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NEW AND EMERGING METHODS

Cristian Tefas, et al.: Metabolic and lipid profiling in inflammatory bowel diseases

The potential of metabolic and lipid profiling in inflammatory bowel diseases: a pilot study

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ABSTRACT

Inflammatory bowel diseases (IBDs) are conditions that still pose significant problems. A third of the patients are either misdiagnosed or a proper diagnosis of Crohn’s disease (CD) or ulcerative colitis (UC) cannot be made. We need new biomarkers, so that we can offer patients the best treatment and keep the disease in an inactive state for as long as possible. Alterations in metabolic profiles have been incriminated in the pathophysiology of IBD. The aim of the present study was to identify molecules that could serve as biomarkers for a positive diagnosis of IBD as well as to discriminate UC from colonic CD. Twenty-two patients with active colonic IBD (UC = 17, CD = 5) and 24 age- and gender-matched healthy controls were enrolled. Plasma lipid and metabolic profiles were quantified using ultra-high performance liquid chromatography combined with mass spectrometry. Univariate and multivariate statistical tests were employed. Six lipid species and seven metabolites were significantly altered in IBD compared to healthy controls, with the majority belonging to glycerophospholipid, linoleic acid, and sphingolipid metabolisms. Five lipid species and only one metabolite were significantly increased in UC compared to CD. This preliminary study suggests that lipid and metabolic profiling of serum can become diagnostic tools for IBD. In addition, they can be used to differentiate between CD and UC.

KEYWORDS: Crohn’s disease; ulcerative colitis; inflammatory bowel disease; IBD; metabolomics; lipidomics
INTRODUCTION

Inflammatory bowel diseases (IBD) are a group of chronic immune-mediated disorders characterized by inflammation of the digestive tract with a relapsing-remitting pattern. Crohn’s disease (CD) and ulcerative colitis (UC) may affect different digestive tract segments, having different clinical manifestations, prognoses and treatments. However, up to 30% of patients with IBD are either misdiagnosed or a proper diagnosis cannot be made even with the current available armamentarium (1,2).

New biomarkers and techniques that offer better diagnosis rates are expected. The current ones - ESR, C-reactive protein, fecal calprotectin or fecal lactoferrin - each has its limitations, the most important being the lack of specificity (2).

Metabolomics deals with the analysis of endo- and exogenous metabolites and realizes molecular fingerprints that reflect specific cellular processes. Different biological samples may be tested, but the most commonly used are plasma or serum. The obtained data reflect the host’s metabolism.

The use of diagnostic metabolomics has recently become a very promising idea. It has been applied to biomarker detection and has yielded valuable information in diabetes, cardiovascular diseases, rheumatoid arthritis or multiple sclerosis (3). There are several thousand metabolites in the tissues and fluids of living organisms and most of these have a well-defined function and could be used for diagnostic or therapeutic purposes.

Recent studies have identified multiple molecules that could be validated as novel biomarkers of IBD (4–6). Inflammation acts through many metabolic pathways and also involves signaling molecules such as polar lipids (e.g. phosphatidylcholine). These are important structural components of cell membranes and are involved in cell differentiation and proinflammatory cytokine release (7). Sphingolipids and their metabolites, such as sphingosine-1-phosphate, also act as signaling mediators in apoptosis, proliferation, response...
to stress, necrosis, inflammation, autophagy, senescence and differentiation (8–13).

Moreover, ceramides, a family of waxy lipid molecules, have been reported to be involved in apoptosis, cell growth arrest, differentiation, cell senescence, cell migration, adhesion and autophagy (7,14). In a previous preliminary report we investigated the potential of lipid profiling to differentiate IBD from healthy subjects (15). After applying univariate and multivariate analysis, six molecules were found to be statistically significant in discriminating patients from healthy controls (15).

We therefore designed this study in order to identify molecules that could be of help in diagnosing IBD. Furthermore, because of clinical, endoscopic and histopathological overlapping between UC and colonic CD, we wanted to identify molecular differences between them.

MATERIALS AND METHODS

Patient data

Blood samples were collected from consecutive patients admitted to a tertiary referral center in Cluj-Napoca, Romania. All included patients had active left sided or extensive UC or active colonic CD. The study was approved by the local ethical review board (approval no. 196/20.04.2016) and written consent was obtained from all enrolled subjects.

The diagnosis was established using clinical, serological, endoscopic and histological data. Patients with proctitis or indeterminate colitis were excluded from the study, as well as patients under 18 or over 70 years old, those having a history of neoplasia, infectious diseases, diabetes mellitus, debilitating mental diseases, primary hyperlipidemia or any familial lipid syndrome, liver or renal failure, or structural damage of the gastrointestinal tract.

Concomitant medication either prescribed for the IBD or for other conditions was taken into consideration and removed from the statistical analysis.
Sample collection and extraction protocol

Blood samples were collected after overnight fasting. After centrifugation at 4000 RPM for 10 minutes, the serum was separated and stored in Eppendorf tubes at -80°C. When needed, the serum samples were thawed at room temperature and vortexed for 20 seconds. For the metabolic determinations, aliquots of 200 µl were mixed with 800 µl MeOH 98% and vortexed again for 20 seconds to precipitate proteins. After that, samples were ultra-sonicated in a water bath for 5 minutes, then kept at -20°C for at least 5 minutes to precipitate proteins and then centrifuged at 5000 RPM for 10 minutes. The resulting supernatant was collected and filtered using a Nylon filter (0.2 µm).

For the lipid determinations, aliquots of 100 µl were mixed with 200 µl MeOH and vortexed again for 20 seconds to precipitate proteins. A volume of 1.66 ml chloroform was added and vortexed for 20 seconds, then 100 µl of water was added to separate the two phases, vortexed for 20 seconds and centrifuged at 10000 RPM for 10 min. The chloroform layer was collected and evaporated under nitrogen and the residue was dissolved in 500 µl Isopropanol:Acetonitrile (90:10), vortexed for 20 seconds, sonicated in a water bath for 15 minutes and filtered using a PTFE filter (0.2 µm).

In order to ensure the quality control (QC) and reproducibility of the data, two pooled samples from all healthy controls (QCC) and all patient samples (QCP) were similarly prepared and placed as every sixth sample.

UHPLC-QTOF-ESI⁺-MS analysis

The separation was done in a Thermo Scientific Ultra-High-Performance Liquid Chromatograph (UHPLC) UltiMate 3000 system, equipped with a Dionex quaternary pump delivery system, using a C₁₈ column (Acclaim Dionex) (5 µm, 120Å, 2.1x100 mm).
For the metabolic determinations, run-to-run time was set at 20 minutes using a gradient program with two mobile phases (A - H2O + 0.1% Formic acid and B - Acetonitrile + 0.1% Formic Acid). The gradient for phase A was 5 to 15% (0 to 3 minutes), 15 to 50% (3 to 6 minutes), 50 to 95 % (6 to 9 minutes), isocratic until the 15th minute, then decreased from 95 to 5% (15 to 20 minutes). The injected volume was 5μl, column temperature was 40 ºC and the flow was set at 0.5ml/min.

For the lipid determinations, run-to-run time was set at 28 minutes using a gradient program with two mobile phases (A-Acetonitrile:H2O (60:40) + 0.1% Formic acid + 10 mM ammonium formiate and B-Isopropanol:Acetonitrile (90:10) + 0.1% Formic Acid + 10 mM ammonium formiate). The gradient for phase A (%) was 75 to 50 (0-4 minutes), then 50 to 3 (4-19 minutes), isocratic at 23 minutes, then increased from 3 to 75% (23 to 28 minutes). The injected volume was 5 μl, column temperature was 55 ºC and the flow was set at 0.26 ml/min.

Mass spectrometry was performed using a detection system provided by MaXis Impact Time-of-Flight (QTOF) MS (Bruker Daltonics, version 2012). The QTOF-ESI+ -MS parameters were: ionization mode ESI positive, MS calibration with sodium formiate, capillary voltage 3500 V, drying gas flow 12 l/min, drying temperature 300°C.

Instrument control and data processing were done using specific software (Chromeleon, TofControl 3.2, HyStar 3.2 and Compass Data Analysis 4.2, provided by Bruker Daltonics).

**Data processing and statistics**

The base peak chromatograms were processed using Compass DataAnalysis 4.2 with the Find Molecular Feature (FMF). Profile Analysis 2.1 (Bruker, Daltonics) software was then used to generate a matrix based on the FMF. Parameters like time alignment, spectral background extraction, MS recalibration, normalization by sum of bucket values in analysis
and 80% bucket filtration were used. For the multivariate and univariate statistical analysis, Profile Analysis as well the MetaboAnalyst online software were used on the final matrix. The unsupervised multivariate Principal Component Analysis (PCA) was first used to evaluate the discrimination between the experimental groups (by scores and loadings) and to detect and eliminate the outliers. Then, Partial Least Squares Discriminant Analysis (PLS-DA) was performed. The relevance of each metabolite was quantified using the Variable Importance in Projection (VIP) score and this was subsequently used to identify the putative biomarkers. Cross validation by Leave One Out Method (LOOCV) was performed on the PLS-DA model to find, minimize the risk of overfitting and validate the results. The same online software was used to perform the univariate analysis in order to calculate the fold change (FC) and t-test p-values. Receiver Operating Characteristic Curves (ROC) and heatmaps, which may provide an intuitive visualization on the differences, were generated. Molecule identification was made according to the Human Metabolomic Database (http://www.hmdb.ca/) and Lipidomic Gateway (http://www.lipidmaps.org).

RESULTS

Patient data

All included patients were diagnosed with extensive UC (n=17, female:male=6:11, mean age 39 years) or colonic CD (n=5, female:male=3:2, mean age 43 years). The control group included 24 subjects (female:male=14:10, mean age 29 years). All patients included in the study had active disease, confirmed by colonoscopy with targeted biopsy. The patients diagnosed with UC had an average clinical severity of 7.6, assessed using the Ulcerative Colitis Diseases Activity Index (UCDAI) and an average endoscopic severity of 2.3, assessed using the Mayo score. Patients with colonic CD had an average clinical severity of 182,
assessed using the Crohn’s Diseases Activity Index (CDAI) and an average endoscopic severity of 12, using the Simple Endoscopic Score for Crohn’s Disease (SES-CD).

**Lipid profiling of colonic inflammatory bowel diseases**

Tetracosanoic acid, phosphatidylcholine (PC), lysophosphatidylcholine (LPC), sphingomyelin (SM) and diacylglycerol were significantly lower in patients with colonic IBD than in healthy controls, as previously reported (15).

The comparison between the UC and CD subgroups was made using multivariate and univariate statistical analysis (Figure 1). The cross validation of PLS-DA analysis showed that $Q^2$ and $R^2$ values were higher than 0.5 and the accuracy was higher than 0.85. All the VIP scores that discriminate between the UC and colonic CD groups are shown in Figure 2. 15 molecules with VIP values above 5 were identified, these being significantly different between the colonic CD and UC groups.

Univariate statistical analysis of CD and UC patients, considering a VIP>1.5, a p-value<0.001 and an AUROC>0.8 revealed five molecules that were significantly different between the two groups, with the corresponding m/z of 280.265, 256.265, 254.249, 511.521 and 507.490. Thus, linoleamide, palmitic amide levels, branched fatty esters of hydroxyl fatty acids as well as three isomers of the hexadecanoic acid (stearyl palmitoleate, palmitoleyl stearate, oleyl palmitate) were higher in patients with colonic CD than in those with UC (Table 1).

**Metabolic profile to discriminate between healthy controls (M) and colonic IBD patients (P) groups**

The final data matrix obtained using FMF was processed by MetaboAnalyst 3.0 and the PCA and PLS-DA plots were compared (Figure 3). The 3D-PCA plot shows that the healthy
subjects’ (M) as well as the patients’ (P) groups were not clustered significantly, the discrimination being characterized by a variability of 51.4% for PC1, 17% for PC2 and 12.4% for PC3 (Figure 3A). Figure 3B includes the 3D-PLS-DA scores plot for the first 3 components (PC1 at 28.5%, PC2 at 37.8%, PC3 at 6.3%) with a significant separation between control and IBD groups.

The PLS-DA analysis - cross validation data showed cumulative values of $R^2 = 79.2\%$ and $Q^2 = 53.6\%$ where $R^2$ (sum of squares captured by the model) indicates the variation shown by all the five components in the model and $Q^2$ (cross validated $R^2$) shows the best accuracy of the model prediction when 5 components were considered (Figure 4A). These scores plot and values indicate good clustering and demonstrate a good distinction between the two groups. From the PLS-DA loading values, the compounds with VIP values above 1.2 were selected, as shown in Figure 4B.

Out of the 13 molecules with VIP scores above 1.2, seven were found to be statistically significant in discriminating patients from controls, as shown in Table 2.

Using Euclidian hierarchical clustering, a dendrogram presenting the samples’ grouping is displayed in Figure 5. A good clusterisation was observed between the patient group and the healthy group.

**Metabolic profile to discriminate between UC and colonic CD patients**

Similarly to the comparison between the healthy subjects and patients, the UC and CD subgroups were compared using multivariate and univariate statistical analysis.

Clusterization and outliers were analyzed using PCA. The discrimination was characterized by 57.5% for PC1, 16% for PC2 and 6.4% for PC3 between UC and CD (Figure 6A). PLS-DA showed discriminations of 30.1% on PC1, 41.2% on PC2 and 5.5% on PC3. The cross
validation of PLS-DA analysis showed that $Q^2$ was negative, $R^2$ was around 0.45 and that the accuracy was 0.6 (Figure 7A).

P-values, FC and FDR values along with AUROC were also calculated. However, given the low number of patients with CD and the low $R^2$ and $Q^2$ values, these did not fit within the confidence interval. Although 15 molecules have good FC values and their VIP scores are higher than 1.4 (Figure 7B), their p and FDR values do not fit the condition to be less than 0.05.

Regarding ROC analysis, from all 15 molecules selected from PLS-DA analysis, only one has an AUROC value higher than 0.8.

The compounds that had the closest acceptable values to possibly discriminate between the two groups are 923.616 (tryglicerides (TG) (58:12)), 568.340 (LPC(22:6)/ceramide (Cer)(d18:0/18:0)), 520.341 (LPC(18:2)), 274.276 (heptanoyl carnitine) and 760.585 (phosphatidylethanolamines (PE)(22:1(13Z)/15:0) /PC(18:1(9Z)/16:0)). However, only one was significantly increased (TG (58:12)) in UC patients compared to colonic CD patients (Table 3).

In parallel, using a serum metabolic pathway analysis, we obtained an overall image of the possible pathways involved in IBD. This revealed that the glycerophospholipid and sphingolipid metabolisms seem to be mainly involved. To discriminate CD from UC groups, the match status was 3/39 for glycerophospholipid pathways ($p<0.01$) and 1/25 for sphingolipid metabolism ($p=0.04$).

**DISCUSSION**

When comparing IBD patients to healthy subjects, the former have lower PC and LPC levels (15). These two, along with PE and lysophosphatidylethanolamines (LPE) are virtually found in all cell membranes and play a role in cellular signaling, division, apoptosis and
inflammation. PC and LPC act as a hydrophilic barrier in the bowel and don’t allow harmful compounds to come into contact with the mucosal cells. Patients with UC seem to have less PC in their intestinal mucus (16). This could be a factor in the development of the disease. Sustained inflammation leading to a compromised integrity of the intestinal mucosal membrane and digestive loss could account for the lower levels of PC. However, TNF-α signaling, activation of NF-κB, mitogen-activated protein kinase (MAPK) pathway and peroxisome proliferator activated receptor (PPAR) signaling seem to be implicated as well (17). Several authors have found decreases in phospholipid levels in patients with IBD (18-20). This however contrasts with the findings of Fan et al, who did not find any association between PC and IBD (21).

Sphingomyelin (SM) is a type of sphingolipid found in cell membranes that has similar functions to PC. Drugs that target specific sphingolipid receptors have been developed, leading to the sequestration of lymphocytes away from sites of chronic inflammation (22). Marked differences in sphingolipid metabolism have been reported, with metabolites being decreased in patients with IBD (19-21,23). These lower values seem to be a consequence of the compound action of TNF-α, NF-κB and IFN-γ, which activate sphingomyelinases (24-26). Interestingly enough, although the lipid metabolism is affected in both patients with UC and those with CD, sphingomyelin appears to be significantly decreased only in patients with UC (20,23).

Compared to other authors (21), we previously reported that cholesteryl esters showed higher values in patients with colonic IBD than in healthy subjects (15). These changes could be explained by lipid mobilization or by increased intestinal permeability.

Fatty acid amides seem to have a role in differentiating UC from colonic CD. These molecules play a role in inflammation, intracellular signaling, pain, immune function, reproduction and appetite (27), and appear to attenuate colitis (28).
Stearyl palmitoleate, palmitoleyl stearate and oleyl palmitate are ester isomers with the same mass and are at the base of branched fatty acid esters of hydroxy fatty acids (FAHFA). In a similar manner to fatty acid amides, FAHFAs protect against colitis by regulating the enteric immune systems and preventing mucosal damage (29). We have shown that both fatty acid amides and FAHFAs are increased in CD as opposed to UC. This might be related to the more profound inflammatory changes encountered in the former.

We have also shown that the main discriminant molecules between healthy subjects and those with IBD are LPC, LPE and ceramides. While some variants of LPC and ceramides are increased, LPE is decreased. An explanation for this is the increased turnover of LPE in order to produce LPC, via S-adenosyl methionine methylation (30,31).

We have shown that while LPC (20:4) and LPC (22:6) increase, LPC (18:2) decreases. This is because of an inverse relationship between the two, as previously stated (32). The pro-inflammatory activity of saturated LPCs (18:2) is antagonized by the actions of polyunsaturated LPCs, (20:4) which are anti-inflammatory lipid mediators.

From a diagnostic standpoint, while LPE and ceramides have a very good discriminatory potential, with an AUROC of over 0.8, LPC has a more modest one, with an AUROC between 0.7 and 0.8.

Hydroperoxylinoleic acid (HPLA), one of the primary products of the major polyunsaturated fatty acids, also has higher values in patients with IBD than in healthy subjects. HPLA is part of the biochemical cascade that leads to the formation of proinflammatory eicosanoids. As previously reported, this cascade is overactivated in patients with IBD; therefore more HPLA is produced (33).

In this metabolomic study we also reported higher levels of TG in patients with IBD than in healthy subjects, a finding that was noted in other studies as well (34-37). Interestingly, TG seem to be elevated more in men than in women (37). Triglyceride levels are regulated by
apolipoprotein C-III. The hypertriglyceridemia encountered in inflammatory states is caused by increased lipoprotein production and decreased lipoprotein clearance. In addition, anti-TNF treatment may influence triglyceride as well as cholesterol levels (38,39). In our study, although we included patients on anti-TNFs, all of them were at the start of the therapy. Therefore, the changes in TG levels were due solely to the underlying IBD. Unfortunately, comparing the metabolic profiles of UC and colonic CD yielded only modest results. This is because of the low number of subjects included in the colonic CD subgroup. A single molecule (TG (58:12)) is significantly different between UC and colonic CD, with an AUROC over 0.8, indicating a good discriminatory potential. This is the first study that, to our knowledge, has shown a significant difference between UC and colonic CD concerning fatty acids and their derivatives. Determining serum markers that allow the diagnosis and monitoring of patients with IBD is extremely important. Avoiding repeated endoscopic examinations in these patients reduces the necessary costs, procedural risks, and the psychological impact of these procedures on patients. The major limitation to our study was the low number of included patients. In order to achieve maximum diagnostic power we chose to include only patients in an active state of the disease, so that eventual molecular changes were as obvious as possible. In addition, we excluded patients with proctitis, as the limited extent of the disease does not translate to marked biochemical changes. However, these criteria, as well as the reduced frequency of IBD in our country, have seriously limited our ability to enroll subjects in the study. Supplementary analysis on disease pattern and severity or type of medication used between different subgroups of patients is possible but with a larger sample size. Therefore, these results will need to be validated in larger, well-designed studies with comprehensively assessed clinical (e.g., medication, comorbidities) and dietary factors.
CONCLUSION

This preliminary indicates that a serum lipid and metabolic profiling has a good potential to detect IBD patients non-invasively, as well as to differentiate between colonic CD and UC. These results also contribute to the improvement of our understanding of the pathophysiological mechanisms of colonic CD and UC.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.
REFERENCES


Table 1. Lipidomic differences between CD and UC. Molecules with a VIP > 1.5, p values < 0.001, FDR<0.1 and AUROC>0.8 are shown.

<table>
<thead>
<tr>
<th>m/z values</th>
<th>Tentative identification</th>
<th>Tendency (CD vs UC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>280.265</td>
<td>Linoleamide</td>
<td>Increase</td>
</tr>
<tr>
<td>256.265</td>
<td>Palmitoylamide</td>
<td>Increase</td>
</tr>
<tr>
<td>254.249</td>
<td>Palmitoleamide</td>
<td>Increase</td>
</tr>
<tr>
<td>511.521</td>
<td>Branched fatty acid esters of hydroxy fatty acids (e.g.</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td>Hexadecanoyloxy-Hexadecanoic acid FAHFA (16:0/O-16:0))</td>
<td></td>
</tr>
<tr>
<td>507.490</td>
<td>Stearyl palmitoleate, palmitoleyl stearate, oleyl palmitate</td>
<td>Increase</td>
</tr>
</tbody>
</table>
Table 2. Metabolomic differences between healthy subjects (M) and IBD patients (P). Molecules with a VIP > 1.2 and FDR > 0.05 are shown.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Tentative identification</th>
<th>VIP</th>
<th>P value</th>
<th>FC (P/M)</th>
<th>AUROC</th>
<th>Tendency (P vs M)</th>
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</thead>
<tbody>
<tr>
<td>554.552</td>
<td>LPE(24:6)</td>
<td>2.636</td>
<td>3.8436E-11</td>
<td>0.5035</td>
<td>0.9777</td>
<td>Decrease</td>
</tr>
<tr>
<td>526.520</td>
<td>LPE(22:6)</td>
<td>2.0115</td>
<td>2.5541E-10</td>
<td>0.5545</td>
<td>0.9577</td>
<td>Decrease</td>
</tr>
<tr>
<td>568.340</td>
<td>LPC(22:6) or Cer(d18:0/18:0)</td>
<td>1.7659</td>
<td>4.0254E-4</td>
<td>1.5359</td>
<td>0.8444</td>
<td>Increase</td>
</tr>
<tr>
<td>544.340</td>
<td>LPC(20:4)</td>
<td>3.8247</td>
<td>9.3683E-4</td>
<td>1.3532</td>
<td>0.7822</td>
<td>Increase</td>
</tr>
<tr>
<td>520.341</td>
<td>LPC(18:2)</td>
<td>15.367</td>
<td>0.00556</td>
<td>0.78254</td>
<td>0.7311</td>
<td>Decrease</td>
</tr>
<tr>
<td>991.674</td>
<td>TG(20:3/22:0/20:3) or TG(20:1/22:4/20:1)</td>
<td>6.1001</td>
<td>0.04617</td>
<td>1.4536</td>
<td>0.7266</td>
<td>Increase</td>
</tr>
<tr>
<td>313.155</td>
<td>Hydroperoxylinoleic acid</td>
<td>1.4226</td>
<td>0.01192</td>
<td>1.5850</td>
<td>0.6800</td>
<td>Increase</td>
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Table 3. Metabolomic differences between UC and CD. The molecules with the best combination of p-value, FC and AUROC are shown.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Identification</th>
<th>p-value</th>
<th>FC (CD/UC)</th>
<th>AUROC</th>
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<tbody>
<tr>
<td>923.616</td>
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<td>804.554</td>
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<td>0.0597</td>
<td>1.5069</td>
<td>0.7909</td>
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<tr>
<td>784.493</td>
<td>SM(d18:1/22:1)</td>
<td>0.1210</td>
<td>0.4956</td>
<td>0.7681</td>
</tr>
<tr>
<td>520.510</td>
<td>LPC(18:2)</td>
<td>0.0698</td>
<td>0.7610</td>
<td>0.7681</td>
</tr>
<tr>
<td>760.585</td>
<td>PE(22:1/15:0)</td>
<td>0.0937</td>
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<td>0.7636</td>
</tr>
<tr>
<td>274.276</td>
<td>Heptanoyl carnitine</td>
<td>0.2302</td>
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<td>0.7090</td>
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<tr>
<td>792.598</td>
<td>PG(16:1/22:5)</td>
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<tr>
<td>522.357</td>
<td>LPC(18:1)</td>
<td>0.50075</td>
<td>0.87377</td>
<td>0.5909</td>
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Figure 1. The PLS-DA 2D scores showing the discrimination between the UC (green) and CD groups (red).
Figure 2. The VIP scores that discriminate between the UC and CD groups. The mini heatmap on the right indicates their concentration variations within different groups.

Figure 3. A. PCA 3D score plot after outliers’ removal. B. PLS-DA 3D score plot to discriminate between the P group (green) and the controls (red).
Figure 4. A. Plots obtained by cross validation method (LOOCV) applied on PLS-DA data. B. VIP values above 1.2 derived from PLS-DA analysis, to compare the variability of 15 individual molecules between M and P groups. The mini-heatmap (green to red colors) shows the relationship between the M and P values for each molecule.
Figure 5. The cluster analysis using Euclidian distance and single clustering algorithm.
Figure 6. A. 3D scores plot of PCA when comparing CD and UC patients. B. PLS-DA analysis when comparing CD and UC patients.

Figure 7. PLSDA analysis- cross validation method (LOOCV) and VIP scores resulted from PLS-DA analysis for CD vs UC.