopy (2D ^1H - ^{13}C HSQC), using standard Bruker pulse programs.

Spectra Fitting. Processed spectra were imported into Chenomx NMR Suite 6.1 software (Chenomx Inc., Edmonton, Canada) for quantification using the “targeted profiling” approach,¹⁹ where individual NMR resonances of interest are mathematically modeled from pure standard metabolite compound spectra stored in an internal database, and this

database is then interrogated to identify and quantify metabolites in complex spectra of mixtures, such as biofluids. Overall 71 compounds in urine, 44 compounds in serum, and 37 compounds in plasma spectra were detectable with sufficient signal-to-noise (Supplementary Table 2S). Spectra were randomly ordered for profiling. Compounds were profiled in order of decreasing typical concentration. Each compound concentration was then normalized to a total concentration of all metabolites in the sample (excluding glucose and lactate for serum and plasma samples and urea, creatinine, and citrate for urine samples because of excessively large volumes that otherwise would have dominated the normalization).

Data Analysis

To reveal patterns in metabolite concentration shifts, multivariate analysis was conducted using SIMCA-P+ 12.0 software (Umetrics, Sweden). Orthogonal projections to latent structures discriminant analysis (OPLS-DA), a supervised pattern recognition approach, were used as a predictive model to identify differences in metabolite composition in samples of UC and CD patients and healthy controls.²⁹ The objective of OPLS-DA is to divide the systematic variation in the X-block (an input data set of metabolite concentrations) into two model parts: one part that models the covariation between the measured data of X variable (metabolite concentrations) and the response of Y variable (in our case binary variables of disease status) within the groups, and a second part which captures systematic variation in X that is unrelated (orthogonal) to Y. Model components that are related to Y are called predictive, while those that are unrelated to Y are called orthogonal. For each OPLS-DA model, 7-fold cross validation (CV) was used to validate the statistical significance of each model dimension. To calculate the area

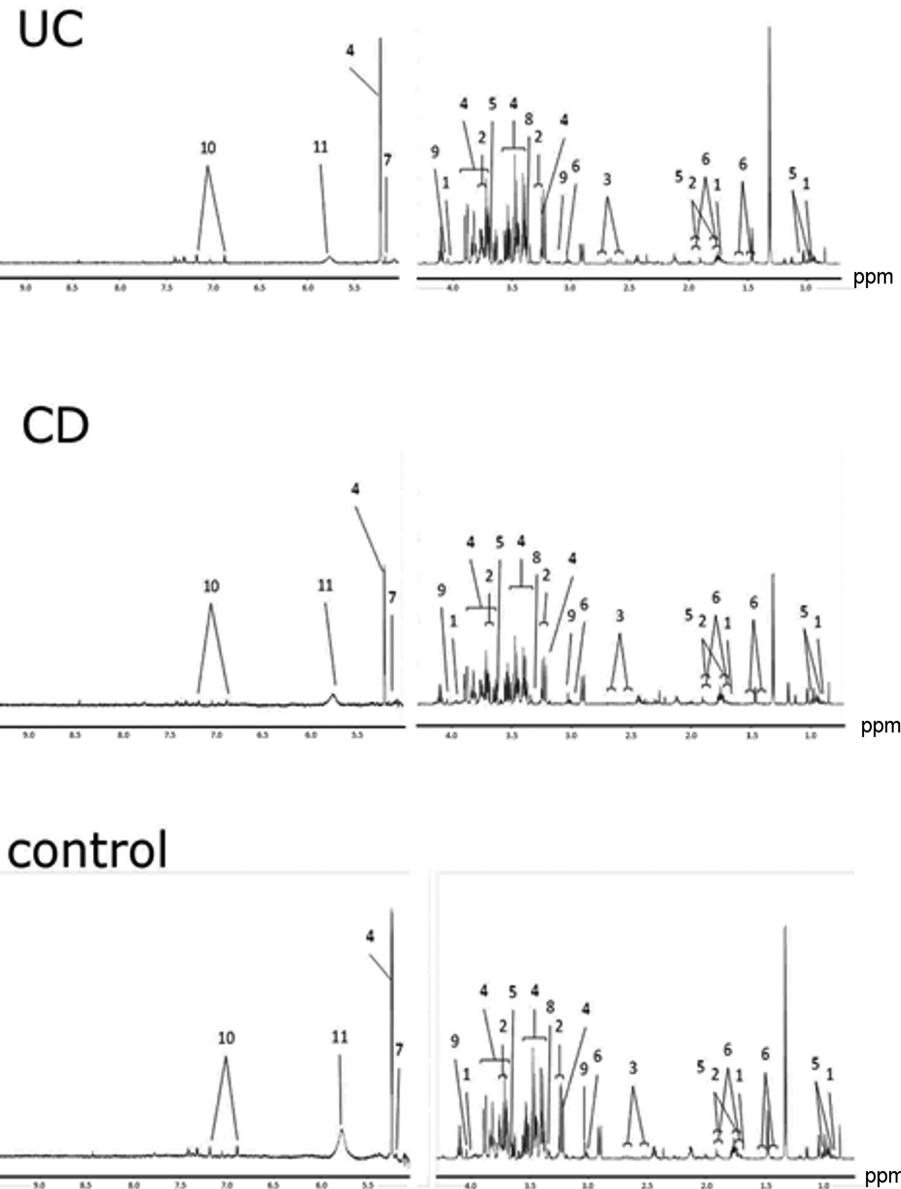


Figure 1. Typical 600 MHz ^1H NMR spectra of serum from patients with ulcerative colitis (UC) and Crohn's disease (CD) and from a healthy control subject. Aromatic region (5.0–9.5 ppm) magnified $\times 4$ compared with the aliphatic region (0.7–4.3 p.p.m.). Metabolites: (1) 2-hydroxybutyrate, (2) arginine, (3) citrate, (4) glucose, (5) isoleucine, (6) lysine, (7) mannose, (8) methanol, (9) creatinine, (10) tyrosine, (11) urea.

under the ROC curve (AUROC), specificity and sensitivity were determined on the basis of sample class prediction during the 7-fold cross validation (Y-predcv, predictive Y variables; SIMCA-P+ software). Calculation of AUROC was performed using the GNU R ROCR package.³⁰

Model Generation

We used multiblock principal component analysis (PCA) and hierarchical OPLS-DA as methods for comparing several blocks (i.e., several PCA models from multiple biofluids) derived from the same “objects” (e.g., subject). This method is ideally used for analyzing variable-rich data sets.³¹ The idea of hierarchical modeling is to group the variables for the purpose of improved clarity and interpretability to reveal how the different blocks (concentration data from different biofluids) are related, which blocks provide overlapping or unique information, and which biofluid measurements are most useful from a predictive viewpoint. This blocking leads to two model levels: the upper

level where the relationships between blocks are modeled and the lower level showing the details of each block. Metabolite concentrations data were divided into three blocks according to the type of sample biofluids (i.e., serum, plasma, and urine) obtained from the same subject. A PCA model was constructed in the lower level for the metabolite concentration data of each type of biofluid. All meaningful scores (t_b) from each PCA model were combined into a super block, T . In the higher level, a hierarchical OPLS-DA was performed on T with t_b denoted as variables and samples denoted as observations. The resulting super scores (t_T) plot shows the relationship between observations, and the super loadings plot (p_T) indicates which scores (t_b) are most influential on the hierarchical OPLS-DA model, and hence facilitates visualization of differences and/or similarities in the metabolic responses of multiple biofluids.

RESULTS

Subject Groups

Participant demographics and disease characteristics in the IBD cohorts are summarized in Table 1. Patients reported that they were taking medications including 5-aminosalicylate (5-ASA) drugs (3 CD and 15 UC), azathiopurine (7 CD and 5 UC), and corticosteroids (15 CD and 16 UC). As subjects from these cohorts were studied during an exacerbation of their disease, as defined by a Harvey-Bradshaw or simplified clinical colitis activity index ≥ 5 , all participants were on some form of medication.

¹H NMR Spectroscopy of Serum, Plasma, and Urine Samples

Inspection of the serologic and urinary NMR spectra revealed the wide variety of metabolite resonances present in the spectra. Representative ¹H NMR spectra of serum samples from UC and CD patients and healthy control subjects are shown in Figure 1. A number of metabolites, including a range of amino acids, saccharides (glucose, maltose, galactose), energy metabolism related molecules (pyruvate, succinate, citrate, lactate, creatine, creatinine), and others (cholines, amines and amides) were identified based on comparison with the *Chenomx* metabolite database, as well as 2D ¹H–¹³C HSQC and ¹H–¹H TOCSY NMR experiments (see a complete list of metabolites in the Supporting Information, Table 2S).

Metabolic Profile Related to IBD

Changes in metabolites of each biofluid from UC and CD patients (with active disease) and from corresponding healthy control subjects were established using an OPLS-DA strategy, comparing ¹H NMR profiled metabolite concentrations between IBD patients and healthy subjects, as well as each disease cohort between each other. Three OPLS-DA models were built for each sample biofluid comparing metabolites between UC and matched control cohorts, CD and matched control cohorts, and between UC and CD cohorts. The quality of the models were determined by the goodness of fit in the X (R^2X) and Y (R^2Y) variables and the predictability based on the fraction correctly predicted in one-seventh cross-validation Q^2Y_{CV} (see Table 2 for the model summary statistics). Clear separation was achieved between samples obtained from disease (both UC and CD) and healthy control subjects for all biofluids examined, as evidenced by the consistently high Q^2Y values for all models (Table 2). Discrimination between the two IBD groups was not clear enough in urinary spectra; therefore no model could be built to discriminate metabolic patterns between CD and UC samples (Table 2). The metabolites responsible for the separation of the disease groups (UC and CD) from the control groups (Figures 2, 3, and 5), and of both disease groups (Figure 4) are summarized by the OPLS-DA regression coefficients and scores plots in Figures 2–5. Only metabolites with statistically significant differences ($p < 0.05$) are shown.

Serum Metabolites in Patients with UC and CD versus Control Subjects. Serum metabolites were significantly altered in IBD. In serum of UC patients (Figure 2A), 21 metabolites showed significant changes in concentration versus control subjects while serum from CD patients (Figure 2C) revealed significant changes of concentrations in 11 metabolites. In both UC and CD, the metabolite profile showed strong increases in methanol, mannose, and amino acids, such as

Table 2. Summary Statistics of the Models Used to Describe Changes in Serum, Plasma, and Urine of UC and CD Patients and Healthy Persons^a

data set	model	A_{pred}	A_{orth}	R^2X	R^2Y	Q^2Y_{CV}
serum	UC:control	1	1	0.29	0.798	0.53
	CD:control	1	4	0.508	0.963	0.84
	CD:UC	1	0	0.142	0.39	0.0249
plasma	UC:control	1	1	0.312	0.772	0.613
	CD:control	1	1	0.381	0.789	0.67
	CD:UC	1	0	0.157	0.386	0.115
urine	UC:control	1	5	0.466	0.997	0.688
	CD:control	1	0	0.143	0.785	0.528
	CD:UC	0	0	0	0	0
hierarchical	UC:control	1	0	0.221	0.749	0.685
	CD:control	1	1	0.366	0.728	0.635
	CD:UC	1	0	0.227	0.376	0.23

^a A_{pred} , number of Y-predictive components; A_{orth} , number of Y-orthogonal components; R^2X , explained variance of X; R^2Y , explained variance of Y; Q^2Y_{CV} , predicted variance of Y estimated using cross-validation. R^2X and R^2Y show how well the model explains the variation in X and Y, respectively. Q^2Y represents the quality and predictive power of the model.

isoleucine. Common to both forms of IBD are decreases in urea (more prominent in UC than CD), citrate and acetate.

Plasma Metabolites in Patients with UC and CD versus Control Subjects. Changes in plasma metabolites differed little from those in serum; this particularly applies for UC patients (Figure 3A), whereas in CD patients (Figure 3C) differences to serum can be seen. In plasma of UC patients, 16 metabolites showed significant changes of concentration, whereas in CD patients, 21 metabolites revealed significantly altered concentrations. As in serum from UC patients, significant increases were measured for mannose, formate, 3-methyl-2-oxovalerate, 2-hydroxybutyrate, creatine, isoleucine, and lysine, while urea, tau-methylhistidine, valine, tyrosine, choline and creatinine were decreased in both serum and plasma as compared to control subjects. Unlike in serum, changes in metabolite concentrations of plasma samples from UC patients resembled more the ones from CD patients. Almost all metabolites that were increased (mannose, 3-methyl-2-oxovalerate, 2-hydroxybutyrate, creatine, isoleucine, lysine) or decreased (urea, tyrosine, tau-methylhistidine, valine, creatinine, choline, betaine) in samples from UC patients were also increased and decreased, respectively, in samples from CD patients as compared to controls. However, there were differences as to the magnitude in the concentration changes in some of these metabolites (from serum and plasma) between samples from CD and UC patients (Figure 4A).

Urine Metabolites in Patients with UC and CD versus Control Subjects. Urine metabolites showed a largely different profile from serum and plasma metabolites, especially in samples of CD patients (Figure 5C). In addition, metabolites in urine also vastly differed between UC and CD; this applies at least to those metabolites that had increased. In urine samples from UC patients (Figure 5A), 23 metabolites showed changes in concentration, whereas in CD patients (Figure 5C), 26 metabolites had altered concentrations. Only a few metabolites that were decreased in UC were also decreased in CD patients (i.e., citrate, succinate, betaine, hippurate, and methanol) as compared to controls. All other metabolites differed between UC and CD. Several saccharides, such as lactose, galactose,

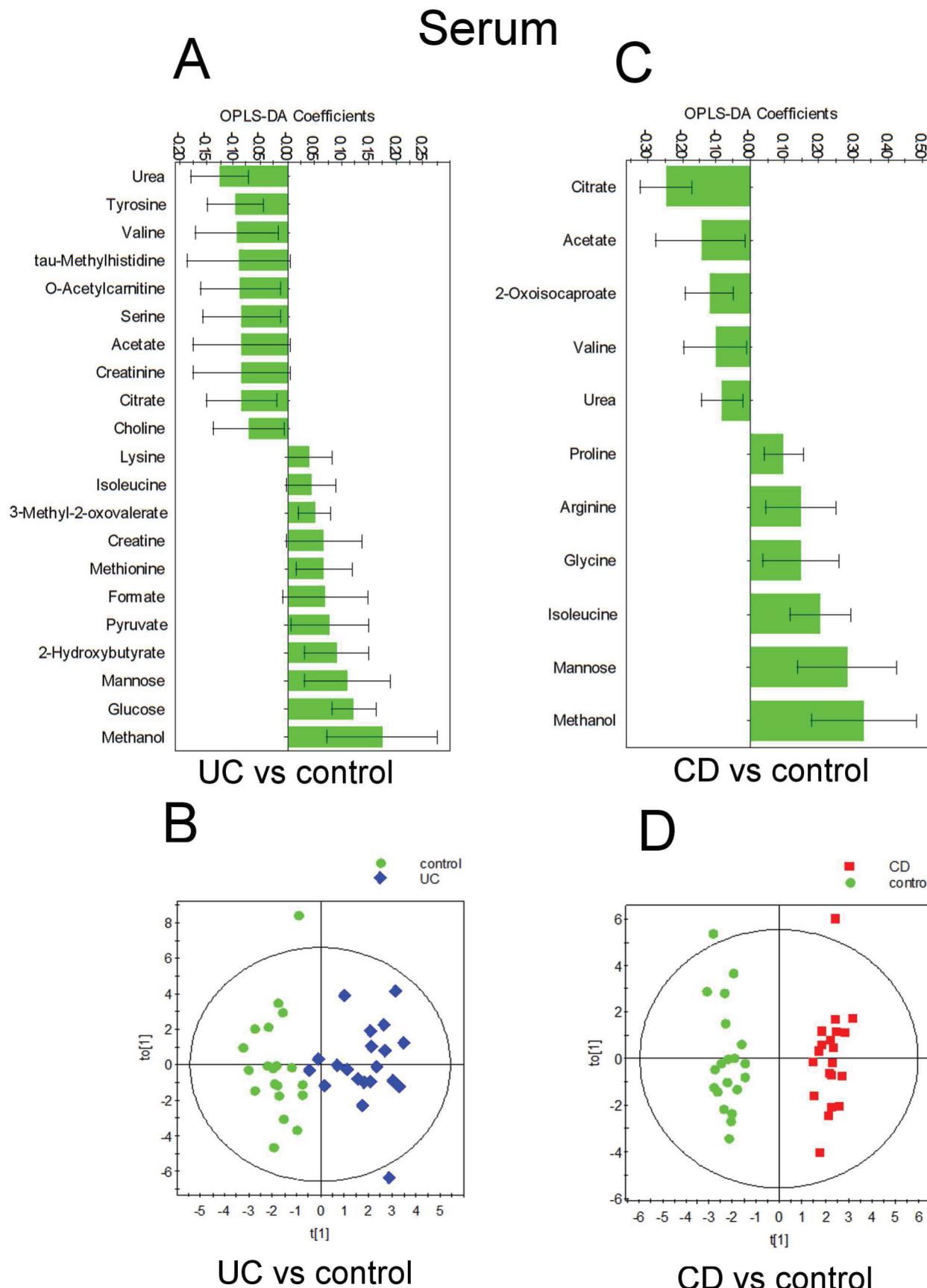
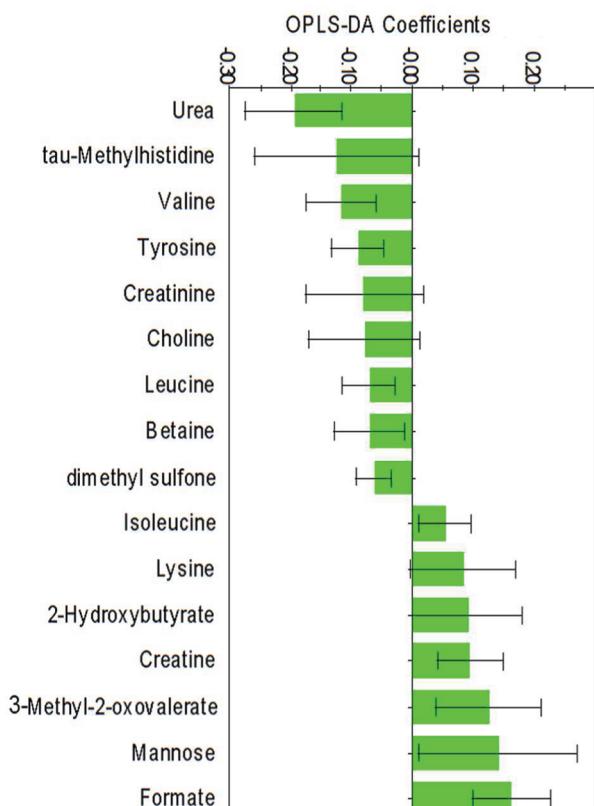


Figure 2. Changes in OPLS-DA coefficients of serum metabolites from patients with (A) ulcerative colitis (UC) and (C) Crohn's disease (CD) as compared to control subjects. Positive bars (\pm SEM) illustrate metabolites significantly increased in UC and CD, whereas negative bars (\pm SEM) denote metabolites significantly higher in control subjects. Panels B and D depict the respective OPLS scores plots.

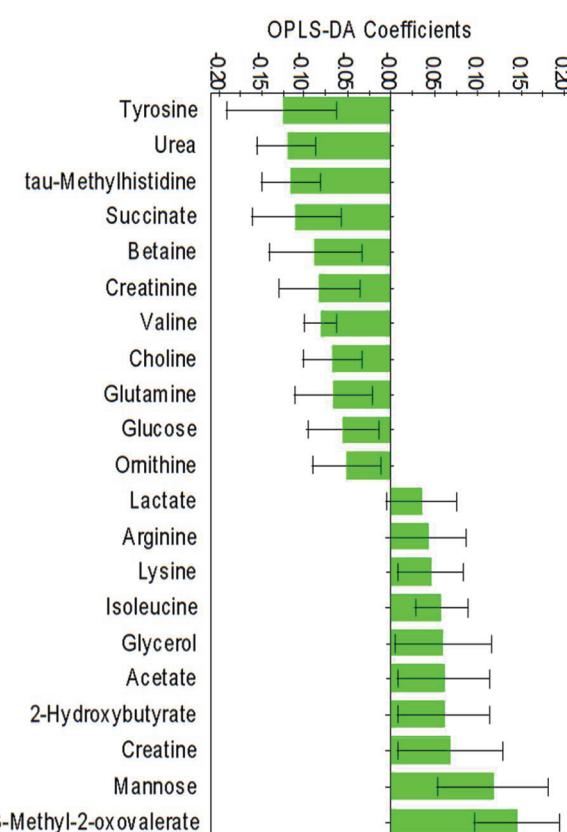
Plasma

A



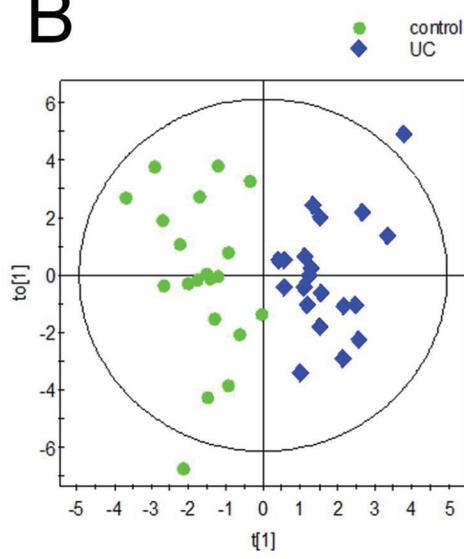
UC vs control

C



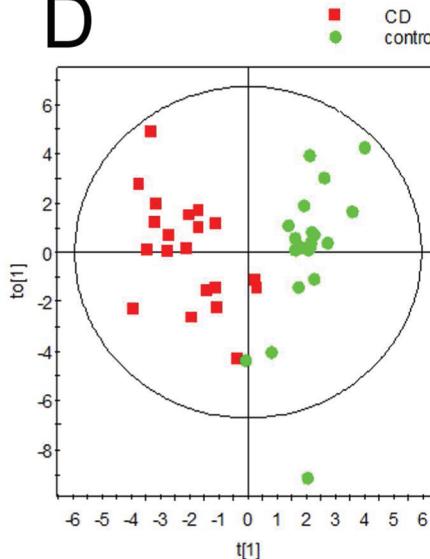
CD vs control

B



UC vs control

D



CD vs control

Figure 3. Changes in OPLS-DA coefficients of plasma metabolites from patients with (A) ulcerative colitis (UC) and (C) Crohn's disease (CD) as compared to control subjects. Positive bars (\pm SEM) illustrate metabolites significantly increased in UC and CD, whereas negative bars (\pm SEM) denote metabolites significantly higher in control subjects. Panels B and D depict the respective scores plots demonstrating good separation of metabolites between IBD patients and control subjects.

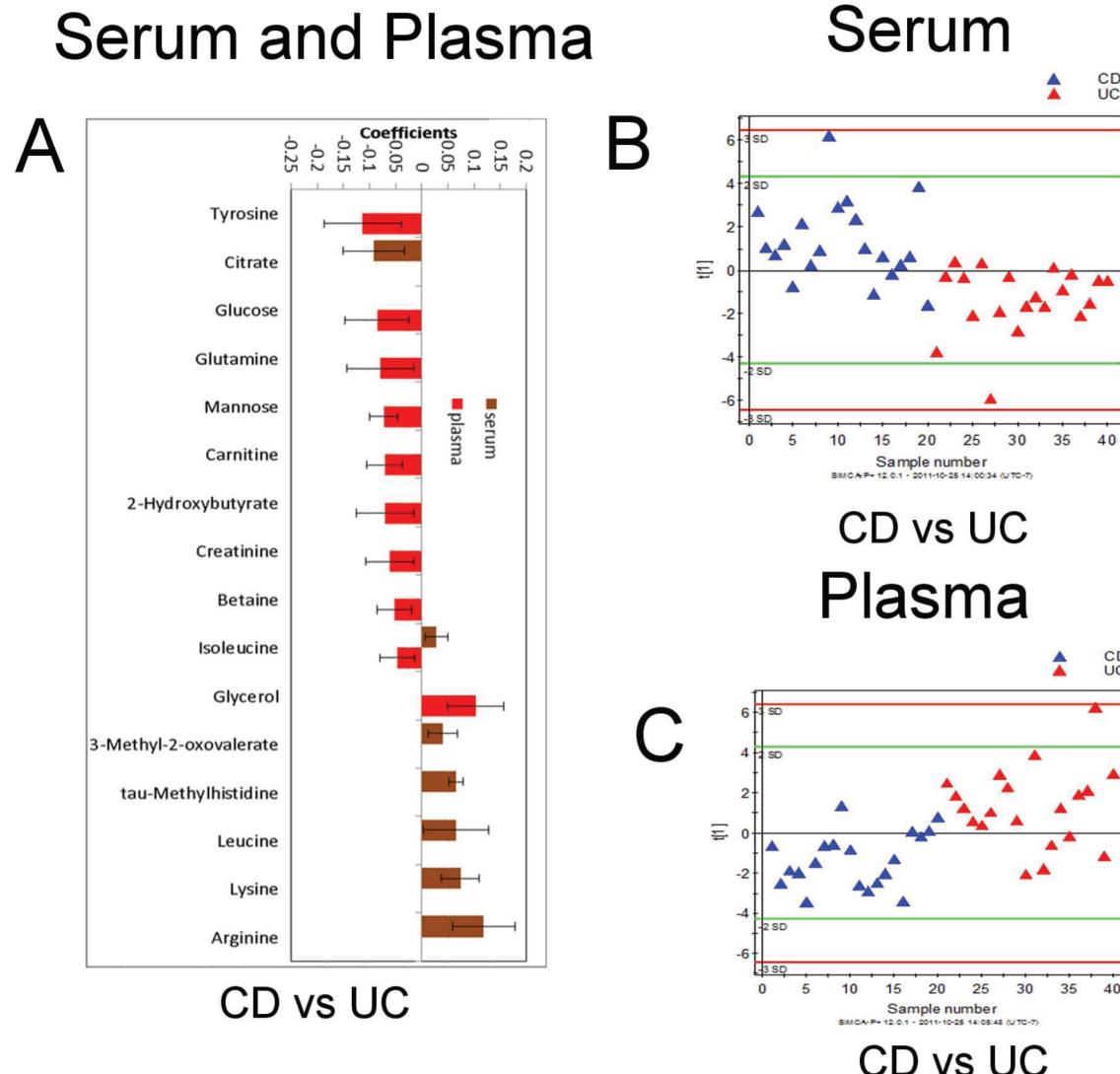


Figure 4. OPLS-DA coefficients (\pm SEM) obtained from serum and plasma samples compared between CD and UC patients (A) to demonstrate significant differences in metabolites that could help differentiating between these two diseases. Panels B and C show the respective OPLS scores plots for CD versus UC in (B) serum and (C) plasma.

maltose, and xylose, however, which were typically increased in CD, were not higher in UC patients. On the other hand, as compared to control subjects, UC patients showed increased levels of mannitol, allantoin, glycylproline, and tryptophan levels, which were missing from CD samples.

Model Validation and AUROC

One important aspect of the data-modeling procedures lies in the predictive ability in terms of specificity and sensitivity in distinguishing disease (UC and CD) from healthy controls and from each other. The multivariate OPLS-DA modeling procedures employed here incorporated a 7-fold cross validation step. In this case, 7 models were built with exactly one-seventh of the data excluded from each model and each sample excluded a single time. The ability of the models to predict those samples not involved in the modeling provided a measure of the overall predictive ability of the metabolite profiling. Using these values (Y_{predcv}), we were able to generate a receiver-operating characteristic (ROC) curve and calculated the area under the ROC curve (AUROC; see Figure 1S in the Supporting Information for an example). Table 3 shows the

results for the constructed models and demonstrates the ability of these modeling procedures to distinguish the cohorts.

PCA and Hierarchical OPLS-DA (Figure 6)

In order to assess the nature of the metabolic response during IBD across several human biofluids, PCA models were constructed individually for each disease subset in each biological matrix, and the significant scores from each model were combined into a single new matrix to include all biofluids (the number of components and percentages of total variance in the data matrix explained by the PCA models are summarized in Table 1S in the Supporting Information). The scores (t_b) values for each model were combined into a “super block” (T), and then OPLS-DA was performed on the super block with unit variance-scaled data, which is termed hierarchical OPLS-DA, to maximize the separation by using class information as the Y variable. The model summary statistics of hierarchical OPLS-DA models are presented in Table 2, and their predictive abilities are in Table 3. The hierarchical OPLS-DA scores plots (Figure 6A and B) showed clear discrimination between inflammatory (UC and CD) and

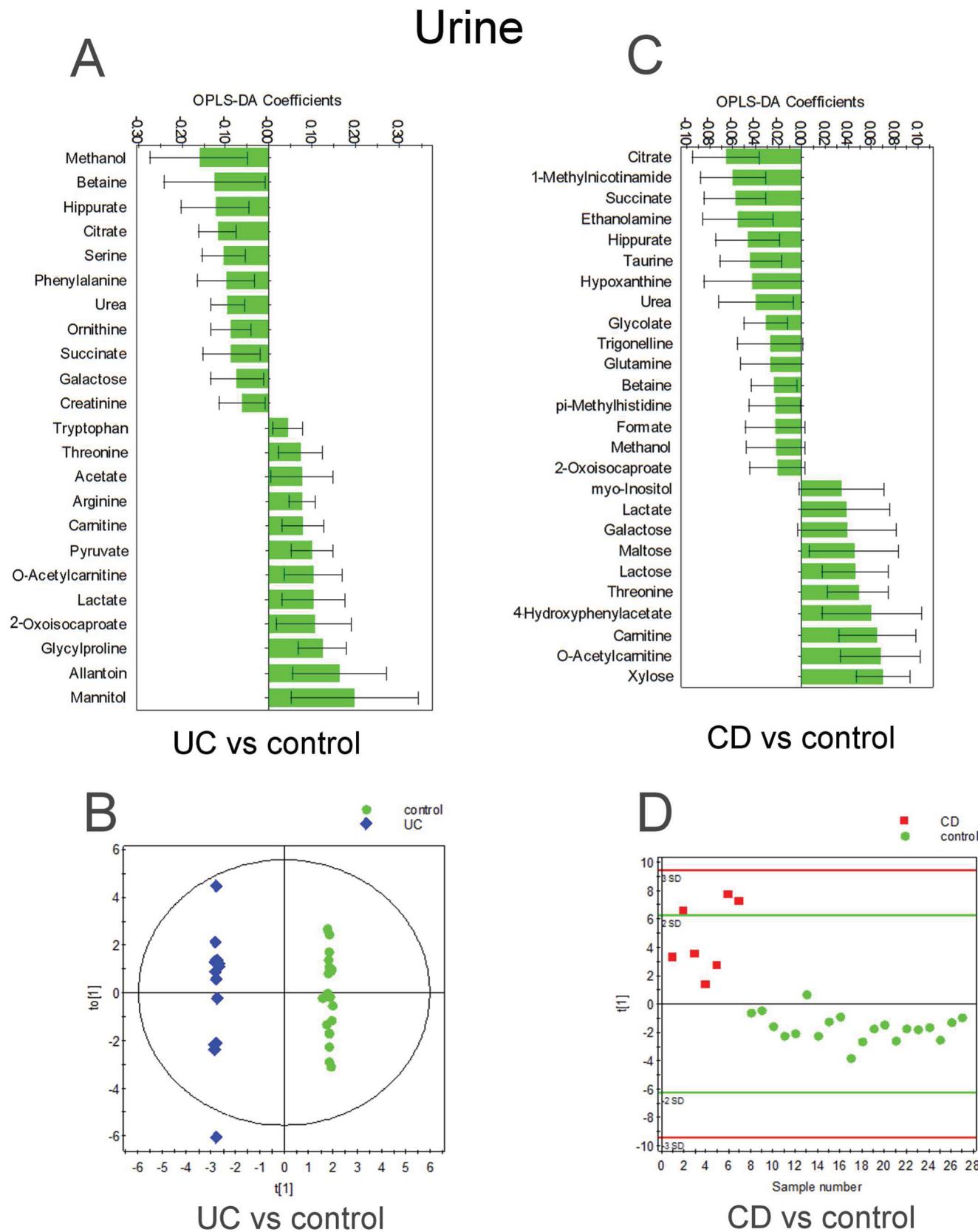


Figure 5. Changes in OPLS-DA coefficients of urine metabolites from patients with (A) ulcerative colitis (UC) and (C) Crohn's disease (CD) as compared to control subjects. Positive bars (\pm SEM) illustrate metabolites significantly increased in UC and CD, whereas negative bars (\pm SEM) denote metabolites significantly higher in control subjects. Panels B and C show the respective OPLS scores plots for (B) UC compared to control subjects and (D) CD compared to control subjects.

Table 3. Predictive Abilities of the Constructed Models^a

		sensitivity:specificity	PPV:NPV	ACC	AUROC
hierarchical	CD:UC	65:65	65:65	65	0.7675
	CD:control	95:90	90:95	93	0.9925
	UC:control	95:90	90:95	93	0.9925
urine	CD:UC	0:0	0:0	0	0
	CD:control	43:100	100:83	85	0.9643
	UC:control	85:100	100:91	94	0.9923
plasma	CD:UC	75:65	68:72	70	0.7325
	CD:control	90:90	90:90	90	0.9825
	UC:control	90:95	95:90	93	0.985
serum	CD:UC	60:50	55:56	55	0.655
	CD:control	95:100	100:95	98	1
	UC:control	80:95	94:83	88	0.9225

^aPPV, positive predictive value; NPV, negative predictive value; ACC, accuracy; AUROC, area under the ROC curve. PPV (NPV) is the proportion of samples with positive (negative) test results that are correctly predicted with the model. Sensitivity (specificity) measures the proportion of actual positives (negatives) that are correctly predicted with the model. Accuracy (ACC) is the proportion of true results (both true positives and true negatives) in all results. The area under curve (AUROC) is equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one.

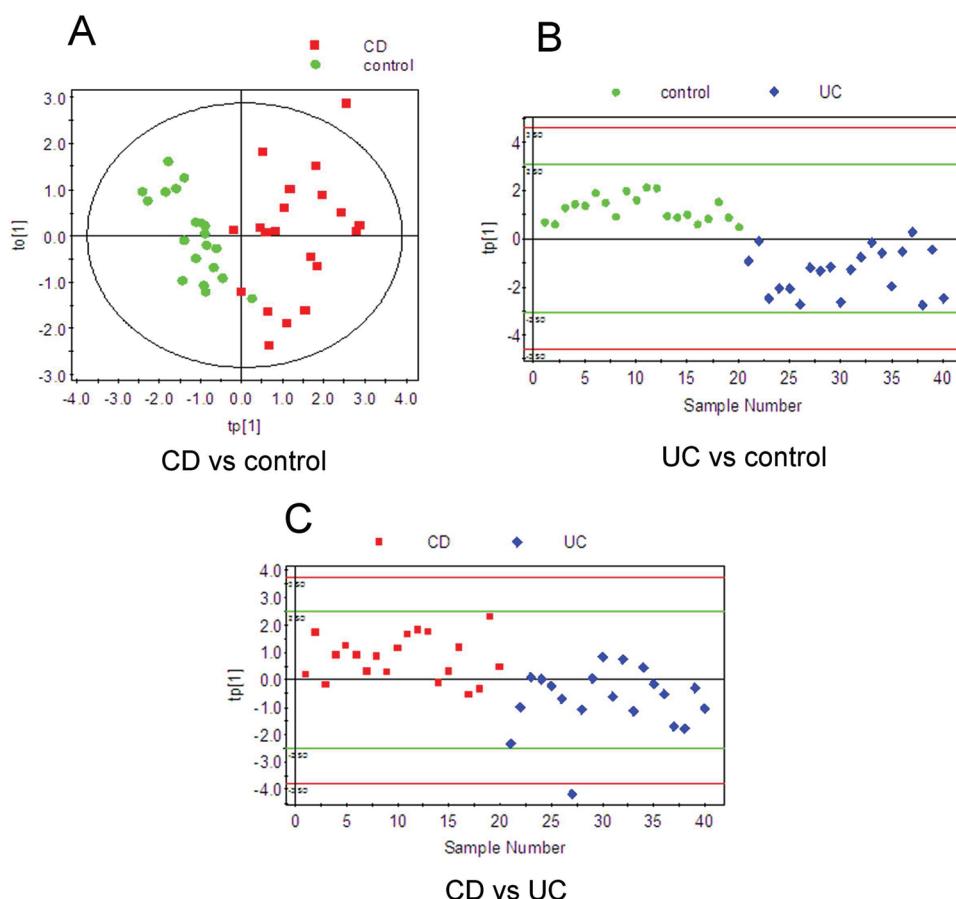


Figure 6. Hierarchical orthogonal projection to latent structure-discriminant analysis (hierarchical OPLS-DA) scores plots (A, B, C) obtained from scores of sub-PCA models that were separately derived from corresponding disease cohorts in the three different biofluids from patients with Crohn's disease (CD, red), ulcerative colitis (UC, blue), and matched healthy individuals (control, green).

healthy control cohorts ($Q^2Y = 0.685$ and 0.635 , respectively, Table 2; AUROC = 0.9925 for both models, Table 3) and weak discrimination between two inflammatory cohorts ($Q^2Y = 0.230$, Table 2; AUROC = 0.7675, Table 3; Figure 6C). Notably, the predictive power of hierarchical UC:CD OPLS-DA model was superior to any similar model for single biofluid (the corresponding loading plots are shown in Figure 2S in the Supporting Information).

DISCUSSION

Quantitative metabolite analysis of biofluids on a large scale offers two important opportunities: first, the chance to discover metabolites associated with the disease that may eventually serve as biomarkers and second, the profile obtained may provide us with an invaluable insight into the pathogenesis of the disease. In the present study, we used the quantitative NMR

“targeted” approach¹⁹ to investigate differences in metabolite concentrations of patients with active IBD in comparison to healthy control subjects. We collected serum, plasma, and urine from each patient with either acute UC or CD and from healthy individuals and determined a set of metabolites in each sample by ¹NMR spectroscopy to discover differences in metabolite concentration. Multiblock principal component analysis (PCA) and hierarchical OPLS-DA were used as statistical methods to discriminate between diseased and non-diseased. As several articles have already described metabolites from urine and colonic mucosal extracts in IBD patients,^{11,20,32,33} we included metabolites from serum and plasma in our study in addition to urinary metabolites. Contrary to plasma, urinary metabolites are highly prone to environmental factors, such as diet, resulting in great intersubject variability.^{21,27} For instance, changes in the bacterial composition of the gut in mice after treatment with antibiotics may alter urinary metabolites.³⁴ We therefore reasoned that testing serum and plasma metabolites may prove as a more reliable approach of quantitative metabolite analysis.

We were able to show that OPLS-DA analysis was sufficient to discriminate between healthy subjects and IBD patients. The Q^2Y values of the CD and UC versus control models for serum, plasma, and urine metabolites were between 0.53 and 0.84, which indicates high reliability and strong predictive power of the models. The predictability of the models to distinguish between CD (or UC) and health was also extremely high and showed an area under the curve (AUROC) of around 0.99. However, our model only discriminated weakly between CD and UC on the ground of plasma or serum metabolites, and for urine, in contrast to other groups,¹¹ no model could actually be created. The reason for this discrepancy could lie in the application of different spectral analysis methods but may also highlight that IBD is a multifactorial disease of unknown etiology with a high variation in phenotypes and severity,³ suggesting that the pathogenesis of active CD is similar to that of active UC on the metabolomic level. As a matter of fact, colonic CD and indeterminate colitis are easily confounded with UC. On the other hand, the small sample size in our study may have accounted for the failure to create a model.

Differences in Metabolite Composition between Serum and Plasma

There were slight differences in the metabolite composition between serum and plasma samples in our IBD patients. This was not unexpected because studies have already shown that metabolites may differ depending on whether they are measured in serum or plasma.^{23,24} Serum derives from coagulated blood, whereas plasma is treated with an anticoagulant such as EDTA or heparin before removal of blood cells. One reason for the differences therefore could be, for example, the release of mediators from platelets during the coagulation processes.^{35,36} A recent study by Yu et al.²⁴ suggests that serum may be more sensitive for biomarker detection compared to plasma, whereas measuring metabolites in plasma may be more reproducible. Nevertheless, despite some differences between serum and plasma metabolite concentrations, both our serum or plasma metabolite-based statistical models were able to separate well between IBD and non-diseased subjects revealing a higher Q^2Y for UC in plasma than in serum, but a higher Q^2Y for CD in serum than in plasma.

Differences in Serum and Plasma Metabolite Levels of UC and CD Patients versus Controls

Amino Acids and Related Metabolites. Both UC and CD have an impact on amino acid metabolism showing increased levels of isoleucine (and its first degradation product 3-methyl-2-oxovalerate), methionine, lysine, glycine, arginine, and proline and decreased levels of valine, tyrosine, and serine as compared to the control cohort. Some of the increased amino acids were also reported to be increased in fecal extracts³⁷ and extracts of colon mucosa³² with the exception of isoleucine, which has apparently low concentrations in colonic mucosa of active CD and UC.³² Methionine is an essential amino acid and a precursor of homocysteine, a metabolite also shown highly elevated in plasma and colonic mucosa from UC and CD patients.³⁸ Interestingly, 2-hydroxybutyrate showed significant increases in serum and plasma of both UC and CD patients as compared to control subjects. 2-Hydroxybutyrate is mainly found in the liver and is highly expressed during oxidative stress when it is needed for the synthesis of the cell antioxidant glutathione.³⁹ It is a byproduct of the pathway from methionine to glutathione.³⁹

Metabolites Related to Energy Household. Serum of UC and plasma of UC and CD patients had elevated levels of creatine but lower levels of creatinine than in control subjects. Creatine is normally involved in the energy supply of mammalian cells. The relatively increased creatine levels that were also seen in our previously described DSS mouse model of UC⁴⁰ may indicate the need of ATP and fatty acids as energy supply during the states of the disease. Creatine may be degraded by intestinal bacteria;⁴¹ therefore, it is conceivable that the elevated creatine levels may even result from reduced bacterial degradation associated with microbial dysbiosis. We could also see high lactate levels in plasma samples of CD patients, although high lactate levels have been rather connected with severe UC.⁴² The decrease in Krebs cycle intermediates (such as succinate and citrate) and molecules involved in energy metabolism (such as acetate) in IBD patients may indicate the demand and rapid utilization of metabolites that feed energy producing pathways.

Metabolites of the Urea Cycle. Many similarities exist between serum and plasma from UC patients, but few in samples from CD patients with regard to downregulated metabolites. Urea showed strong decreases in UC as well as in CD patients, which is in contrast with previous findings of urea production in IBD patients.⁴³ In plasma from CD patients, we detected decreased levels of ornithine, which is also part of the urea cycle. The decrease in urea and ornithine in CD patients as compared to the control cohort may indicate disturbances in the urea cycle. In addition, these patients had a higher level of arginine, another component of the urea cycle, than the control individuals. High serum arginine levels that correlate with disease severity have been recently described for UC.⁴⁴

Monosaccharides and Other Metabolites. Among monosaccharides, higher levels of mannose and glucose were detected in IBD patients as compared to control subjects. Increased mannose was also observed in our DSS colitis mouse model,⁴⁰ while another group has found high levels of glucose in extracts of macroscopically unininvolved colonic mucosa of IBD patients.³³ In both CD and in UC, we noticed, relative to control subjects, a decrease in choline and its oxidized product betaine. Also other metabolomic studies of IBD have revealed a downregulation of choline,^{20,32} which is an essential nutrient.⁴⁵ Its deficiency has been connected with the development of

nonalcoholic fatty liver.⁴⁶ It is interesting that methanol was highly increased in both UC and CD. Methanol is produced endogenously in humans.⁴⁷ It may be produced in reasonable amounts in the colon through degradation of fiber pectin (which is contained in fruit, vegetables, jellies, milk products, etc.) and taken up by the circulation.⁴⁸ The human colonic flora is able to degrade pectins,^{49,50} and methanol is released into the blood upon this degradation.⁵¹ Since populations of the colonic microflora are deranged in IBD, either as a cause or a consequence,⁵² it is possible that an overgrowth of pectin-degrading bacteria may contribute to an increased methanol content. On one hand, increased formate levels (compared to control subjects) in serum of UC patients are in accordance with increased methanol levels. Methanol is converted into formate in the liver via formaldehyde by alcohol dehydrogenase, a process that also leads to the production of free radicals.⁵³ On the other hand, formate may be formed by intestinal bacteria, such as enterobacteria.⁵⁴ A role for *Enterobacteriaceae* in the etiology of IBD has been recently put forward.⁵⁵

Differences in Urine Metabolite Levels of UC and CD Patients versus Controls

We have previously used a metabolite analysis approach⁴⁰ and performed ¹H NMR spectroscopy to determine whether mice, which had developed an experimental form of colitis following DSS administration, could be discriminated from healthy controls. By multivariate statistical evaluation we were able to separate between diseased and healthy mice. As we have already observed in this mouse model of IBD, the metabolite profile of urine is different to that of serum/plasma and revealed different sets of metabolites. The origin of many urine metabolites in UC and CD patients may be related to the intestinal microflora.^{11,20} In our present study, urine metabolites showed some differences between CD and UC, however, it was not enough to create a model for distinguishing between these two forms of IBD. Similar to serum and plasma of UC and CD patients, Krebs cycle intermediates (such as citrate and succinate), betaine and urea levels were lower than in the control cohort. We also observed low hippurate levels in UC and CD, which is well in accordance with a study by Williams et al.¹¹ Low hippurate could indicate disturbances in the gut microbiome of IBD. For instance, decreases in *Clostridia* are widely found in CD patients.⁵⁵ The gut microbiome structure and the urinary metabolite profile have been recently investigated by Li et al.,⁵⁶ and these authors were able to correlate the presence of *Clostridia* with hippurate levels. Some similarities of the urine metabolome also exist with our DSS mouse model.⁴⁰ As in the DSS model, we found, relative to the control cohort, higher levels of allantoin and tryptophan in UC and higher levels of lactate and carnitine in UC and CD. Allantoin is a metabolite of uric acid and it is regularly detected in human urine.⁵⁷ It correlates with dietary purine uptake,⁵⁸ indicating disturbances in the purine metabolism in IBD. Tryptophan, which is the precursor of serotonin, is a widely expressed metabolite and transmitter throughout the gut. Tryptophan hydroxylase is decreased in rectal biopsies from patients with UC,⁵⁹ highlighting the possibility that tryptophan levels have gone up because of the reduced levels of its metabolizing enzyme. High levels of tryptophan could contribute to the role of serotonin as a pathogenic mediator in IBD. Unlike in serum and plasma, we noticed a decrease in methanol in both CD and UC relative to controls, which could be explained by the fact

that most methanol was taken up by the circulation as already shown.⁴⁸

Finally, CD patients exhibited an increase in sugars, such as xylose, maltose, galactose, and lactose compared to the control cohort. The changes of these metabolites are not quite clear. Xylose, for instance, can be broken down in the gut from dietary fiber (e.g., hemicellulose and cellulose) by cellulolytic microflora in the colon including *Enterococcus* sp.⁶⁰ As a fact, *Enterococcus* sp. is found in great abundance in CD patients and could thus contribute to higher xylose production and urine levels seen in our CD patients.⁶¹

CONCLUSION

Our study shows that quantitative metabolite analysis of serum, plasma, and urine from CD and UC patients can be used to discriminate between healthy and diseased subjects. We were additionally able to confirm the detection of metabolites described by other groups, suggesting that these overlapping results may be very important in the future for the determination of biomarkers (an overview of metabolite differences in experimental IBD models and human IBD is given by Lin et al.⁶²). In order to discriminate between CD from UC, use of serum and plasma may be of advantage because no model could be constructed for urine samples. This points to a possible limitation of this study, which may have been the small sample size. In summary, however, our study indicates that metabolic profiling is a powerful tool to identify intestinal inflammation and may be useful in the management of IBD and in clinical studies exploring disease pathogenesis.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>

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Notes

The authors declare no competing financial interest.

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