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Metabolomic Signatures in Pediatric Crohn's Disease Patients with Mild or Quiescent Disease Treated with Partial Enteral Nutrition: A Feasibility Study

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Abstract

Little is known about the metabolic response of pediatric Crohn's disease (CD) patients to partial enteral nutrition (PEN) therapy and the impact of disease activity and inflammation. We analyzed plasma samples from a nonrandomized controlled intervention study investigating the effect of partial enteral nutrition (PEN) on bone health and growth throughout one year with untargeted metabolomics using high-performance liquid chromatography (HPLC) coupled with high-resolution mass spectrometry (HRMS). Thirty-four paired samples from two time points (baseline and 12 months) were analyzed. Patients (median age: 13.9 years, range: 7–18.9 years, 44% females) were in remission or had mild disease activity. The intervention group received a casein-based formula for 12 months, providing ~25% of estimated daily energy requirements. Sparse partial least squares discriminant analysis (splsda) was applied for group discrimination and identifying sources of variation to identify the impact of PEN. We also investigated the correlation of metabolites with inflammation markers, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and fecal calprotectin. After 12 months, our results show substantial difference between PEN and non-PEN groups in the metabolome of CD patients in remission or with mild disease activity. Inflammatory markers were associated with individual compounds and chemical classes such as isoprenoids and phospholipids. Identified compounds comprise metabolites produced by human or bacterial metabolism, as well as xenobiotics recognized as flavoring agents and environmental contaminants and their biotransformation products. Further longitudinal studies that also include patients with higher disease activity are warranted to evaluate the suitability of these metabolic biomarkers for predicting disease activity.

Keywords

enteral nutrition, inflammatory markers, pediatric inflammatory bowel disease, untargeted metabolomics, xenobiotics

Introduction

Enteral nutrition (EN) therapy is effective in pediatric Crohn's disease (CD) patients. Most published studies investigated exclusive enteral nutrition (EEN) or, more recently, specific exclusion diets.[1] Partial enteral nutrition (PEN), or nutritional supplementation with liquid formulas that provides 35–50% of habitual caloric intake with continued consumption of a normal diet, has also been applied as a dietary therapy for improving the nutritional status of children with CD.[2–4] Although PEN was not effective in inducing disease remission, it can help to maintain remission in patients who were initially treated with EEN.[3,5–8] Data on the long-term efficacy of PEN for maintenance of remission in pediatric CD patients are inconclusive, and PEN's mechanisms of action are not understood.[9] Hypotheses were generated on the impact of EN intervention on the gut microbiome,

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mucosal integrity, and the immune system, the three components of the current inflammatory bowel disease (IBD) paradigm.[10] Some studies investigated dietary effects on the intestinal microbiota by tracing changes in the levels of some bacterial metabolites, while others investigated the potential impact on gene regulation for cell growth and inflammatory pathways.[10–17] Studying the effects on the metabolome, characterizing small molecules that are intermediate or end products of biochemical reactions, may provide insights on an individual's genome and its interactions with environmental exposures that affect cellular metabolism and functions.[18,19] We have previously applied targeted metabolomics to investigate the impact of PEN therapy on the metabolic profile of pediatric CD patients in remission.[20] In the present work, we assess the feasibility of liquid chromatography–quadrupole-time-of-flight tandem mass spectrometry (LC-QTOF-MS/MS) to explore a more global picture of impacts of PEN therapy and to investigate the potential of the technique in CD biomarker research, especially in patients in remission.

While targeted metabolomics quantifies a defined set of metabolites of interest, untargeted mass spectrometry (MS)-based metabolomics enables comprehensive profiling of up to 4000 distinct molecules involved in different metabolic pathways in a few microliters of plasma.[21] The untargeted approach may be applied in the study of conditions when the affected metabolic pathways are unknown.[21,22] A few studies[23–26] applied targeted and untargeted metabolomics to identify signatures for CD; however, none has been conducted to evaluate the impact of PEN adjunctive therapy on the metabolic profiles of pediatric CD in remission or mild disease activity. We also investigated associations between metabolome and markers reflecting the degree of inflammation.

Materials and Methods

Patient Cohort and Samples

This study was performed with samples obtained in a prospective nonrandomized controlled intervention trial[20] to assess the effect of PEN on bone–muscle geometry in CD patients aged 6–19 years with quiescent or mild disease based on the mathematically weighted pediatric CD activity index (wPCDAI).[27] Patients in the intervention group received a casein-based complete liquid formula (Modulen IBD, Nestlé, Frankfurt/Main, Germany), providing for a duration of 12 months ~25% of daily energy requirements, estimated based on reference values from the German, Austrian, and Swiss nutrition societies for children, adolescents, and adults.[28] Patients who did not agree to PEN treatment were assigned to the control group and followed by the same protocol. Both groups continued their medical maintenance treatment. Disease activity, biochemical parameters, and ethylenediaminetetraacetic acid (EDTA) plasma were collected at baseline and then every 3 months for up to one-year follow-up. Forty-two patients were recruited between February 2016 and March 2017 in the Department of Pediatrics of the two university hospitals in Munich (LMU Munich and Technical University Munich). Samples from baseline (t0) and after 12 months of follow-up (t12) were selected for analysis. Paired samples were available from 34 CD patients. CRP, ESR, fecal calprotectin, and leucocyte count [white blood cells (WBCs)] were determined as part of routine clinical care.

Sample Preparation

Blood was drawn in EDTA–blood collection tubes (S Monovette, Sarstedt, Nümbrecht, Germany), and tubes were placed immediately on crushed ice. Within 30 min, plasma was separated from cells in a precooled centrifuge, aliquoted in cryotubes, and stored at −80 °C until further analysis. Preanalytical handling of samples was kept identical throughout the study period. On the day of analysis, samples were thawed on ice. For protein precipitation and sample extraction, 100 µL of plasma were transferred into a 1.5 mL Eppendorf tube, and 900 µL of ice-cold high-performance liquid chromatography (HPLC)-grade methanol was added, vortexed for 30 s, and set on freezer at −20 °C for 30 min. The tubes were centrifuged at 4000×g for 10 min, and the supernatant was filtered using a polytetrafluoroethylene (PTFE) 45 µm 96-well filter plate. The filtrate was transferred to vials and kept at −80 °C prior to analysis. To prepare a quality control (QC) sample, 100 µL of each sample extract was pooled together for analysis. Plasma QC samples were used to provide a representative sample containing all plasma samples. QC sample was injected in the beginning of the analysis and then after every fifth study sample to guarantee reproducibility.

Analytical Method: LC-QTOF-MS(/MS)

The LC-QTOF-MS(/MS) experiments were performed on a 1290 Infinity II HPLC system coupled to a 6545 Q-TOF (both from Agilent, Santa Clara, CA). Chromatographic separation was achieved using a Poroshell 120 EC-C18 (2.1× 150 mm, 2.7 µm) column (Agilent). The column oven was set to 40 °C; the autosampler was set to 10 °C. Analysis was performed with the instrument in the 2 GHz, extended dynamic range in the positive (POS) and negative (NEG) ionization modes using an Agilent Jet Stream (AJS) electrospray ionization (ESI) ion source, using the following operation parameters: capillary voltage: 3500 V (POS) / 4000 V (NEG); nozzle voltage 0 V (POS) / 500 V (NEG); nebulizer pressure: 40 psi (POS and NEG); gas temperature: 290 °C (POS and NEG); sheath gas flow: 12 L/min (POS and NEG); sheath gas temperature: 380 °C (POS and

NEG); fragmentor voltage: 170 V (POS and NEG); skimmer voltage: 65 V; and octupole radio frequency (RF): 750 V (POS and NEG). Data were acquired using MassHunter Acquisition B.09.00 software (Agilent) by injecting 6 μL of extract in a 16 min run at a flow rate of 0.4 mL/min using a gradient with the following mobile phases: A, water (0.1% formic acid); and B, methanol (0.1% formic acid). Gradient elution was performed with an initial mixture of 5% B and 95% A, then increased to 60% B throughout 4 min, to 99% B at 12 min, held until 14 min, returned to 5% B at 15.1 min, and held to 16 min.

LC-QTOF-MS Analysis

The QC samples were injected in triplicate in the full-scan MS acquiring mode from 100 to 1050 m/z, in the POS and NEG ion modes, to create an inclusion list to be used in the auto MS/MS mode. The data obtained in the MS experiment from the QC samples were extracted using the batch recursive feature extraction algorithm in MassHunter Profinder B.08.00 software (Agilent), then the features were evaluated individually among the replicates to ensure reproducibility and exported as CEF (Cluster Exchange Format) files. Mass Profiler software (Agilent) was used for alignment of features using retention time (RT) tolerance of up to 0.3 min and mass tolerance of ±15 ppm. Features with 100% occurrence in the replicates were used to create a target MS/MS inclusion list.

LC-QTOF-MS/MS Analysis

Data were acquired using data-dependent acquisition (DDA) (auto MS/MS mode) using the features present in the target MS/MS inclusion list as preferred for fragmentation, using a delta m/z of 15 ppm and delta RT of 0.15 min. A collision cell operates with fixed collision energies of 10, 20, and 40 eV, using nitrogen as collision gas. The acquisition mass ranges used were 100 to 1050 m/z at 4 spectra/s in the MS and 50–800 m/z at 3 spectra/s in the time of flight (TOF) for the fragments generated in the collision cell (MS/MS). The precursor threshold was set as 1000 counts (absolute) and 0.01% (relative) with active exclusion of two spectra for 0.2 min and five maximum precursors per cycle.

Data Processing

Samples acquired on auto MS/MS acquisition mode were extracted using the batch recursive feature extraction algorithm in Profinder B.08.00. The features were evaluated individually, and data were exported as CEF files. Mass Profiler Professional (Agilent) was used for alignment, normalization, and quality control. Raw data were normalized using a quantile algorithm, and features with less than 100% occurrence between the QCs and with a coefficient of variance higher than 25% were excluded. Principal component analysis was used to perform a QC on samples to exclude any outlier by visual inspection. Identification of features was performed by library search using Mass Hunter METLIN Personal Compound Database and Library (PCDL) (Agilent) at the MS/MS level. The features that did not match with the library compounds had their formulas generated by a molecular formula generator (MFG) algorithm. Analysis of the QC samples in the full-scan MS acquisition mode resulted in the detection of 2599 features in the negative-ion mode and 2074 features in the positive-ion mode. A preferred precursor mass list was built from these features that was used in the auto MS/MS acquisition mode for the study samples. After QC, a total of 322/140 features was putatively identified using MS/MS data, and 52 and 85 features had the formula generated in the negative and positive ion modes, respectively. The data were then exported in CSV (comma-separated values) format for statistical analysis.

Statistical Analysis

Statistical analysis was performed independently on the generated datasets comprising 374 and 225 annotated compound peak lists in NEG and POS modes, respectively. Each dataset was composed of paired sample data for control (Non-PEN) and intervention (PEN) groups at two time points (baseline, and after 12 months of treatment with or without PEN). As a first step, sparse partial least squares discriminant analysis (splsda) analyses were performed using the absolute concentrations at the two time points to visualize the group separation. Then, to eliminate observed baseline differences, relative concentrations were used to build the model, which were calculated by dividing the metabolites' concentration data for the second time point (12 months) by the respective baseline concentrations. For model optimization, a tuning process using M-fold cross validation (CV) was carried out for the selection of parameters giving the best model performance [number of principal components (PCs) and key metabolites to keep in each PC (keep X)]. Accordingly, three PCs were selected for the POS and NEG ion mode datasets with a keep X of (30, 9, 8) and (24, 34, 34) key metabolites for the two PCs, respectively.

Another aspect of the analysis was to investigate which metabolites correlated most with markers used in assessing clinical and biochemical disease activity, comprising CRP, ESR, WBCs, and fecal calprotectin. For this purpose, an integrative analysis [sparse partial least squares (spls)] model was applied on the within-group matrices of the matched datasets. Respectively, multilevel spls models were built for both positive and negative modes, and tuning was performed for selection of the optimum features giving the best model performance in terms of regression coefficient (R^2), mean squared error of prediction (MSEP), root

Table 1. Baseline Patient Characteristics: Subcohort of a Nonrandomized Intervention Trial on PEN in Pediatric CD Patients.[20]

Patient Characteristics		Non-PEN ($n = 18$)	PEN ($n = 16$)	p Value
Gender (male/female)		11/7	8/8	n/a
Age at diagnosis (years)		8.7 ± 3.6	10.8 ± 2.6	0.06
Age at study inclusion (years)		12.9 ± 3.2	14.6 ± 1.9	0.07
Time (years) of IBD until study inclusion		4.2 ± 2.8	3.7 ± 2.5	0.65
Positive family history		5/18	6/16	n/a
Extra-intestinal involvement		4/18	5/16	n/a
Disease location	L1 Terminal ileum	2/18	1/16	n/a
	L2 Colon	7/18	4/16	n/a
	L3 Ileocolonic	9/18	11/16	n/a
	+L4 (Upper GI tract)	16/18	8/16	n/a
Disease behavior	B1 Nonstricturing, nonpenetrating	17/18	14/16	n/a
	B2 Stricturing	1/18	2/16	n/a
	B3 Penetrating	0/18	0/16	n/a
	Perianal involvement	7/18	3/16	n/a
Therapy at baseline	Azathioprine	10/18	7/16	n/a
	5-Aminosalicylates	3/18	4/16	n/a
	Infliximab	12/18	11/16	n/a
	Methotrexate	2/18	2/16	n/a
	Adalimumab	1/18	0/16	n/a
Disease activity	Remission (wPCDAI < 12.5)	16/18	14/16	n/a
	Mild disease (wPCDAI $\geq 12.5 \leq 40$)	2/18	2/16	n/a

CD: Crohn's disease; GI: gastrointestinal; IBD: inflammatory bowel disease; PEN: partial enteral nutrition; wPCDAI: weighted pediatric CD activity index.
IBD phenotype was determined according to disease activity according to wPCDAI.[27]

mean squared error of prediction (RMSEP), and prediction residual error sum of squares (PRESS). Then, correlations between the metabolome and the inflammation markers were visualized using a clustered image map (CIM) and correlation circles plots. The entire data analysis was performed in R using the mixOmics package (http://cran.r-project.org).[29]

Ethics

Ethical Committees of LMU Munich (no. 690-15) and Technical University Munich (no. 316/16 S) reviewed the study protocol. Written informed consents of adult patients and of minor patients' parents or caregivers, and age-appropriate assent of patients, have been obtained. The study was registered at the German Clinical Trials Registry (no. DRKS00010278).

Results

Impact of Partial Enteral Nutrition

We analyzed 34 paired samples obtained at baseline and after 12 months from the control (Non-PEN, $n = 18$) and intervention (PEN, $n = 16$) groups, respectively. Patient details are shown in **Table 1**. Results of the splsda analyses using the absolute concentrations at the two time points showed significant baseline differences between the non-randomized control (Non-PEN) and intervention (PEN) groups, which were evident in group separation, especially using the POS data (**Suppl. Fig. S1**). Differences at baseline make it difficult to determine whether the separation in the subsequent time point was due to the treatment or just an extension of the encountered baseline differences (e.g., age difference before initiation of treatment). Similar to our previous approach using targeted metabolomics,[28] we used relative concentrations to build the splsda models, as previously described in the Materials and Methods section. By applying relative concentration data, almost complete separation was obtained between the treatment groups at t12, which was evident in the score plot (**Fig. 1**). Using the POS-mode data, all samples of the two groups (Non-PEN and PEN) were completely separated on the first PC (PC1) except two samples, one of which was lying exactly on PC1 (**Fig. 1A**). In contrast, a less efficient separation was obtained using the NEG-mode data, in which six out of 34 samples were not separated on PC1 and were located with the other treatment group on the same side of PC1 (**Fig. 1B**). Thus, the models were able to accurately separate 94% and 82% of samples using the POS and NEG modes,

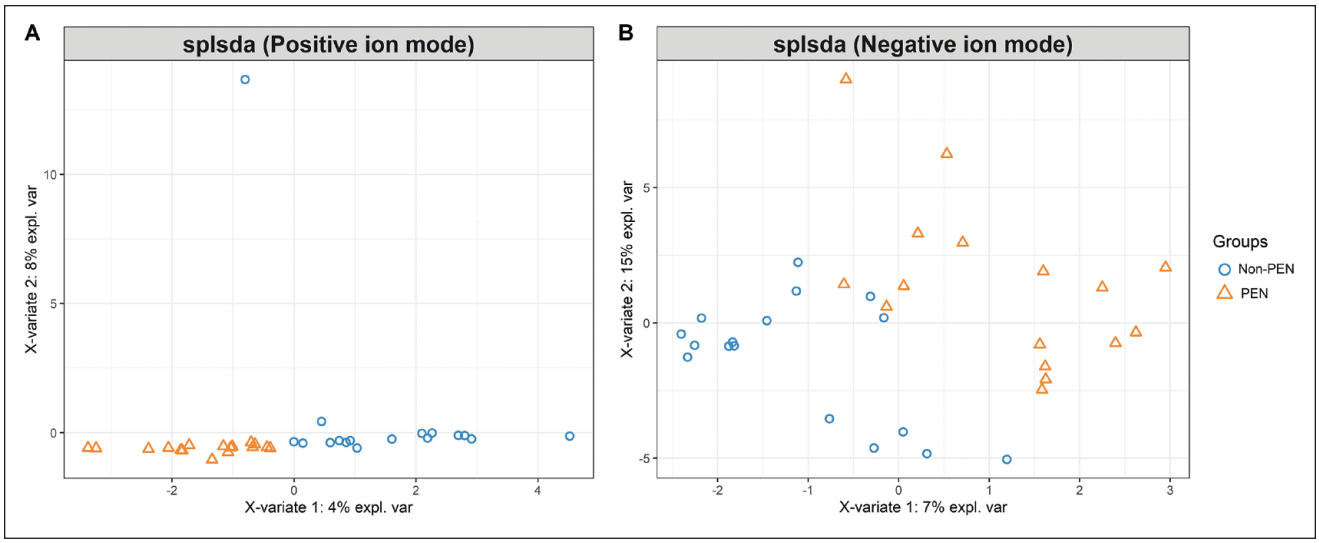

Figure 1. Sparse partial least squares discriminant analysis (splsda) score plot of the relative concentration data of the plasma metabolome of patient samples for the control [Non-PEN (partial enteral nutrition)] and intervention (PEN) groups in the (A) positive-ion (POS) and (B) negative-ion (NEG) modes at 12 months post intervention (t12).

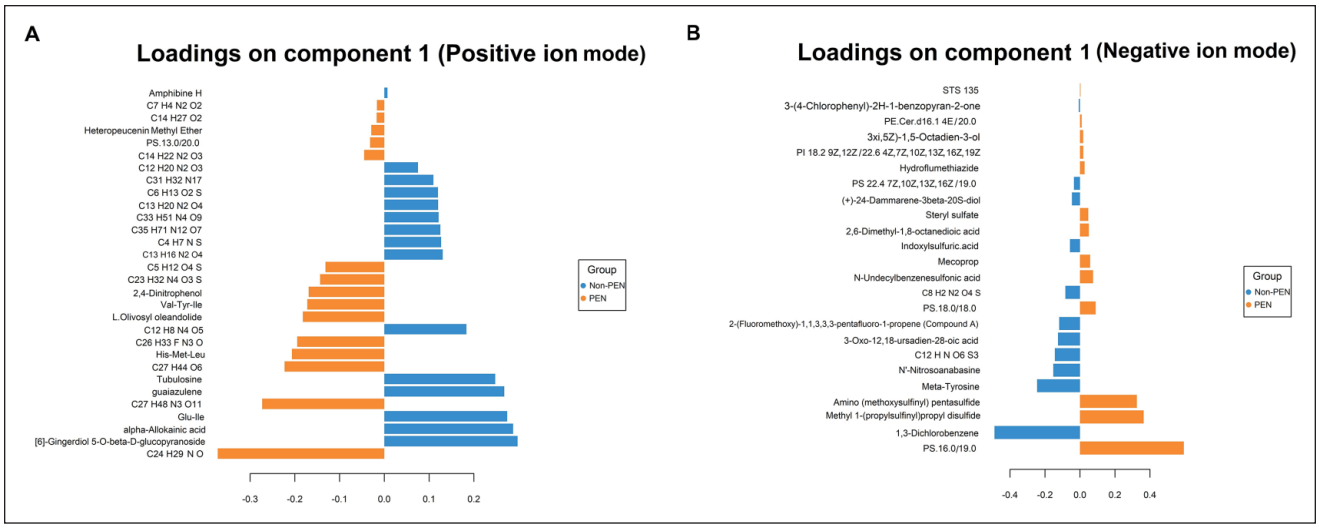

Figure 2. The loading plot represents the key features selected for the first principal component (PC1) of the sparse partial least squares discriminant analysis (splsda) models in the (A) positive-ion (POS) and (B) negative-ion (NEG) modes. Colors indicate the group in which the mean concentrations of the metabolite are maximal.

respectively. The selected features (key metabolites) for PC1 using both POS- and NEG-mode data are shown in **Figure 2**. In the POS mode, more than half of the key metabolites driving the group separation were unidentifed, including the metabolite showing the highest variable importance (**Fig. 2A**). Among the key features showing the highest importance, however, are [6]-gingerdiol 5-O-beta-D-glucopyranoside, alpha-allokainic acid, guaiazulene, tubulosine, L-olivosyl oleandolide, 2,4-dinitrophenol, and three peptides (Glu-Ile, His-Met-Leu, and Val-Tyr-Ile). The control (Non-PEN) group showed higher levels of the first five compounds and the peptide Glu-Ile, while the intervention group (PEN) showed higher levels of L-olivosyl oleandolide, 2,4-dinitrophenol, and the other two peptides (His-Met-Leu and Val-Tyr-Ile). In the NEG data, the five major metabolites driving the group separation were PS 16.0/19.0, 1,3-dichlorobenzene, methyl 1-(propylsulfinyl) propyl disulfide, amino (methoxysulfinyl) pentasulfide, and meta-tyrosine (**Fig. 2B**). The intervention group showed higher levels of principally PS 16.0/19.0 in addition to

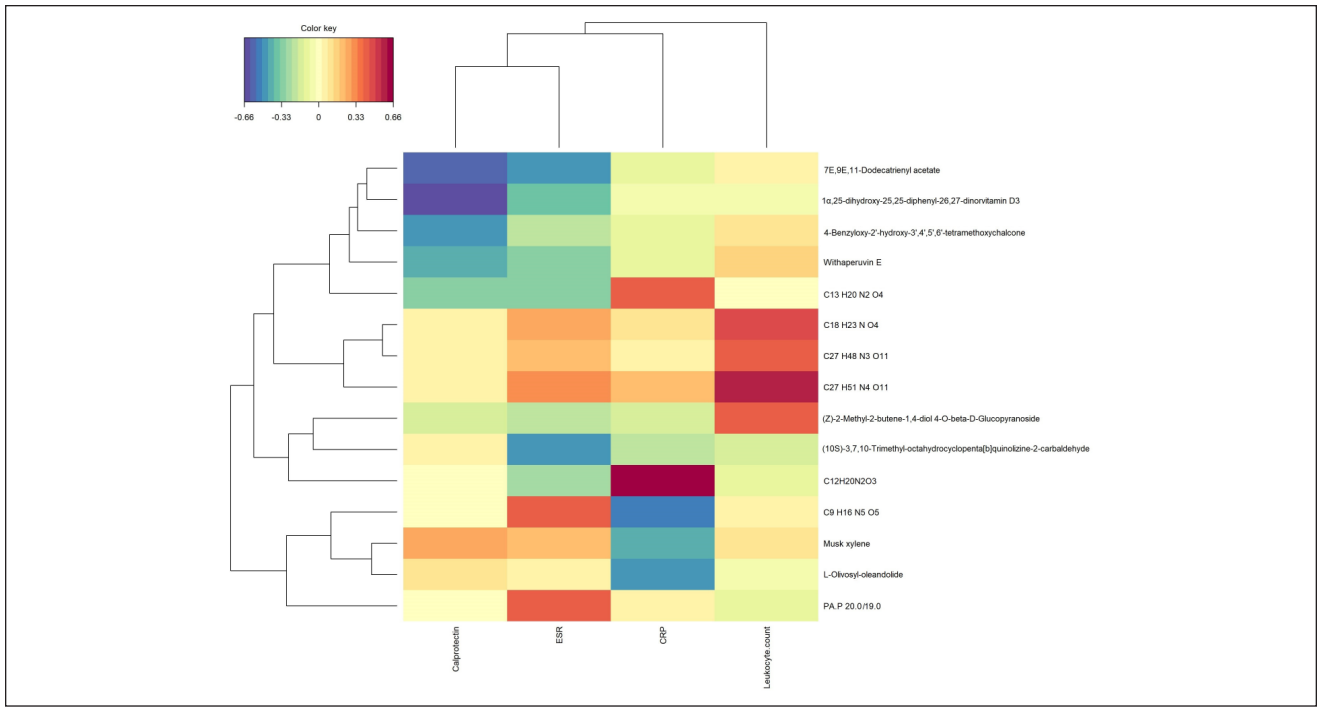

Figure 3. Clustered image map (CIM) obtained by a sparse partial least squares (spls) model using metabolomics data in positive-ion mode. The plot shows pairwise correlations between the metabolite species and biochemical markers, applying a threshold value of 0.4. The red and blue colors indicate positive and negative correlations, respectively, whereas yellow indicates small correlation values. The metabolites and the biochemical markers are clustered on the left and the top sides of the CIM, respectively.

methyl 1-(propylsulfinyl)propyl disulfide and amino (methoxysulfinyl) pentasulfide. Other phospholipids (PLs) were found among the discriminating components between the control and intervention groups, such as PS(18:0/18:0), PI[18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)], PE-Cer[d16:1(4E)/20:0], and PS[22:4(7Z,10Z,13Z,16Z)/19:0], which showed higher levels in the intervention group (PEN) relative to the control group (Non-PEN) in all compounds except for PS[22:4(7Z,10Z,13Z,16Z)/19:0]. The control group showed higher levels of 1,3-dichlorobenzene.

Associations of Inflammatory Biomarkers and the Metabolome Scanned in Positive-and Negative-Ion Modes

Based on the clinical disease activity score of wPCDAI,[27] 30 patients were in remission, and four patients had mild disease (**Table 1**). Accordingly, elevated levels of systemic inflammation markers, such as CRP (≥1 mg/dL),[30] ESR (>15 mm/h for boys and >20 mm/h for girls),[31] and WBC count ($>11\times10^9$/L),[32] were found in only single patients, but more patients showed increased levels of fecal calprotectin (>50 mg/L),[33] a sensitive biomarker of intestinal inflammation (**Suppl. Fig. S4**).

Positive-Ion Mode. Using the POS-mode data, a spls model was built after thoroughly selecting the number of PCs and features, which yielded R^2 values of 0.58, 0.52, 0.40, and 0.54 for CRP, ESR, WBC count, and calprotectin, respectively. Considering the obtained R^2 values, the models were considered of moderate to high predictive accuracy for all inflammation markers.[34] Selection of the optimal model dimensions and features comprising each dimension was tuned to obtain the best model performance assessed in terms of highest R^2 and lowest MSEP and RMSEP.

The CIM plot shows three major clusters, the first of which comprises the WBCs, while the second comprises CRP, and the third comprises ESR and calprotectin (**Suppl. Fig. S2**). To facilitate the interpretation, we investigated the obtained correlations exceeding an arbitrary threshold of 0.4, as shown in **Figure 3**.

We observed a cluster of metabolites all negatively associated with both ESR and fecal calprotectin; however, a higher correlation was obtained with the latter. This cluster comprised five metabolites, one of them not recognized by matching with the PCDL library search (C13H20N2O4); the remaining four metabolites were denoted as 7E,9E 11-dodecatrienyl acetate, 1α,25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3, 4-benzyl-oxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxychalcone, and withaperuvin E. These four metabolites were also negatively associated with CRP, albeit to a lesser extent. Generally, a global view of the CIM plot shows that only ESR and calprotectin majorly shared the same directions of associations with the key metabolites

selected by the model, except for two metabolites: (10S)-3,7,10-trimethyl-1,3a,4,8,9,9a,10,10a-octahydrocyclopenta[b] quinolizine-2-carbaldehyde and an unidentified compound (C12H20N2O3).

CRP is associated negatively with a metabolite cluster comprising L-olivosyl oleandolide, musk xylene, and an unknown metabolite (C9H16N5O5), which were, however, positively associated with fecal calprotectin and ESR. Another metabolite cluster of three unannotated compounds (C18H23NO4, C27H48N3O11, and C27H51N4O11) positively associated with all the investigated inflammatory markers, particularly the WBC count, which showed the closest positive association. Apart from the clusters discussed, we detected high positive correlations of an unidentified compound (C12H20N2O3) with CRP. The lowest inverse correlation was found between calprotectin and 1α,25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3.

Negative-Ion Mode. Using the NEG-mode data, the investigated inflammatory markers showed the same clustering behavior as that seen using the POS-mode data, in which ESR and calprotectin formed one cluster, which together with CRP gave rise to another cluster, while the WBC count clustered separately (**Suppl. Fig. S3**). To focus on only the higher meaningful correlations, we applied a correlation threshold value of 0.4, and then we replotted the CIM (**Fig. 4**).

A cluster of lipid metabolites comprising long-chain fatty acids with four PLs (PS 21:0/18:0, PS 22:2/19:1, PS 18:4/20:0, and PE 22:6/21:0) and one 2-hydroxy ceramide (Cer t18:0/24:0, 2-OH) showed a close inverse correlation with ESR and CRP, and to a lesser extent with calprotectin, but almost no correlation for WBC count except for PE 22:6/21:0. ESR, CRP, and calprotectin also showed similar patterns of correlation with 1-chloro-2,2-bis(4′-chlorophenyl)ethylene and an unannotated compound (C16H4N2O9S2). The greatest discrepancies between CRP and ESR were inverse correlations of CRP to saccharin and 4-dodecyl benzene sulfonic acid, while these correlated positively to ESR. Similar to ESR, calprotectin correlated positively with 4-dodecyl benzene sulfonic acid, albeit with a much closer correlation. Calprotectin showed a close inverse association with a cluster of three metabolites—1-chloro-2,2-bis(4′-chlorophenyl)ethylene, 3-methylbutyl-3-oxobutanoate, and 1-(9H-pyrido[3,4-b]indol-1-yl)-1,4-butanediol—which were also negatively correlated with CRP but to a lesser extent. Apart from the previously mentioned clusters, we detected close positive and negative correlations of WBC count with 1,3-dichloropropanol (1,3-DCP) and acetyl-N-formyl-5-methoxykynurenamine (AFMK), respectively. The number of PCs and features used in building the spls model between the metabolome in the NEG mode and the inflammation markers was optimized using the previously mentioned model performance parameters. The model could predict all the inflammatory markers with high accuracy, with R^2 values of 0.56, 0.73, 0.45, and 0.76 for CRP, ESR, WBC count, and calprotectin, respectively.

Discussion

Impact of PEN Treatment

Our results show a remarkable effect of 12 months of PEN treatment, compared to a normal self-selected diet, on the metabolome of pediatric CD patients in remission or with mild disease activity. This was evident in the substantial separation between the PEN and non-PEN groups, especially using the POS-mode relative concentration data. These results agree with our previous findings in the same patients using targeted metabolomics,[20] in which we detected significant differences during the one year of nutritional intervention, especially after 3 months of PEN treatment. In the present work, however, only samples from baseline and t12 were analyzed.

In the POS mode, more than half of the compounds responsible for the separation were unidentified, including the most important one (**Fig. 2A**). The remaining metabolites were mostly single species rather than metabolite groups, which could be food constituents present as additives, spices, or contaminants such as [6]-gingerdiol 5-O-beta-D-glucopyranoside,[35] guaiazulene,[36] tubulosine,[37] 1,4-dinitrophenol,[38] or bacterial metabolites such as L-olivosyl oleandolide.[39] There were, however, a number of peptides comprising essential amino acids (Glu-Ile, His-Met-Leu, and Val-Tyr-Ile), whose difference between the groups (Non-PEN and PEN) could be relevant to the consumption of the formula. Modulen IBD is a whole-protein casein-based formula; therefore, an impact on patients' protein and peptide levels may be expected in patients receiving the formula.

In the NEG-mode data, we observed that the metabolite most driving the group separation was PS 16.0/19.0, a phosphatidyl serine comprising palmitic acid. This finding supports our previous findings using the targeted metabolomics, in which the wide majority of the metabolites driving the group separation in targeted analysis were PCs, mostly comprising palmitic acid, the major fatty acid (FA) representing >30% of the total FA in the formula provided to the PEN patients.[40] As shown in the loadings plot (**Fig. 2B**), more PL species showed higher levels in the intervention group, which indicates a possible increase in PL synthesis with the formula consumption. Similar to the POS data, some of the key metabolites for the group discrimination were single species like 1,3-dichlorobenzene, which is the second most important loading. 1,3-Dichlorobenzene is used in herbicides, insecticides, medicine, and dyes, and therefore it can be a potential environmental contaminant from different sources such as food, water, or air pollution.[41] Two compounds,

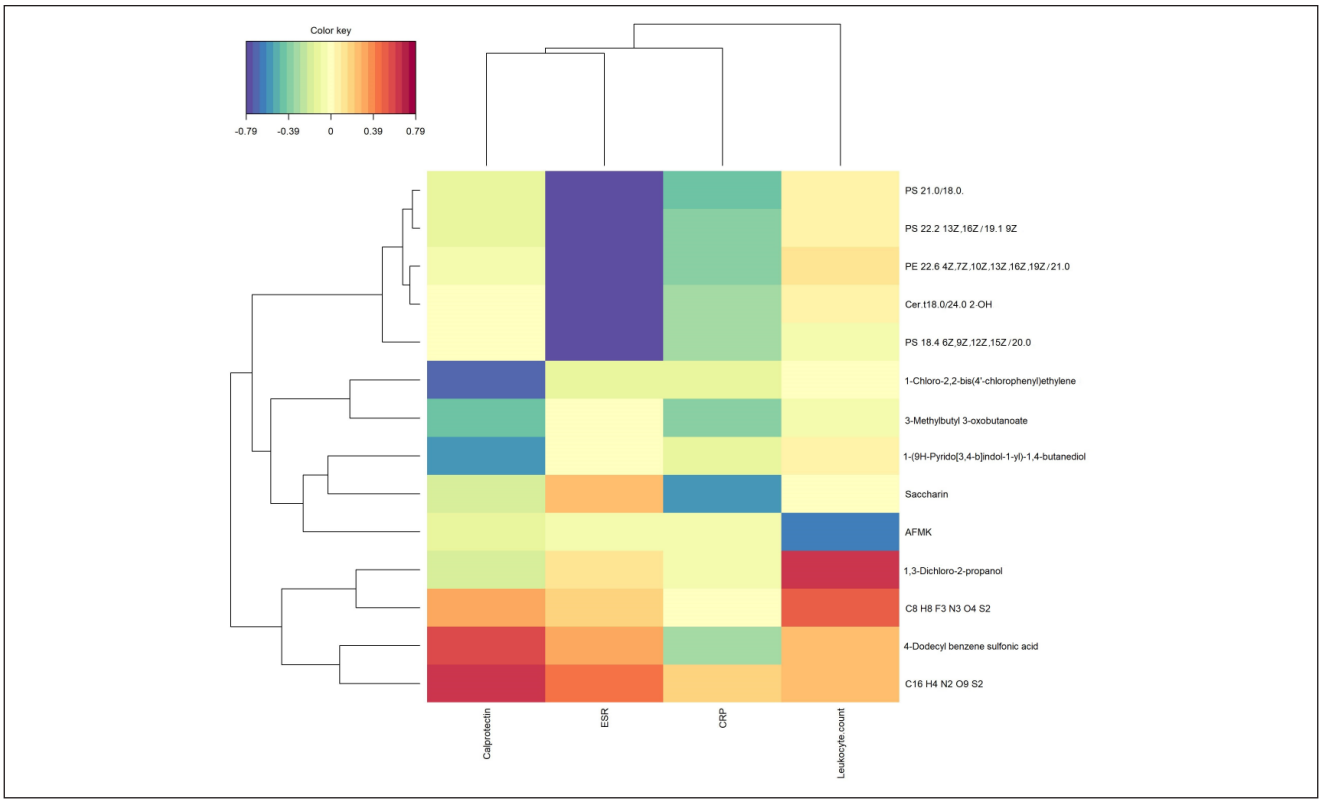

Figure 4. Clustered image map (CIM) obtained using a sparse partial least squares (spls) model using metabolomics data in negative-ion mode, showing pairwise correlations between the metabolite species and biochemical markers applying a threshold value of 0.4. The red and blue colors indicate positive and negative correlations, respectively, whereas yellow indicates small correlation values. The metabolites and the biochemical markers are clustered on the left and the top sides of the CIM, respectively.

methyl 1-(propylsulfinyl)propyl disulfide and amino (methoxysulfinyl) pentasulfide, are also recognized as food constituents and used as potential biomarkers for the consumption of specific foods.[42,43] In contrast, m-Tyr is a product of nonenzymatic free radical oxidative modification of phenylalanine (Phe) residues. It is a rare metabolite present in low concentrations under physiologic circumstances, and its elevated levels were linked to protein damage.[44] m-Tyr was identified among the most discriminating metabolites between the Non-PEN and PEN groups post intervention, with higher levels in the control group. This could imply the amelioration of the protein damage in subjects receiving the formula, relative to subjects in the control group. Clinically, PEN therapy was found to improve growth in a PEN subgroup of pre- and early pubertal patients with growth potential, as previously reported by our group.[20] In this sense, the present work using the untargeted approach confirmed the results obtained by the targeted approach and was able to detect more metabolic differences associated with the consumption of the formula. These findings support the combined use of targeted and untargeted metabolomics as complementary strategies to maximize information on patient samples.

Associations of Inflammatory Biomarkers and the Metabolome

The patients included in the cohort were clinically stable, either in remission or with low wPCDAI scores (**Suppl. Fig. S4**). Accordingly, levels of inflammatory markers were generally low and in the normal range for the majority of the subjects. Nonetheless, we analyzed the association of clinical markers with metabolites as a hypothesis-free explorative study on potential metabolic alterations at a relatively low inflammatory state.

Positive-Ion Mode

Our results show associations between inflammatory markers and clusters of metabolites, as well as single metabolite species related to inflammation. One major metabolite cluster was composed of chemically and structurally related compounds comprising one or more isoprenoid units, which showed similar inverse correlation patterns with ESR and fecal calprotectin. The members of this cluster were dodecatrienyl acetate (sesquiterpenoid), 25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3 (triterpenoid), withaperuvin

E (sesquiterpene lactone), and 4-benzyl-oxy-2′-hydroxy-3′,4′,5′,6′-tetramethoxychalcone (flavone derivative). Isoprenoids have been previously related to treatment and prevention of autoimmune diseases by acting as reduced nicotinamide adenine dinucleotide phosphate (NADPH) activators, thus providing normal or increased levels of NADPH oxidase, while decreased NADPH oxidase activity has been related to disease progression.[45] In other reports, diterpenoids and triterpenoids were reported at significantly lower levels in CD patients than in healthy controls.[46] Among this cluster, 25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3, a derivative of vitamin D3, showed the highest inverse correlation with fecal calprotectin. Vitamin D deficiency was repeatedly reported in patients with IBD; however, it is controversial whether it is merely a consequence of the disease or contributes to the inflammatory condition.[47] Decreased vitamin D3 levels may be attributed to less outdoor activity and sunshine exposure due to lower general well-being and possibly also to reduced lipid absorption, both associated with chronic intestinal disease. Withaperuvin E is a plant-derived steroid[48] that belongs to a family of compounds with potential anti-inflammatory activity mediated by inhibition of tumor necrosis factor alpha (TNF-α)-induced nuclear factor kappa B (NF-κB) activity.[49] 4-Benzyl-oxy-2′-hydroxy-3′,4′,5′,6′-tetrametho xychalcone is a flavone derivative with activity against ulcers, gastritis, and IBD.[50]

Another cluster comprising L-olivosyl oleandolide (a bacterial metabolite),[39] musk xylene, and an unknown metabolite (C9H16N5O5) was inversely correlated with CRP, and alternatively positively correlated with fecal calprotectin and ESR. There were no reports linking either of these compounds to CD; however, concerns were raised regarding the toxic and carcinogenic effects of musk xylene, a synthetic fragrance used as a fixative in cosmetics and perfumes and a potential water contaminant.[51] In the POS-mode data, some of the strong correlations could not be interpreted due to the unknown identities of the compounds, including the C12H20N2O3 correlated with CRP and the cluster of metabolites closely correlated with the WBC count, of which three members were unannotated.

Negative-Ion Mode

Inflammatory markers were associated with a lipid cluster comprising phospholipids and ceramides, with xenobiotics comprising sweeteners, flavors, food additives, and contaminants. This reflects that inflammation in CD may be triggered or modulated by exogenous and endogenous factors.

Similar to the results obtained using data in the POS mode, a group of structurally related metabolites comprising phospholipids containing long-chain fatty acids (LCFAs) and ceramides clustered together, showing high negative correlations with ESR and CRP, the two most recognized inflammatory markers in IBD. Calprotectin showed inverse or no correlation with the same cluster. Other studies have linked CD to disturbed lipid metabolism, especially regarding sphingolipids and phospholipids.[52,53] These compounds play crucial roles in maintaining barrier function as well as modulating inflammation and immunity.[54] Moreover, ceramides decrease the release of TNF and induce autophagy, a process strongly implicated in the pathogenesis of CD, in addition to their impact on cell signaling.[55–57] Elevated levels of phospholipase A2 (PLA2) were reported in serum and colonic mucosa of IBD patients.[58] PLA2 are enzymes hydrolyzing phospholipids into lysophospholipids, which were proposed to be involved in the inflammatory process and pathogenesis of IBD.[59,60] ESR, CRP, and calprotectin correlated negatively with 1-chloro-2,2-bis(4′-chlorophenyl)ethylene; however, we found no previous reports linking 1-chloro-2,2-bis chlorophenyl ethylene to IBD to provide an explanation for this finding. The artificial sweetener saccharin was positively associated with ESR. Artificial sweeteners, like saccharin and sucralose, were previously linked to increased IBD risk, with a proposed inhibition of gut bacteria and digestive proteases, and thus enhanced digestion of the mucus layer and gut barrier considered the "bacteria-protease-mucus-barrier hypothesis."[61,62] It is difficult, however, to understand why saccharin correlates in different directions with CRP and ESR, respectively. Inconsistency in the inflammatory markers' response to inflammation and their lack of accuracy have been reported frequently.[63,64] Similar to saccharin, 4-dodecyl benzene sulfonic acid was positively associated with ESR but negatively associated with CRP. It was also positively correlated with calprotectin and the WBC count. 4-Dodecyl benzene sulfonic acid is a xenobiotic belonging to the family of benzenesulfonic acids and derivatives, with increased levels previously linked to IBD.[46,65] 4-Dodecylbenzenesulfonic acid is a major compound in laundry detergents,[66] and its sodium salt is applied in antimicrobial formulations for treating organic vegetables and fruits.[67]

A cluster of three metabolites—1-chloro-2,2-bis(4′-chlorophenyl)ethylene, 3-methylbutyl 3-oxobutanoate, and 1-(9H-pyrido[3,4-b]indol-1-yl)-1,4-butanediol—showed a close inverse association with calprotectin, principally the first of these three. Although information on the source of 1-chloro-2,2-bis(4′-chlorophenyl)ethylene is lacking in the published literature, the most usual way for exposure to the other two species is dietary intake. 3-Methylbutyl 3-oxobutanoate is a sweet ethereal flavoring agent,[68] while 1-(9H-pyrido[3,4-b]indol-1-yl)-1,4-butanediol is a natural β-carboline indole alkaloid derived from plant origins.[69] β-Carboline alkaloids may occur in many plant-derived foods, thermally processed protein-rich foods, and beverages,[70] and were demonstrated to exhibit anti-inflammatory activity via potent inhibition of nitric oxide (NO), TNFα, and interleukin-6 (IL6).[71,72]

1,3-DCP, an unannotated compound (C8H8F3N3O4S2), and AFMK were the three main compounds showing high correlations with the WBC count. The first two compounds were positively correlated with WBCs, while AFMK was inversely correlated with WBCs. 1,3-DCP is an organochlorine compound that may occur as a food and water contaminant.[73,74] 1,3-DCP can be metabolized either by cytochrome P450 2E1 (CYP2E1) into cytotoxic compounds, or by bacterial enzymes into epichlorohydrin, 3-MCPD, glycidol, and glycerol, which exert their toxicity by cellular glutathione depletion through conjugation with glutathione and may also induce a loss of mitochondrial function.[75] AFMK acts as an antioxidant and was reported to have anti-inflammatory effects mediated by the decrease in TNFα and interleukin-8 (IL8) production by monocytes.[76] As an oxidation product of melatonin, it could be endogenously produced or be derived from exogenous melatonin found in some plant- and animal-derived foods. Melatonin was reported to have beneficial effects in inflammatory and enteroimmune diseases.[77,78]

Strengths and Limitations

The strengths of our study are the use of a powerful analytical platform and a longitudinal observation throughout a period of one year following a standardized study design. The limitations are the limited sample size and a nonrandomized assignment of patients to the study arms, which was chosen since a randomized approach was not feasible in the children and adolescents studied.[20] The inclusion of only patients in remission with a relatively low level of inflammation may have decreased the likelihood of identifying metabolic responses that are altered by higher disease activity or that may serve as a trigger for inflammation. Around 25% of the markers detected in this study remain uncharacterized, of which some were closely correlated with inflammatory markers or key metabolites in studying the impact of PEN therapy; thus, the findings could not be fully interpreted.

Conclusions

It appears worthwhile to explore the combination of targeted and untargeted approaches in monitoring treatment effects in CD. The diversity of the metabolites detected by the untargeted technique, comprising products of human or bacterial metabolism, xenobiotics recognized as flavoring agents, environmental contaminants, and their biotransformation products, found associated with inflammatory markers in pediatric CD patients points to further opportunities for applying metabolomics in biomarker research and in understanding mechanistic pathways involved in the disease. Further studies are warranted in patients with a broader range of disease activity and abnormal inflammatory markers to explore whether the present findings can be replicated, identify the unknown compounds, and address their possible contribution to the etiopathogenesis of CD.

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