Untargeted Metabolomics and Inflammatory Markers Profiling in Children With Crohn's Disease and Ulcerative Colitis—A Preliminary Study

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Background: Metabolic profiling might be used to identify disease biomarkers. The aim of our study was to determine the usefulness of untargeted metabolomics analysis to detect differences in serum metabolites between newly diagnosed and untreated pediatric patients with Crohn's disease (CD) or ulcerative colitis (UC) in comparison with a control group (Ctr). Moreover, we investigated the potential of profiling metabolomics and inflammatory markers to improve the noninvasive diagnosis of CD and UC in children.

Methods: Metabolic fingerprinting of serum samples was estimated with liquid chromatography coupled with mass spectrometry in children with CD (n = 9; median age, 14 years), UC (n = 10; median age, 13.5 years), and controls (n = 10; median age, 12.5 years).

Results: The majority of chemically annotated metabolites belonged to phospholipids and were downregulated in CD and UC compared with the Ctr. Only 1 metabolite, lactosylceramide 18:1/16:0 (LacCer 18:1/16:0), significantly discriminated CD from UC patients. Interestingly, combining LacCer 18:1/16:0 with other inflammatory markers resulted in a significant increase in the area under the curve with the highest specificity and sensitivity.

Conclusions: Using serum untargeted metabolomics, we have shown that LacCer 18:1/16:0 is a very unique metabolite for CD patients.

Key Words: untargeted metabolomics, Crohn's disease, ulcerative colitis

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) represent inflammatory bowel diseases (IBDs) characterized by multifactorial pathogenesis involving genetic, environmental, and microbial factors. The exact mechanisms of these diseases are still unknown; hence, there is no causative treatment. The diagnosis and monitoring of IBD are mainly based on invasive procedures like endoscopy and only a few inflammatory biomarkers like erythrocyte sedimentation rate (ESR), albumin, C-reactive protein (CRP), and fecal calprotectin (FC), which are very sensitive but moderately specific indicators of intestinal inflammation.[1, 2] Therefore, there is an urgent need to find the specific diagnostic and noninvasive biomarkers for these diseases. Metabolomics, a new branch of science, deals with analysis of the metabolites present in human specimens in various states of health and disease. It has been shown that metabolomics might be used to identify disease biomarkers. Actually, all biological samples may be tested, but the most commonly used are serum, urine, and feces, because they are obtained from patients in relatively noninvasive procedures. Depending on the type of biological material tested, various metabolites can be discovered.[3] The fecal metabolome reflects the host–microbiota interactions, in contrast to serum, which more closely captures the host's metabolism. Recent findings confirmed alterations in the number of metabolites, mainly related to lipids and amino acids, in the serum of patients with IBD. However, the majority of studies included adult individuals with ongoing treatment, and only 1 study focused on the pediatric population.[4–6] Therefore, the aim of our study was to determine the usefulness of untargeted metabolomics analysis in the comparing and contrasting of blood metabolite profiles between newly diagnosed and untreated pediatric patients with CD or UC in comparison with healthy individuals (controls). The second aspect of the study was to investigate the

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potential of metabolomics and inflammatory marker profiling for improving the noninvasive diagnosis of IBD in children.

METHODS

Patient Selection

The investigation included newly diagnosed children with UC (n = 10; median age, 13.5 years), CD (n = 9; median age, 14 years), and 10 patients without symptoms of IBD, confirmed with normal fecal calprotectin level (noninvasive marker of IBD), who belonged to the control group (Ctr; median age, 12.5 years). Disease activity was estimated according to the Pediatric Crohn's Disease Activity Index (PCDAI) and the Pediatric Ulcerative Colitis Activity Index (PUCAI). No target therapy was administered before sample collection. There were no differences between controls, UC patients, or CD patients in terms of median age or sex. The characteristics of patients and controls are shown in Table 3.

Measurement of Serum and Fecal Inflammatory Markers

To avoid dietary influence on serum markers and metabolites, blood was collected after overnight fasting. Serum CRP (mg/L) and albumin (g/dL) levels were determined by immunoturbidimetry (Roche Diagnostics, IN, USA). The ESR was evaluated according to the Westergren method (mm/h). Complete blood count was measured using a Hematology Analyzer (Sysmex, Japan).

All fecal samples were collected during hospitalization, frozen immediately after receipt, and stored at –80°C until analysis. Fecal calprotectin (FC) concentration was determined by an enzyme-linked immunosorbent assay kit (IDK Calprotectin, Immundiagnostik, Bensheim, Germany) according to the manufacturer's instructions. The upper normal limit was determined as 50 µg of calprotectin per 1 gram of feces.

SERUM METABOLIC FINGERPRINTING

Chemicals and reagents

Purified water was obtained using the Milli-Q Integral 3 system (Millipore SAS, Molsheim, France). Zomepirac sodium salt (used as the internal standard [IS]), arachidonic acid, docosahexaenoic acid, lysophosphatidylethanolamine (LPE) 18:0, phosphatidylethanolamine (PE) 16:0/22:6, LS-MS-grade acetonitrile, methanol, formic acid, and LC-grade ethanol were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The API-TOF reference mass solution kit (G1969-850001) and tuning solutions, ESI-L low-concentration tuning mix (G1969-85000), and ESI-TOF Biopolymer Analysis reference masses (G1969-850003) were purchased from Agilent Technologies (Santa Clara, CA, USA).

Sample treatment and analysis

Serum samples were obtained from whole blood, which was collected in 2.4-mL Clotting Activator vacuum system tubes. The tubes were stored in the vertical position at room temperature to permit the formation of a clot during 60 minutes. At the end of the clotting time, blood samples were centrifuged in a horizontal rotor (swing-out head) for 10 minutes at 1300*g* at room temperature. Then serum fractions (0.5 mL each) were collected in Eppendorf tubes and stored at –80°C until the day of analysis.

On the day of analysis, samples were thawed on ice. Protein precipitation and metabolite extraction were performed by vortex-mixing (for 1 minute) 1 volume of the serum sample with 4 volumes of freezing cold (–20°C) methanol/ethanol (1:1) mixture containing 1 ppm zomepirac. After extraction, samples were stored on ice for 10 minutes and centrifuged at 21,000×*g* for 10 minutes at 4°C. The supernatant was filtered through a 0.22-µm nylon filter into glass vials. Quality control (QC) samples were prepared by mixing an equal volume of all samples. Obtained mixture was prepared following the same procedure as the rest of the samples.

Samples were randomly analyzed by a liquid chromatography coupled with mass spectrometry (LC-MS) system consisting of 1290 Infinity LC with a degasser, 2 binary pumps, and a thermostated autosampler coupled to a 6550 iFunnel Q-TOF-MS detector (both Agilent Technologies, Santa Clara, CA, USA). Analyses were performed in positive (ESI+) and negative (ESI-) ion modes, whereby 0.5 µL of sample was injected into a thermostated (60°C) Zorbax Extend- C18 RRHT (2.1×50 mm, 1.8-µm particle size, Agilent Technologies) chromatographic column. The flow rate was 0.6 mL/min with solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The chromatographic gradient started at 5% of phase B for the first minute, followed by an increase of phase B to 80% (from 1 to 7 minutes) and to 100% (from 7 to 11.5 minutes). After reaching 100%, the gradient returned to initial conditions (5% phase B) in 0.5 minutes, which were kept from 12 to 15 minutes. The mass spectrometer was operated in full scan mode from mass (m/z) 50–1000. The capillary voltage was set to 3 kV for positive and 4 kV for negative ionization mode. Nozzle voltage was 1000 V. The drying gas flow rate was 12 L/min at 250°C and gas nebulizer at 52 psig; the fragmentor voltage was 250 V for positive and negative ionization modes. Data were collected in centroid mode at a scan rate of 1.5 scan per second. Accurate mass measurements were obtained by means of calibrant solution delivery using a dual-nebulizer ESI source. A calibrating solution containing reference masses at m/z 121.0509 (protonated purine) and m/z 922.0098 (protonated hexakis [1H,1H,3H-tetrafluoropropoxy] phosphazine or HP-921) in positive ion mode or m/z 119.0363 (proton abstracted purine) and m/z 966.0007 (formate adduct of HP-921) in negative ion mode was continuously introduced

by an isocratic pump (Agilent, Santa Clara, CA, USA) at a flow rate of 0.5 mL/min (1:100 split).

LC-MS data treatment

The raw data collected by the analytical instrumentation were cleaned of background noise and unrelated ions by the molecular feature extraction (MFE) tool in Mass Hunter Qualitative Analysis Software (B.07.00, Agilent, Santa Clara, CA, USA). The MFE creates a list of all possible components described by mass, retention time (RT), and abundance. The limit for the background noise for data extraction by MFE was set to 2000 and 1000 counts for positive and negative ion mode, respectively. To identify co-eluting adducts of the same feature, the following adduct settings were applied: +H, +Na, +K in positive ion mode and −H, +HCOO, +Cl for negative ion mode. Dehydration neutral losses were also allowed in both ionization modes. Sample alignment and data filtering were performed using Mass Profiler Professional 12.6.1 (Agilent, Santa Clara, CA, USA). Parameters applied for the alignment were 1% for RT and 15 ppm for the mass variation. In the quality assurance (QA) procedure, metabolic features detected in >50% in QC samples with the coefficient of variation (CV) <20% were kept for further data treatment.

Metabolite identification

Identification of metabolites was performed based on the tandem mass spectroscopy (MS/MS) fragmentation, as previously described.[7] Accurate masses of features were searched against the METLIN, KEGG, LIPIDMAPS, and HMDB databases, which were simultaneously accessed by CEU Mass Mediator (http://ceumass.eps.uspceu.es/mediator/).[8] The identity of metabolites was confirmed by matching the experimental MS/MS spectra to MS/MS spectra from databases or fragmentation spectra and retention time obtained for the metabolite's standard. Experiments were repeated with identical chromatographic conditions to the primary analysis. Ions were targeted for collision-induced dissociation (CID) fragmentation on the fly based on the previously determined accurate mass and retention time. Phospholipids were identified based on a previously described characteristic fragmentation pattern.[9] Characteristic fragments of identified metabolites together with coefficients of signal variation in studied groups (including QCs) are presented in Supplementary Table 1.

Statistical analysis

Multivariate statistics were used to evaluate data quality by checking the location of the QC samples on principal component analysis (PCA) plots and to observe sample discrimination on partial least squares discriminant analysis (PLS-DA) plots. Multivariate calculations and plots were performed by using SIMCA−P + 13.0.3.0 (Umetrics, Umea, Sweden).

To select statistically significant metabolic features, univariate statistics were used. Depending on the normality of the data distribution (assessed by the Shapiro-Wilk test), the *t* test or Mann-Whitney nonparametric *U* test was used. Obtained *P* values were corrected by Benjamini-Hochberg false discovery rate (FDR). The level of statistical significance was set at 95% ($P < 0.05$). Univariate statistical analyses were performed in GNU Octave 4.0.3, which was also used for Spearman's rank correlation analyses. The significance of the difference in the inflammatory metabolites was evaluated with the Mann-Whitney *U* test using Statistica (version 13.1) software. The diagnostic value of the markers combined with LacCer 18:1/16:0 intensity was estimated using receiver operating characteristic (ROC) curve analyses. ROC analysis was performed using MedCalc, version 18 (MedCalc Software, Ostend, Belgium). The performance of the models was compared by applying the nonparametric method of Delong et al.[10] The specificity and sensitivity were determined according to the sample class prediction using the 7-fold cross-validation predicted values of the fitted Y-predcv (implemented in SIMCA-P+ 13.0.3.0 software) for observations in the model.

Ethical Considerations

The study was approved by the Medical Ethics Committee of the Medical University of Bialystok (R-I-002/308/2014) and conformed to the tenets of the Declaration of Helsinki. Either the patients or their parents gave their written informed consent for participation in the study.

RESULTS

Metabolic Fingerprinting of Serum Samples

Serum samples obtained from newly diagnosed children with CD or UC and controls were fingerprinted, and the obtained data underwent a filtering and QA procedure. As a result, 2 data sets were obtained with 576 metabolic features in positive and 601 in negative ionization modes. Based on those data, PCA was performed to check the classification of the QC samples. Close clustering of QCs on obtained PCA models (Fig. 1A and B) indicates the system's stability and performance during analyses. To illustrate the classification of the studied samples based on obtained serum fingerprints, a PLS-DA was performed. Figure 1 shows clear (panel C) and adequate (panel D) separation of CD, UC, and Ctr samples based on data obtained from positive and negative ion modes, respectively. Interestingly, primary data modeling resulted in the exclusion of 1 patient from the UC group (data not shown) due to distinct separation on PLS-DA models. This patient was additionally diagnosed with celiac disease, another autoimmune disease, a few months later.

Statistical analysis resulted in 121 significant metabolic features discriminating the IBD (CD+UC) group from the

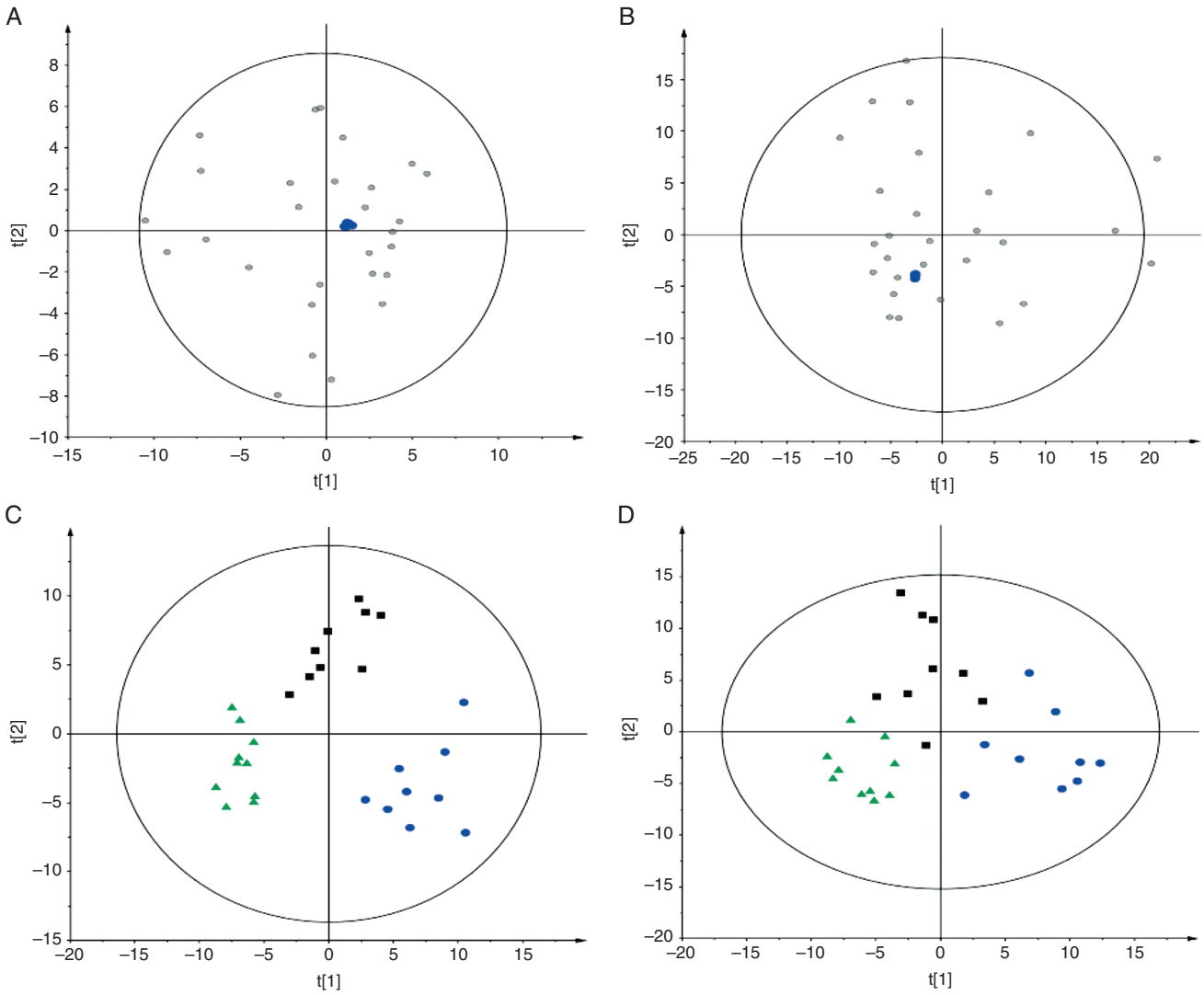

FIGURE 1. PCA and PLS-DA score plots of the serum fingerprints of CD and UC patients and Ctr. Panels A (positive ion mode, $R^2 = 0.578$, $Q^2 = 0.228$) and B (negative ion mode, $R^2 = 0.303$, $Q^2 = 0.049$) show the classification of QC samples (blue dots) on PCA models. Panels C (positive ion mode, $R^2 = 0.45$, $Q^2 = 0.267$) and B (negative ion mode, $R^2 = 0.42$, $Q^2 = 0.22$) show classifications of CD (blue dot), UC (black square), and Ctr (green triangles) samples on PLS-DA models.

Ctr group in negative ion mode and 66 in positive ion mode, respectively. The removal of metabolic features lacking chemical annotation resulted in 36 discriminatory metabolites in negative ion mode and 19 in positive ion mode. In the case of CD vs Ctr, the summarized results from both ion modes found 165 significant metabolic features, 48 having been identified. Similarly, in UC vs Ctr, 50 of 169 significant metabolic features were identified. The metabolites significant in the performed comparisons are summarized in Table 1. For these metabolites, coefficients of signal variation in the studied groups (including QCs) are presented in Supplementary Table 1. The majority of chemically annotated metabolites belonged to phospholipids and were downregulated in the CD and UC groups compared with the Ctr group. Among other metabolites, lysophosphatidylcholine (LPC; 18:1), phosphatidylcholine (PC; 36:6; 18:2/18:0; 16:0/18:1; 16:0/20:3; 16:0/20:4; 20:4/22:5), PE (P-16:0/22:6), sphingomyelin (SM; 32:0; 32:2; 33:2; 34:2), and LPE (18:2) were found to be significantly lower in the serum of CD patients in comparison with the Ctr group. The intensities of the significant lipids identified as LPC, PC, and SM were summed, and their levels in the studied groups are presented in Figure 2. Docosahexaenoic acid, arachidonic acid, and LPC (16:0; 16:1; 17:1; 18:0; 20:2; 20:3; 20:4; 22:4) were the downregulated metabolites, which allowed us to distinguish UC patients from the Ctr group. Only 1 metabolite, lactosylceramide (18:1/16:0; LacCer 18:1/16:0), significantly discriminated CD from UC patients (Fig. 2). Some of the significant metabolites remained unidentified. Table 2

TABLE 1. Serum Metabolites Significantly Discriminating Studied Groups

Metabolites	Percent Change			
	CD vs Ctr	UC vs Ctr	CD vs UC	IBD vs Ctr
A, Phospholipids				
LPC (14:0) sn-2	–49.8	–57.8	NS	–53.8
LPC (14:0) sn-1	–50.2	–53.2	NS	–51.7
LPC (15:0)	NS	NS	NS	–32.0
LPC (16:0)	NS	**–25.8**	NS	–24.5
LPC (16:1) sn-2	–40.5	–35.4	NS	–37.9
LPC (16:1) sn-1	NS	**–32.2**	NS	–30.8
LPC (17:1)	NS	**–40.9**	NS	–34.9
LPC (18:0) sn-2	NS	**–26.4**	NS	NS
LPC (18:0) sn-1	NS	**–26.2**	NS	NS
LPC (18:1) sn-2	**–24.9**	NS	NS	–21.0
LPC (18:1) sn-1	**–30.9**	NS	NS	–28.5
LPC (18:2) sn-2	–42.8	–39.4	NS	–41.1
LPC (18:2) sn-1	–44.0	–27.6	NS	–35.8
LPC (18:3)	–45.2	–49.5	NS	–47.2
LPC (20:0)	NS	NS	NS	–31.0
LPC (20:2)	NS	**–32.5**	NS	–29.8
LPC (20:3)	NS	**–28.8**	NS	–28.8
LPC (20:4)	NS	**–27.2**	NS	–24.9
LPC (20:5)	–43.6	–54.3	NS	–49.0
LPC (22:4)	NS	**–30.6**	NS	–28.5
LPC (22:5)	–38.5	–31.5	NS	–35.0
LPC (22:6)	–38.5	–31.5	NS	–35.0
LPA (18:2)	NS	NS	NS	–36.3
LPA (20:4)	NS	NS	NS	–27.3
LPE (15:1)	–32.2	–35.1	NS	–33.6
LPE (18:0)[a]	–20.2	–21.9	NS	NS
LPE (18:2)	**–34.1**	NS	NS	NS
PC (34:4)	–62.3	–66.2	NS	–64.3
PC (36:6)	**–33.5**	NS	NS	NS
PC (18:2/18:0)	**–34.2**	NS	NS	NS
PC (16:0/18:1)	**–30.5**	NS	NS	NS
PC (16:0/19:1)	–36.1	–24.8	NS	–30.4
PC (16:0/20:4)	**–59.8**	NS	NS	–42.7
PC (16:0/20:3)	**–38.1**	NS	NS	–25.5
PC (20:4/22:5)	**–36.1**	NS	NS	NS
PE (16:0/22:6)[a]	**–38.2**	NS	NS	NS
SM (30:1)	–51.8	–47.3	NS	–49.5
SM (32:0)	**–45.0**	NS	NS	NS
SM (32:1)	–38.8	–34.1	NS	–36.5
SM (32:2)	**–52.0**	NS	NS	–44.0
SM (33:1)	–32.4	–30.0	NS	–31.2
SM (33:2)	**–25.3**	NS	NS	NS
SM (34:1)	NS	NS	NS	–22.9
SM (34:2)	**–31.6**	NS	NS	–23.2
B, Other metabolites				
Lactosylceramide (18:1/16:0)	**70.6**	NS	**40.0**	46.2
Arachidonic acid[a]	NS	**–34.4**	NS	–28.6
Docosahexaenoic acid[a]	NS	**–54.6**	NS	NS

Each column represents a comparison of 2 different groups (CDvsCtr, UCvsCtr, CDvsUC, IBDvsCtr). A positive value means that an abundance of a particular metabolite was higher, whereas a negative value indicates that abundance was lower in 1 group vs another group. Values in bold indicate that a particular metabolite was found significant only in the Crohn's disease (the second column) or ulcerative colitis group (the third column). Depending on the normality of the data distribution (assessed by the Shapiro-Wilk test), the *t* test or Mann-Whitney nonparametric *U* test was used. Only values of statistically significant changes ($P < 0.05$ after Benjamini-Hochberg false discovery rate correction) are presented.
Abbreviations: LPA, lysophosphatidic acid; NS, not significant.
[a]Identity of these metabolites was confirmed by LC-MS/MS analysis of the standards.

presents the most significant metabolites with good-quality fragmentation spectra. For these metabolites, coefficients of signal variation in the studied groups (including QCs) are presented in Supplementary Table 2.

Metabolic Pathways Analysis

To test if the metabolic profiles discriminating children with IBD from the control group match any metabolic pathways, a pathway analysis was performed using MetaboAnalyst 4.0.[11] *Homo sapiens* pathway library, which contains 80 metabolic pathways, was selected. The calculated *P* value was established based on pathway enrichment analysis, whereas the pathway impact value was based on pathway topology analysis. As shown in Supplementary Figure 1, glycerophospholipid metabolism was the most significantly altered metabolic pathway. A similar result was obtained when metabolites discriminating patients with CD from controls were included in the analysis. Additionally, the sphingolipid metabolism also seemed to be the relevant pathway involved in CD (data not shown). Pathway analysis of serum metabolites significant in UC patients in comparison with the control group revealed none of the pathways as significantly altered (data not shown).

Correlation Analysis Between Metabolites and Selected Inflammatory Markers

The median values of selected inflammatory markers in each group are presented in Table 3. Patients with CD manifested higher median values of ESR, CRP, white blood count (WBC), platelet count (PLT), albumin, and FC than Ctr, whereas children with UC presented significantly enhanced

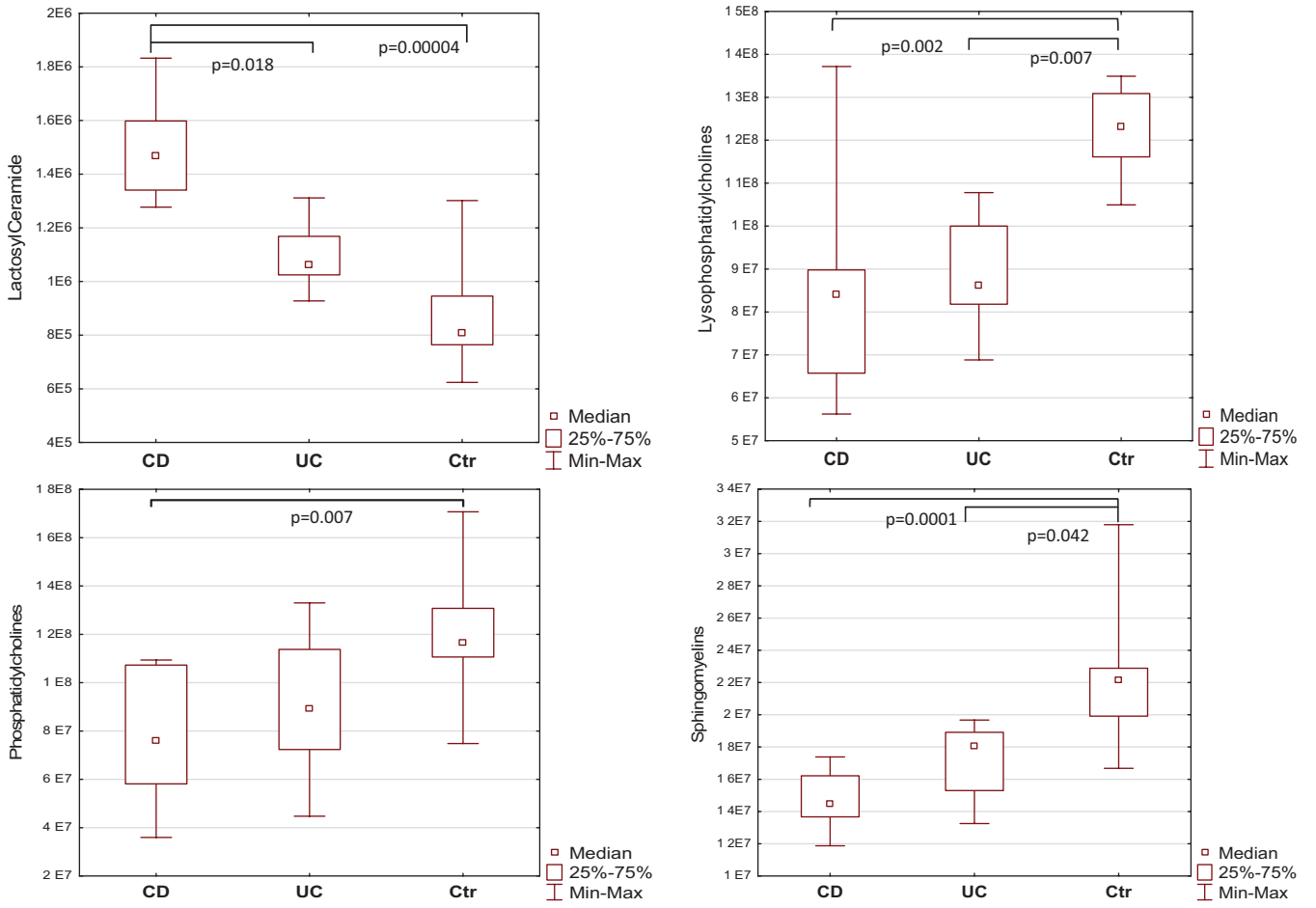

FIGURE 2. Difference in lactoserylceramide 18:1/16:0, lysophosphatidylcholines, phosphatidylcholines, and sphingomyelins intensity detection between study groups. The statistical difference was analyzed by Mann-Whitney *U* test. *P* values <0.05 were considered significant.

TABLE 2. Unidentified Metabolic Features Significantly Discriminating Studied Groups

Ion Mode	Name	Neutral Monoisotopic Mass, Da	RT, min	Percent Change				Fragmentation ions, m/z
				CD vs Ctr	UC vs Ctr	CD vs UC	IBD vs Ctr	
Negative	Unknown_1	540.4384	7.4	–75.1	**–50.6**	NS	–54.0	539.4306, 315.254, 223.17
Positive	Unknown_2	187.067	0.26	NS	–27.2	NS	–29.3	188.0719, 170.059, 146.06, 118.065
Positive	Unknown_3	562.4207	7.4	**–79.1**	NS	NS	–59.6	563.4298, 339.252, 247.168
Positive	Unknown_4	607.4656	7.5	**58.1**	NS	NS	37.0	608.4734, 309.279
Positive	Unknown_5	695.517	7.5	**44.9**	NS	NS	NS	696.5248, 309.281

Each column represents comparison of 2 different groups. In comparisons with the Ctr group, a positive value means that the intensity of a particular metabolite was higher, and a negative value mean that the intensity was lower in the studied group in comparison with the Ctr group. In comparing CD vs UC, a positive value means that the intensity of a particular metabolite was higher, and a negative value means that the intensity was lower in the CD group in comparison with the UC group. Bold formatting indicates that that particular metabolite was detected significantly only in the Crohn's disease group (the first column) or ulcerative colitis group (the second column). Depending on the normality of the data distribution (assessed by the Shapiro-Wilk test), the *t* test or Mann-Whitney nonparametric *U* test was used. For metabolite, intensity with $P < 0.05$ was considered statistically significant after Benjamini-Hochberg false discovery rate correction.
Abbreviation: NS, not significant.

ESR, CRP, WBC, and FC compared with Ctr. Significant correlations between metabolites and CRP, ESR, PLT, WBC, albumin, mean platelet volume (MPV), red blood cell distribution width (RDW), ferritin, FC, and disease activity score (PCDAI/PUCAI) were found in the CD and UC groups. The results are presented as heatmaps in Supplementary Figure

TABLE 3. Demographic and Clinical Characteristic of Patients With CD, UC, or Ctr

	Ctr	UC	*P* UC vs Ctr	CD	*P* CD vs Ctr
No. patients	10	9	-	9	-
Median age, y	12.5 (10–16)	13.5 (8–16)	NS	14 (8–16)	NS
Male sex	5	4	-	4	-
Disease duration, mo	n.a.	0	-	0	-
Disease activity index	n.a.	PUCAI	-	PCDAI	-
Median (range)		37.5 (10–60)	-	30 (10–42.5)	-
Paris classification, No. patients					
Location - L1/L2/L3	n.a.	n.a.	-	7/0/2	-
Behavior - B1/B2/B2B3	n.a.	n.a.	-	3/5/1	-
Growth - G0/G1	n.a.	n.a.	-	7 / 2	-
Extent - E1/E2/E3/E4	n.a.	2/5/0/2	-	n.a.	-
Mayo Endoscopic Score I/II/III	n.a.	0/3/6	-	n.a.	-
ESR, median (range), mm/h	3 (2–9)	8 (5–38)	0.02	41 (8–68)	0.00009
CRP, median (range), mg/L	0.35 (0.1–1.7)	3 (0.45–11.9)	0.001	33 (8.1–156)	0.00002
WBC, median (range), 10^3/μL	6.1 (1.7–7.9)	8.23 (5.7–18.9)	0.04	8.3 (6.1–13.8)	0.008
PLT, median (range), 10^3/μL	248 (206–417)	287 (234–506)	NS	412 (210–543)	0.02
RDW, median (range), fL	40 (34.4–42.1)	39.5 (37–71.4)	NS	39.1 (33.6–42.9)	NS
MPV, median (range), fL	10.3 (9.6–11.4)	10.2 (8.7–12.6)	NS	9.8 (8.6–19.3)	NS
Ferritin, median (range), ng/mL	34 (24.3–47.3)	35.2 (5.7–113.8)	NS	42.2 (5.3–170.6)	NS
Albumin, median (range), g/dL	4.8 (4.2–5.5)	4.53 (2.78–4.91)	NS	4.2 (3.2–4.8)	0.002
FC, median (range), μg/g	14.9 (4.8–51.6)	1799 (415–2673)	0.00002	1989 (522–2607)	0.00002

The statistical difference was analyzed by Mann-Whitney *U* test. *P* values <0.05 were considered significant.
Abbreviations: B1, nonstricturing, nonpenetrating; B2, stricturing; B2B3, stricturing, penetrating; E1, ulcerative proctitis; E2, left-sided colitis; E3, extensive colitis (hepatic flexure distally); E4, pancolitis; G0, no evidence of growth delay; G1, growth delay; L1, distal 1/3 ileum; L2, colonic; L3, ileocolonic; n.a., not applicable; NS, not significant.

2. Interestingly, only a few metabolites were associated with more than 1 inflammatory marker. In the CD group, significant correlations were found between PC (36:6), MPV (R = 0.683, P = 0.04) and ferritin (R = –0.800, P = 0.009). In UC group the strong correlations of LPC (20:0) with ESR (R = –0.728, P = 0.026), WBC (R = –0.700, P = 0.036), PUCAI (R = –0.823, P = 0.006) were noted. Moreover, the significant correlations of SM (32:2) with WBC (R = –0.683, P = 0.042), PLT (R = –0.683, P = 0.042) and albumin (R = 0.766, P = 0.016) were also found. Interestingly, the unidentified metabolite with a neutral monoisotopic mass of 187.067 was associated with the main inflammatory markers, FC (R = –0.766, P = 0.016) and albumin (R = 0.750, P = 0.02), in the CD group, and another compound with a neutral monoisotopic mass of 607.4656 correlated with FC (R = 0.700, P = 0.036), RDW (R = 0.766, P = 0.016), and PUCAI (R = 0.697, P = 0.037) in children with UC.

Integration of Significant Metabolites and Inflammatory Markers

To test if the metabolic profiles combined with inflammatory markers may more accurately differentiate CD patients from UC patients than metabolites or biomarkers analyzed separately, the PLS-DA models were created. As shown in Table 4, the clinical serum markers separated CD patients with the same variance (R^2 = 0.68) as metabolites (R^2 = 0.68), but with a better predictive value (Q^2 = 0.534 vs Q^2 = 0.39). Integration of the significant metabolites and clinical markers only slightly improved the separation model by enhancing variance (R^2 = 0.737), but the predictive value remained lower compared with the PLS-DA model with clinical markers.

TABLE 4. Comparison of PLS-DA Models Differentiating Metabolic Fingerprints of CD and UC Children

Variables	R2	Q2	*P*
Clinical markers[a]	0.68	0.534	0.002
Metabolites[b]	0.68	0.39	0.08
Metabolites[b] + clinical markers[a]	0.737	0.46	0.01

[a]Clinical markers: ESR, CRP, PLT, albumin, WBC, FC.
[b]Metabolites included in Table 1.

As a specific clinical marker for CD has not been found yet, we tested if LacCer 18:1/16:0 alone or in combination with other inflammatory markers may improve discrimination results

TABLE 5. Evaluation of Diagnostic Efficiency of Selected Metabolites and Inflammatory Markers

Variables	AUC (95% CI)	Sensitivity, %	Specificity, %	PPV, %	NPV, %	ACC, %	*P*
CRP	0.988 (0.793–1.000)	100	88.9	75.0	100.0	87.5	< 0.0001
ESR	0.895 (0.661–0.988)	88.9	77.8	57.2	95.5	76.4	< 0.0001
FC	0.568 (0.318–0.794)	100	22.2	30.0	100.0	65.0	0.6
FC+ESR+CRP+albumin	0.988 (0.793–1.000)	100	88.9	75.0	100.0	87.5	< 0.0001
LacCer	0.988 (0.793–1.000)	100	88.9	75.0	100.0	87.5	< 0.0001
LacCer+FC	0.988 (0.793–1.000)	100	88.9	75.0	100.0	87.5	< 0.0001
LacCer+CRP+ESR	1.000 (0.815–1.000)	100	100	100.0	100.0	100.0	< 0.0001
LacCer+CRP+ESR+FC+albumin	1.000 (0.815–1.000)	100	100	100.0	100.0	100.0	< 0.0001

The empirical (nonparametric) method by DeLong et al.[10] was used to estimate the area under the curve.
Abbreviation: NPV, negative predictive value.

between CD and UC in a ROC curve analysis. FC alone, a well-known specific marker of bowel inflammation, had the lowest area under the curve (AUC; 0.568) and positive predictive value (PPV; 30%), but the addition of any of the tested serum inflammatory markers to FC increased the AUC to 0.98 and PPV to 75%, as shown in Table 5. Interestingly, combining LacCer 18:1/16:0 with other inflammatory markers resulted in a significant increase in AUC (1.0), with the highest PPV (100%), accuracy (ACC; 100%), specificity (100%), and sensitivity (100%).

DISCUSSION

Metabolic profiling is an innovative research approach allowing the discovery of disease pathogenesis and its biomarkers. Recently, a few studies have provided new insights into metabolic alterations in IBD.[5, 6, 12, 13] However, using the untargeted metabolite profiling for the first time made our study innovative, and thus our results may complement current knowledge about IBD biomarkers. Furthermore, all serum samples were derived from newly diagnosed children before treatment was started. Our metabolomics analysis using liquid chromatography tandem mass spectroscopy (LC-MS/MS) relevantly differentiated pediatric patients with IBD from the pediatric control group. Moreover, a combination of the significant metabolites and inflammatory markers improved the discrimination model compared with the metabolic profiles.

The most important finding of our study was the detection of lactosylceramide 18:1/16:0 (LacCer), which seemed to be the best discriminator between patients with CD and UC. Its significantly enhanced intensity was found only in CD patients. To our knowledge, this is the first report about upregulation of LacCer in serum of patients with CD. Stevens et al. presented data concerning the presence of LacCer in bowel tissues.[14] Using scanning densitometry, the "strong presence" of LacCer in mucosa of intestinal biopsies taken from CD patients was detected, contrary to biopsies taken from ulcerative colitis, indeterminate colitis, or control individuals. Moreover, LacCer was not found in biopsies from uninvolved parts of the bowels of patients with CD, celiac disease, or microscopic colitis. The intense detection of LacCer only in inflamed CD mucosa may be explained by the presence of a high concentration of LacCer in neutrophils. However, neutrophils also infiltrate the mucosa of ulcerative colitis. The authors suggested that in patients with CD, a novel source of LacCer is present in the mucosa or their normal glycosphingolipid metabolism is disturbed. Therefore, they concluded that it may be used as a marker to distinguish patients with CD from those with UC, indeterminate colitis, or microscopic colitis. It is worth noting that in our study the discrimination model combining LacCer with inflammatory biomarkers, which were found previously as the most specific in CD,[2] resulted in a significant increase in AUC with the highest specificity and sensitivity. LacCer, also known as CD17, is expressed on granulocytes, monocytes, and platelets and is involved in cell–cell interactions, intracellular signaling transmission, generation of nitric oxide, and phagocytosis. LacCer synthesis is mediated by TNF-alpha, proinflammatory cytokine, a well-known target in IBD therapy. In turn, LacCer, via the generation of superoxide, upregulates the expression of the cell adhesion molecule intercellular cell adhesion molecule–1 (ICAM-1) on the surface of endothelial cells, facilitating the adhesion of neutrophils.[14–16] Moreover, LacCer activates phospholipase A_2 (PLA_2) and the release of arachidonic acid (AA), an inflammatory mediator.[17] LacCer and AA have been proposed to be lipid second messengers in inflammatory disease. As an alteration in lipid compound mediates the various biological processes, including inflammation, apoptosis, or proliferation, it has already been suggested that perturbation

in lipid metabolism may contribute to the development of IBD. Our study revealed a decreased intensity of AA and docosahexaenoic acid in children with UC, but not CD, compared with Ctr. This stands in opposition to the Esteve-Comas et al. report concerning adult patients with ongoing treatment.[18] In this study, an increased plasma concentration of docosahexaenoic acid in UC and CD patients compared with controls was noted. However, the level of docosahexaenoic acid gradually decreased as disease activity increased, but never below the values of healthy controls. No significant difference of AA plasma concentrations between IBD patients and controls was found. The authors suggested that in active inflammation the increased biosynthesis of PUFAs is a result of the enhanced fatty acid consumption as increased levels of AA and DHA in the colonic tissues of UC and CD patients with active disease were detected in other studies.[19, 20] This phenomenon may be partially explained by increased tissue phospholipid turnover and an elevated PLA2 level, which hydrolyzes AA.[21] Analysis of colonic biopsies from patients with IBD who were clinically in remission displayed significantly less PC and LPC among UC patients, compared with those with CD and controls.[22, 23] However, results concerning mucosal lipid content in inflamed tissue and inactive colitis are inconsistent and depend on the method used for detection.[13, 22, 24] We also noted decreased LPC compounds in children with newly diagnosed UC, but no difference in the majority of identified PC was found; nevertheless, we tested serum, not tissue, of young patients. In contrast, in CD patients, most of PC compounds were downregulated, and some of them correlated with disease activity and chosen serum inflammatory markers.

Previously reported targeted metabolomics highlighted the altered folate and pterine biosynthesis as the significant metabolic pathway in CD and UC.[4] Our analysis pointed to perturbation of glycerophospholipid metabolism as the most relevant metabolic pathway observed in the IBD group, especially in CD patients.

The limitation of the present study was the small number of participants. Thus, these promising findings should be validated in a multicenter study.

CONCLUSIONS

In conclusion, using untargeted metabolite profiling in serum, we have shown that both CD and UC have an impact on the lipid metabolism. Our results have revealed that LacCer 18:1/16:0 is a very unique metabolite significantly increased in the serum of CD patients. Moreover, a combination of LacCer 18:1/16:0 with certain inflammatory markers is capable of discriminating children with CD from UC with high specificity and sensitivity.

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