Metabolic Alteration in Plasma and Biopsies From Patients With IBD

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Background: Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract, with periods of latency alternating with phases of exacerbation, and include 2 forms: Crohn disease (CD) and ulcerative colitis (UC). Although the etiology of IBD is still unclear, the identification and understanding of pathophysiological mechanisms underlying IBD could reveal newly targeted intestinal alterations and determine therapeutic approaches.

Methods: In this study, by using gas chromatography-mass spectrometry, we characterized plasma and biopsies from the metabolomics profiles of patients with IBD compared with those of a control group.

Results: The results showed a different metabolomics profile between patients with CD (n = 50) and patients with UC (n = 82) compared with the control group (n = 51). Multivariate statistical analysis of the identified metabolites in CD and UC showed changes in energetic metabolism, and lactic acid and ornithine in particular were altered in both plasma and colon biopsies. Moreover, metabolic changes were evidenced between the normal ileum and colon tissues. These differences disappeared when we compared the inflamed ileum and colon tissues, suggesting a common metabolism.

Conclusions: This study showed how the metabolomics profile could be a potential tool to identify intestinal alterations associated with IBD and may have application in precision medicine and for better defining the pathogenesis of the disease.

Key Words: inflammatory bowel diseases, Crohn disease, ulcerative colitis, metabolomics, gas chromatography-mass spectrometry

INTRODUCTION

The intestinal epithelium is normally characterized by a mild and controlled inflammatory state.[1, 2] However, whatever the mechanism supporting it, inflammation may proceed unconditionally with epithelial tissue rupture and bowel dysfunction. Inflammatory bowel diseases (IBD), which include Crohn disease (CD) and ulcerative colitis (UC), are a typical severe example of uncontrollable inflammation.[3] The etiology of chronic IBD is not clear. The hypothesis currently most accredited is that IBD is the result of an inappropriate and exaggerated immune response of T cells mediated toward the normal constituents of the resident mucosal intestinal microflora in genetically predisposed individuals, to which is added the influence of environmental factors that together induce the onset or reactivation of the disease. Moreover, gut microbiota alteration is considered an emerging new factor involved in the pathogenesis of IBD.[4-6]

Treatment choices for IBD are currently quite limited and mainly focused on disease reduction and maintenance in remission rather than on a cure.[7, 8] Considering the poor therapeutic efficacy and numerous adverse effects of modern therapy,[9] it is very important to understand the pathological mechanism that underlies IBD development and maintenance. A previous study investigated the correlation between microbiota and metabolome in fecal samples from patients with IBD.[10] Recently, Borrien et al[4] showed proteomic, metabolomic, and microbial biomarkers and an altered metabolomic profile in patients with CD and with UC. The study highlighted that both the metabolomics and microbiological profile were significantly altered in patients with IBD compared with those of healthy patients.

In this complementary study, the changes in metabolic pathways that seem to occur in the intestinal inflammation process were evaluated in biopsies and plasma samples coming from the same patient cohort. To date, IBD etiology is still partly unknown, and the possibility of detecting and understanding changes in metabolism represents an intriguing perspective for the elucidation of IBD pathogenesis. To achieve this goal, both biopsies and plasma samples from patients affected by CD and UC were analyzed through a combined gas chromatography-mass spectrometry (GC-MS)

and metabolomics approach. An untargeted approach was applied to detect different classes of hydrophilic metabolites. Moreover, to select potential biomarkers that could discriminate among different IBDs, a multivariate statistical analysis was performed.

MATERIALS AND METHODS

Patients

Patients were recruited at the Department of Gastroenterology of the University Hospital of Cagliari, Sardinia, Italy. The institutional ethics committee of the University Hospital of Cagliari, Italy) approved the study, and written consent was obtained from all participants. A total of 132 patients were admitted to the study: 50 patients affected by CD and 82 patients affected by UC. The details of the diagnosis for each patient are reported in a previous article.[10] Disease activity was confirmed based on well-established criteria, including endoscopic grading according to the Crohn's disease endoscopic index of severity (CDEIS) and Rutgeerts scores for patients with CD and the Mayo score for patients with UC.[11] Moreover, a group of healthy volunteers (n = 51) was matched for age, body mass index, and freedom from comorbidity. The clinical data on the participants are reported in Table 1. The exclusion criteria were being older than age 80 years or younger than age 20 years, recent use of antibiotics or probiotics, and pregnancy.

A blood sample was collected from each patient and transported to the laboratory within 3 hours from collection. Colonoscopy for disease rating was performed on patients with IBD, and small samples of tissue were removed from the areas endoscopically inflamed and noninflamed. Colon biopsies were classified into 3 groups: normal colon tissue, CD-affected colon tissue, and UC-affected colon tissue. Ileum biopsies were classified as normal ileum and CD-affected ileum because the ileum was not involved in patients with UC.

Plasma Samples

Blood samples were collected in tubes with EDTA and subsequently centrifuged at 3600*g* for 10 minutes. Next, an aliquot of plasma was recovered and centrifuged at 5500*g* for 10 minutes at 4°C, and 400 µL of supernatant were transferred to an Eppendorf tube. The extraction of hydrophilic metabolites from plasma samples was conducted using a Folch modified method.[12] Each plasma sample was dissolved in 1.2 mL of a chloroform/methanol solution (1:1, v/v) containing succinic

TABLE 1. Clinical Data of Patients With IBD and Healthy Control Patients for Metabolomics Analysis

Groups	Healthy	CD	UC
Age (y)*	40.7 ± 13	48.8 ± 13	47.3 ± 12
Body mass index	23.2 ± 3.04	21.76 ± 3.8	24.6 ± 4.8
Sex			
Male	31	24	44
Female	20	26	38
Therapy			
Mesalazine/salazopyrin	—	8	54
Steroids	—	—	—
Azathioprine	—	9	15
Infliximab/adalimumab	—	27	9
No IBD therapy	51	6	4
Lesion localization			
Ileo-colon	—	19	—
Colon	—	3	—
Ileum	—	24	—
Ileo-cecum	—	3	—
Rectum sigmoid	—	1	21
Rectum	—	—	16
Descending colon	—	—	6
Rectum-sigmoid-descending colon	—	—	14
Rectum-sigmoid-descending-transverse colon	—	—	5
Pancolitis	—	—	20
Total	51	50	82

*Mean ± standard deviation.

acid-2,2,3,3-d4 as the internal standard (Sigma-Aldrich) and 175 mL of Milli-Q water. The plasma solution was centrifuged at 5500*g* for 30 minutes at 4°C, and 150 µL of the hydrophilic phases was recovered for GC-MS analysis. The hydrophilic phases were dried with a speed vacuum concentrator (Eppendorf, Hamburg, Germany) and derivatized with 50 µL of a solution of methoxamine in pyridine (10 mg/mL; Sigma-Aldrich). After 1 hour at 70°C, 100 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich) was added. After 1 hour at room temperature, the samples were resuspended with 150 µL of hexane (Sigma-Aldrich) and filtered with syringes and Acrodisc filters with 0.45 mm polytetrafluoroethylene (PTFE) membranes (Sigma-Aldrich). Quality control samples were created by pooling all the samples under evaluation.

Biopsy Samples

Approximately 2 mg of tissue was extracted with 1 mL of cold methanol/water solution (80:20) and then vortexed.[13] Next, the samples were centrifuged at 2500*g* for 5 minutes at 4°C, and 700 µL of supernatant was dried using a speed vacuum concentrator overnight. The samples were derivatized with 50 µL of a solution of methoxamine in pyridine (Sigma-Aldrich); after 1 hour at 70°C, 100 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich) was added and left at room temperature for one hour. Finally, samples were resuspended with 50 µL of hexane (Sigma-Aldrich). Quality control samples were created by using a pool of all samples.

GC-MS Analysis

One microliter of the derivatized sample was injected splitless into a 7890A gas chromatograph coupled with a 5975C Network mass spectrometer (Agilent Technologies, Santa Clara, CA). The technical details have already been described in previous articles.[14] Metabolites were identified by using the standard National Institute of Standards and Technology and Golm Metabolome Database mass spectra libraries and by comparison with external standard. Peak detection and deconvolution, filtering, and normalization were performed using a pipeline on Knime.[15]

Multivariate Statistical Analysis

Multivariate statistical analysis (MVA) was conducted using SIMCA software (version 15.0, Umetrics, Umea, Sweden). Raw data were organized in matrices, and datasets were normalized to the total area of chromatograms and scaled using UV scaling. For the multivariate statistical analyses, 2 procedures were employed: principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Research has shown that PCA is important for the exploration of sample distributions without prior classification and enables the detection and then the exclusion of any outliers, defined as observations located outside the 95% confidence region of the model.[16] We used OPLS-DA to maximize the covariance between the measured data of the X-variable response of the Y-variable within the groups; in addition, we employed OPLS-DA to discriminate between patients affected by CD and UC and healthy patients. A 7-fold cross-validation and a permutation test were used to evaluate the quality of the OPLS-DA models. In the permutation test, the Y-block was permuted 400 times using the same number of components as determined previously in a partial least-squares discriminant analysis model. An R^2Y intercept value <0.3 to 0.4 and a Q^2Y intercept value <0.05 were indicative of a valid model.[17] The estimated predictive power of the models was expressed as reported by Eriksson et al.[18] To identify the metabolites playing a role in class separation, variables with a Variables Important in the Projection (VIP) value >1 together with the S-plot were considered.

Univariate Statistical Analysis

Univariate statistical analysis of the data was conducted using GraphPad Prism software (version 7.01, GraphPad Software, Inc.). The Mann-Whitney *U* test was used to evaluate the statistical significance of metabolites, and a P value < 0.05 was considered statistically significant. To acquire the level of significance for multiple testing, the Benjamini-Hochberg adjustment was applied to the obtained P values.[19]

RESULTS

Plasma Analysis

A total of 39 metabolites were identified in plasma samples from patients with CD, patients with UC, and healthy control patients using GC-MS. To highlight possible metabolic differences among the plasma samples and to identify potential outliers (outside the 95% confidence limit), a PCA analysis (Supplementary Fig. 1) was initially carried out on the GC-MS dataset. The analysis did not reveal any strong outliers. Next, a supervised analysis was conducted on the same dataset. The OPLS-DA models resulting from the comparisons of the 3 samples obtained using multivariate statistical analysis are shown in Fig. 1. The quality parameters of the OPLS-DA models and the values of the respective permutation test are reported in Supplementary Table 1 and indicate the statistical validity of the analysis. The first OPLS-DA model (Fig. 1A) was built comparing patients with UC and healthy control patients and showed good statistical values (Q^2 = 0.610). Comparable results were also obtained between patients with CD and healthy control patients (Q^2 = 0.609), and the resulting OPLS-DA score plot is shown in Fig. 1B. Another OPLS-DA model (data not shown) was built to understand whether the plasma profile could distinguish between patients with CD and patients with UC. The results of this OPLS-DA analysis did not show good results (Q^2 = -0.224), indicating an intrinsic similarity in metabolic profiles between the 2 groups (Supplementary Table 1). Given this strong similarity, the 2 groups were considered as a

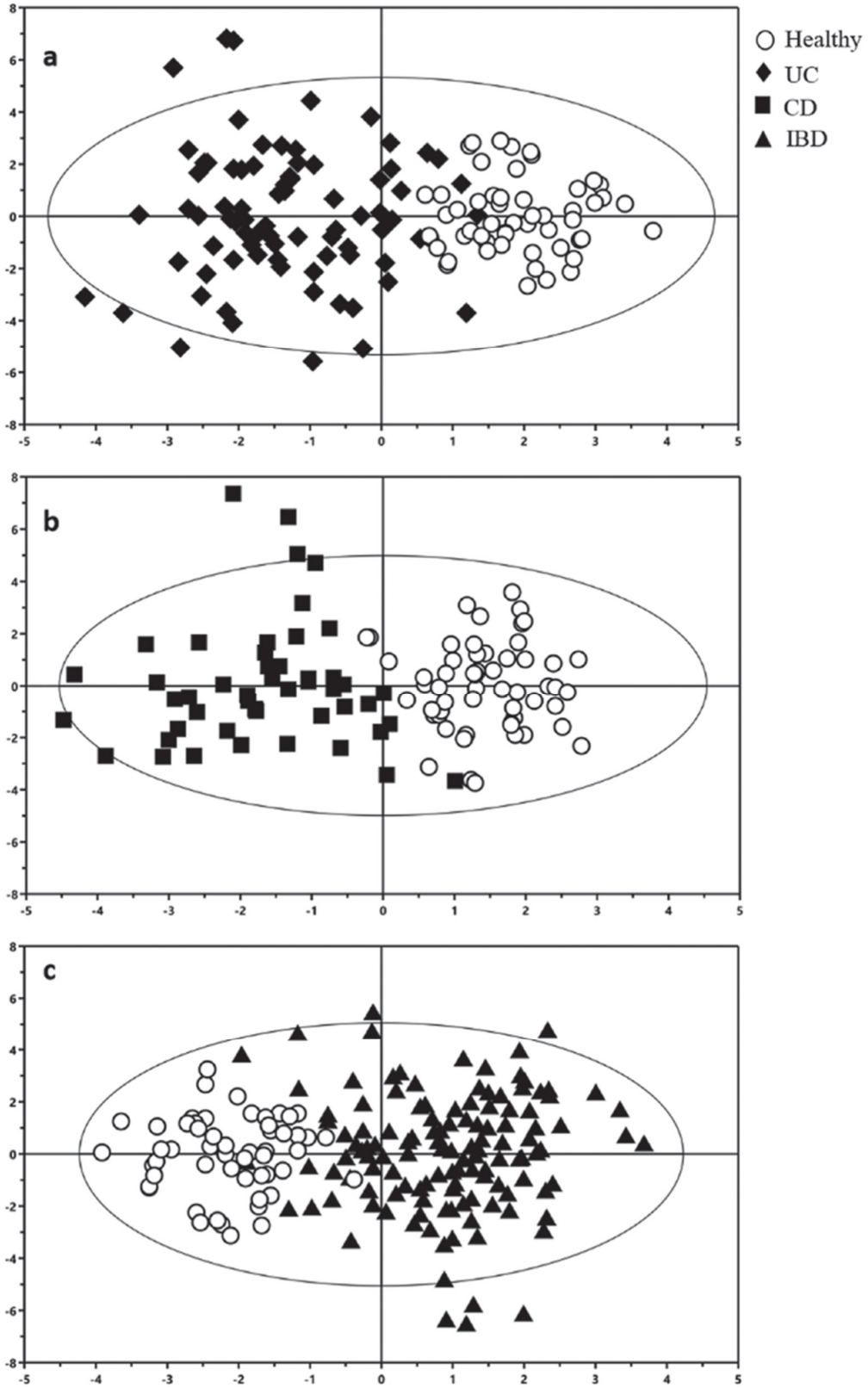

FIGURE 1. OPLS-DA score plot of the comparison between UC and healthy plasma (A), CD vs healthy plasma (B), and IBD vs healthy plasma (C). Models were established with one predictive (x axis) and one orthogonal (y axis) component.

unique class. The OPLS-DA of patients with IBD (CD + UC) vs that of healthy control patients is shown in Fig. 1C and the statistical parameters are reported in Supplementary Table 1.

To identify the potential metabolites that could distinguish patients with IBD from healthy control patients, an S-plot was built (Supplementary Fig. 2). The statistical significance of the discriminated metabolites was evaluated with a Mann-Whitney *U* test with a Benjamini-Hochberg correction. The GC-MS parameters and the VIP values of discriminant metabolites are shown in Supplementary Table 2A. The relative concentrations of the discriminant metabolites were compared using box and whisker plots (Fig. 2), and the *P*-values are shown in Supplementary Table 2B. As shown in Fig. 2, IBD samples were characterized by higher concentrations of 2-hydroxybutyric acid, 3-hydroxybutyric acid, citric acid, lactic acid, lysine, and uric acid and lower concentrations of ornithine, proline, threonine, urea, and 1,5-anydro-d-sorbitol compared to control samples. The altered metabolites in IBD plasma were all involved in the energetic metabolism as part of different metabolic pathways such as glycolysis, ketone bodies, purine metabolisms, the tricarboxylic acid (TCA) cycle, and the urea cycle.

Biopsy Analysis

In biopsy samples from the colon and ileum of patients with CD and with UC, 50 metabolites were identified. As with the previous plasma analysis, a PCA analysis was conducted on all samples (Supplementary Fig. 3), and no sample was excluded for the supervised analyses. All the OPLS-DA models were performed as described above for the plasma samples. As a first step, we compared inflamed and normal colon biopsies from patients affected by UC (Fig. 3A), and the analysis showed good statistical parameters ($Q^2 = 0.508$; Supplementary Table 3). A similar analysis was conducted for inflamed and normal colon biopsies of patients with CD ($Q^2 = 0.314$; Fig. 3B). Subsequently, we investigated the biopsies deriving from the inflamed and normal ileum of patients with CD (Fig. 3C). The statistical parameters are reported in Supplementary Table 3 and revealed, as expected, a different metabolic profile between inflamed and normal ileum ($Q^2 = 0.239$).

To evaluate the effect of inflammation on the differing metabolism of the colon and ileum in patients with CD, different OPLS-DA models were constructed. The statistical parameters of the OPLS-DA models are shown in Supplementary Table 3. In particular, a comparison between normal ileum and colon and between inflamed ileum and colon was conducted (Figs. 3D, E). These results showed that the metabolic differences observed between the normal colon and normal ileum ($Q^2 = 0.558$) in patients with CD were not detected in their inflamed counterparts ($Q^2 = –0.310$). To investigate possible differences in the colon biopsies of patients with UC or CD, an OPLS-DA model was created. This comparison did not indicate significant statistical results ($Q^2 = –0.406$), suggesting an intrinsic similarity in the metabolic profiles (Fig. 3F and Supplementary Table 3). The metabolomic analysis of intestinal biopsies, as seen previously for the plasma analysis, did not allow us to discriminate patients with CD from patients with UC. So even in this case, patients with CD and patients with UC were analyzed as a single class. The resulting OPLS-DA between normal and IBD-affected colon is shown in Fig. 3G, and the statistical parameters are summarized in Supplementary Table 3.

Discriminant metabolites identified were highlighted by S-plot (Supplementary Fig. 4). A Mann-Whitney *U* test with a Benjamini-Hochberg correction was performed to uncover significant concentration differences (Fig. 4). The GC-MS parameters and the VIP values of discriminant metabolites are shown in Supplementary Table 2A. As shown in Fig. 4, by comparing the normal colon and the colon affected by IBD, levels of aspartic acid, glutamic acid, glutamine, glycine, and ornithine were significantly higher, and levels of fumaric acid, glycerol-phosphate, lactic acid, myo-inositol, and oleic acid decreased. The *P* values of discriminant metabolites are shown in Supplementary Table 2B. The altered metabolites in the IBD colon were all involved in the energetic metabolism as part of different metabolic pathways such as glycolysis, the TCA cycle, and the urea cycle (Fig. 5).

DISCUSSION

One of the objectives of untargeted metabolomics is the possibility of identifying new biological markers and better understanding the different pathophysiological mechanisms underlying various human diseases.[20] The study of the metabolomic profile of plasma samples and inflamed and noninflamed tissue biopsies (colon for patients with UC and colon and ileum for patients with CD) allowed us to distinguish patients with CD and with UC from healthy control patients. Regarding CD, we focused on understanding the effects of IBD on ileum and colon metabolism. When the ileum and the colon are characterized by IBD, their metabolomic profile is practically superimposable, as shown in Fig. 3E. Meanwhile, it was not possible to differentiate CD and UC at the plasma and colon biopsy level. These results confirm the intrinsic similarity between these 2 pathological conditions, which have been reported in previous research.[10] In some cases, it has been reported that it is not feasible to have a clinical differentiation between CD and UC.[21] Therefore, because of their similar metabolic profile, CD and UC samples were analyzed together as IBD samples.

Despite the results reported in this study and in previous work[10] that could not differentiate patients with UC and patients with CD based on their hydrophilic metabolic profiles, a study conducted on the lipid profile of the plasma of children affected by CD and UC showed that it is possible to discriminate between these 2 IBD types by measuring the metabolite

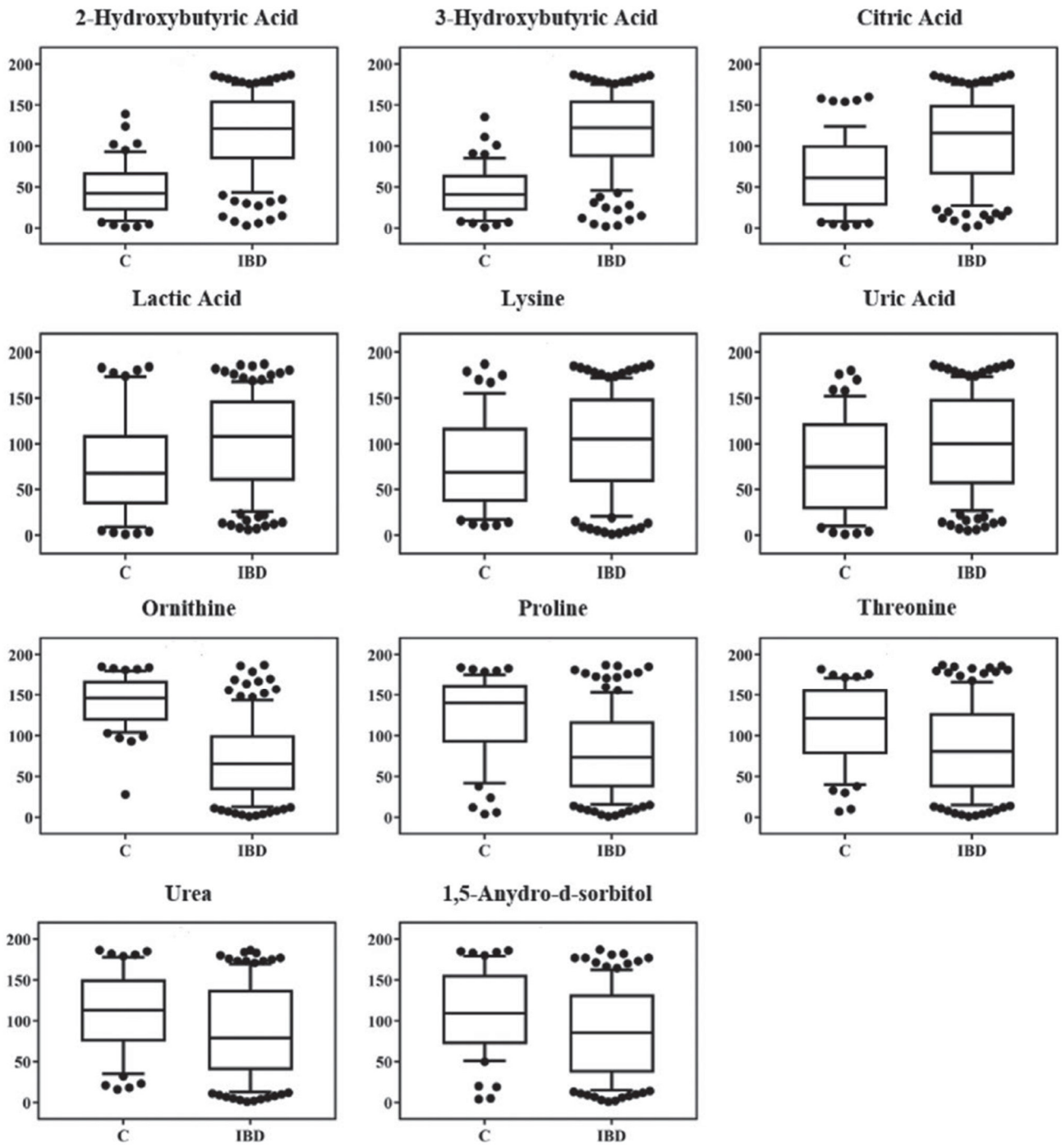

FIGURE 2. Statistically significant metabolites in IBD vs healthy plasma samples (C) comparison. Discriminant metabolites obtained with multivariate statistical analysis underwent a Mann-Whitney U test with a Benjamini-Hochberg correction to determine which metabolites were statistically significantly varied. The resulting metabolites are shown and expressed on the y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted).

Lactosylceramide 18:1/16:0 with liquid chromatography-mass spectrometry.[22] These contrasting results could depend on the different analytical methods used. The univariate statistical analysis in this study allowed us to identify 2 metabolites, lactic acid and ornithine, altered in both plasma and colon biopsies. In particular, the concentration of lactic acid was increased in plasma and decreased in the colon of patients with IBD. During inflammatory activation, lactic acid production

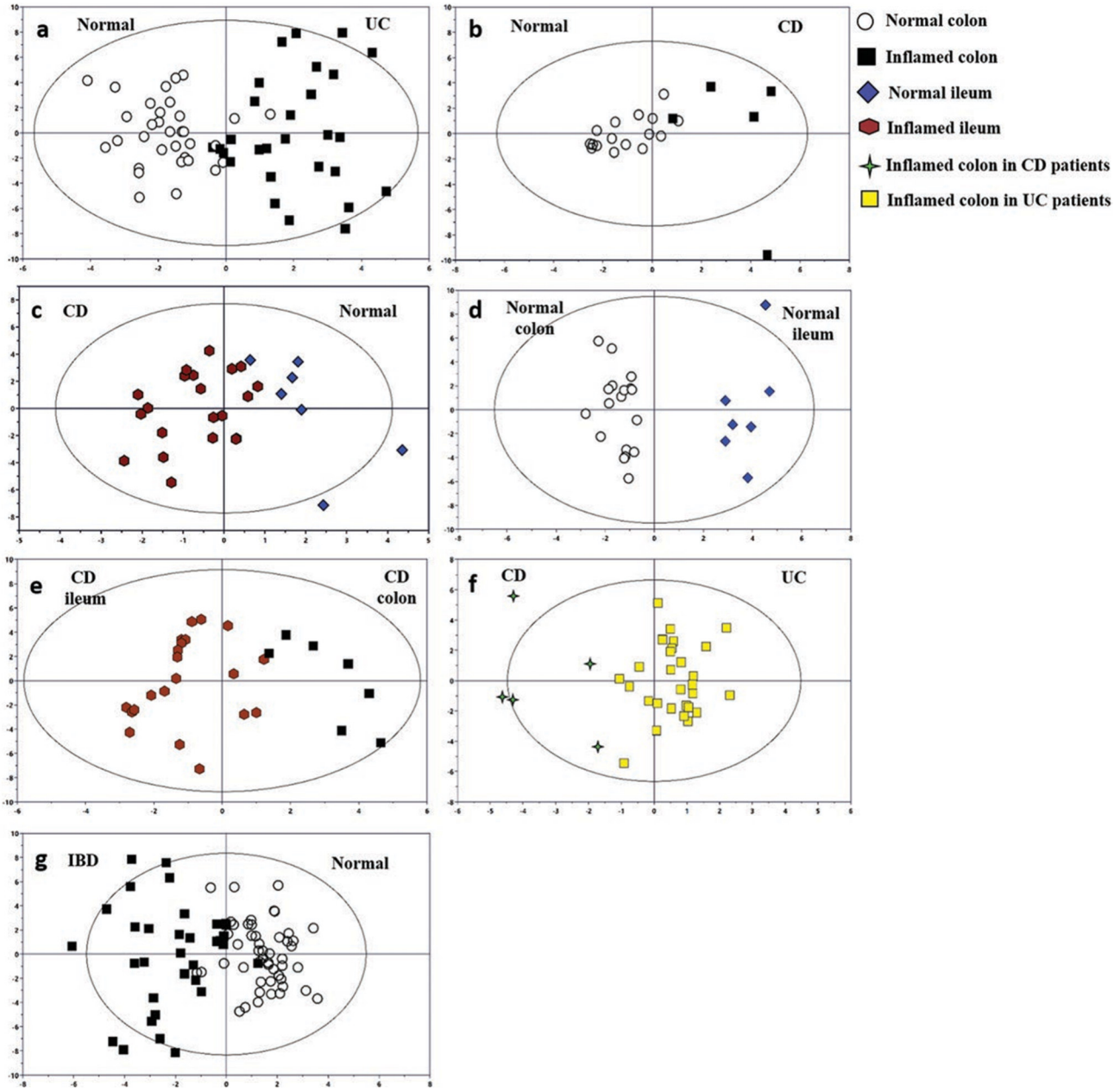

FIGURE 3. OPLS-DA score plot of biopsy samples. Normal colon vs inflamed colon in UC (A), normal colon vs inflamed colon in CD (B), normal ileum vs inflamed ileum in CD (C), normal ileum vs normal colon in CD (D), inflamed ileum vs inflamed colon in CD (E), CD inflamed colon vs UC inflamed colon (F), and normal colon vs inflamed colon in IBD (G). The OPLS-DA models were established with one predictive (x axis) and one orthogonal (y axis) component.

is increased by innate immune cells. Extracellular lactic acid induces metabolic reprogramming in innate immune cells, resulting in a reduction of glycolysis and an increase in the oxidative rates of monocytes immediately after exposure to lactate.[23] Furthermore, the alteration of lactic acid is in agreement with a study reporting an increase in the concentration of lactic acid in the blood because of the overexpression of monocarboxylate transporter 4 as a consequence of inflammation in the intestinal mucosal epithelial tissue.[24] On the other hand, ornithine was decreased in plasma and increased in the colon of IBD. Ornithine and urea levels were lower in plasma of patients with IBD, indicating a possible involvement of the urea cycle in the inflammation-induced shift of the metabolism.

Furthermore, the plasma metabolomic profile of patients with IBD in our study was characterized by increased

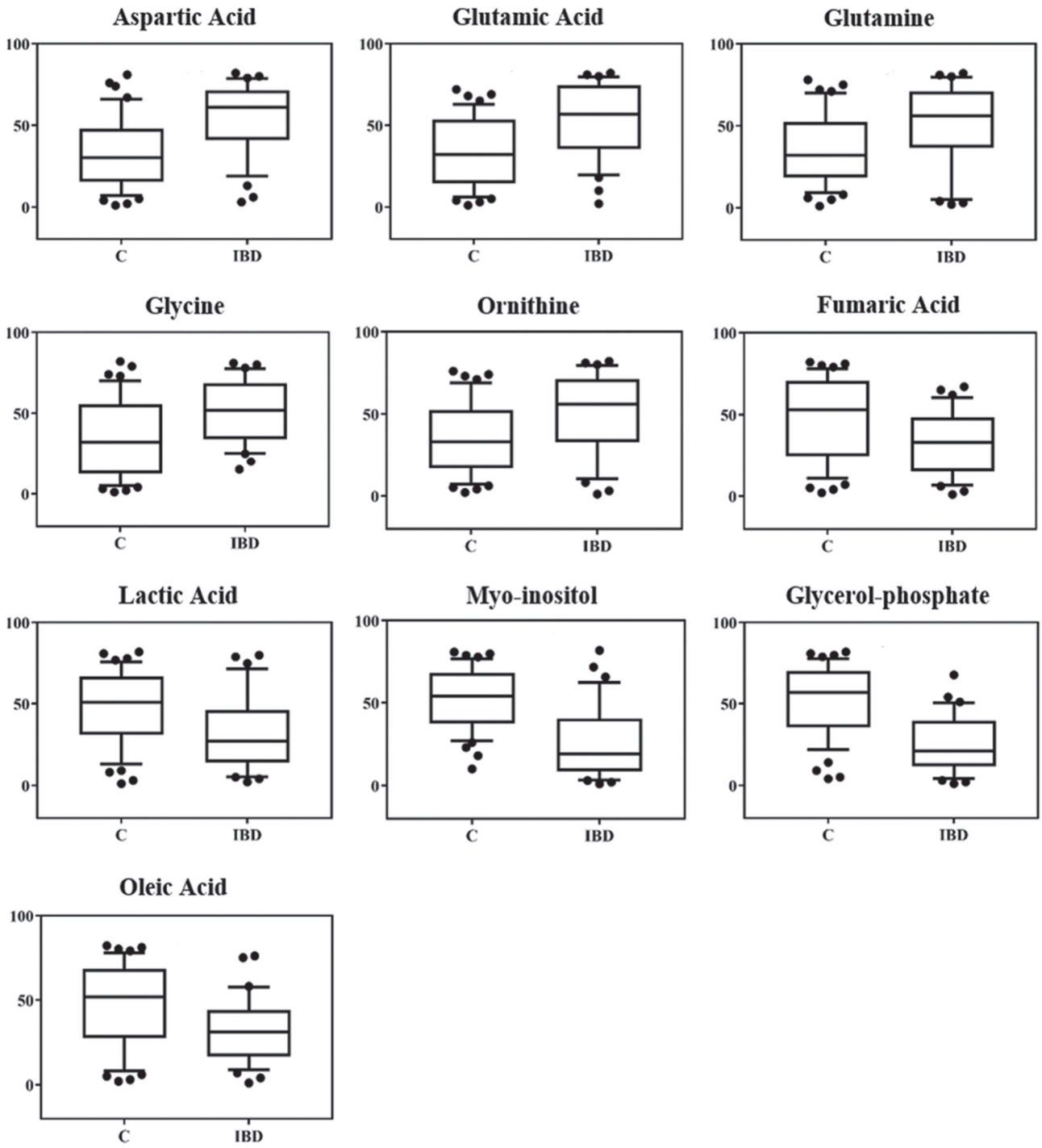

FIGURE 4. Comaprison of statistically significant metabolites in colon affected by IBD vs normal colon (C). Discriminant metabolites obtained with multivariate statistical analysis underwent a Mann-Whitney U test with a Benjamini-Hochberg correction to determine which metabolites were statistically significantly varied. The resulting metabolites are shown and expressed on the y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted).

levels of 2-hydroxybutyric acid and a decrease of threonine. 2-hydroxybutyric acid is considered an early marker for altered glucose control. The biochemical mechanisms involve increased lipolysis and oxidative stress.[25] Many chronic diseases are associated with oxidative stress and a variety of protein oxidations. Different studies have reported a relationship between oxidative stress, inflammation, suggesting that oxidative stress may play a pathogenic role in chronic inflammatory diseases such as IBD.[26]

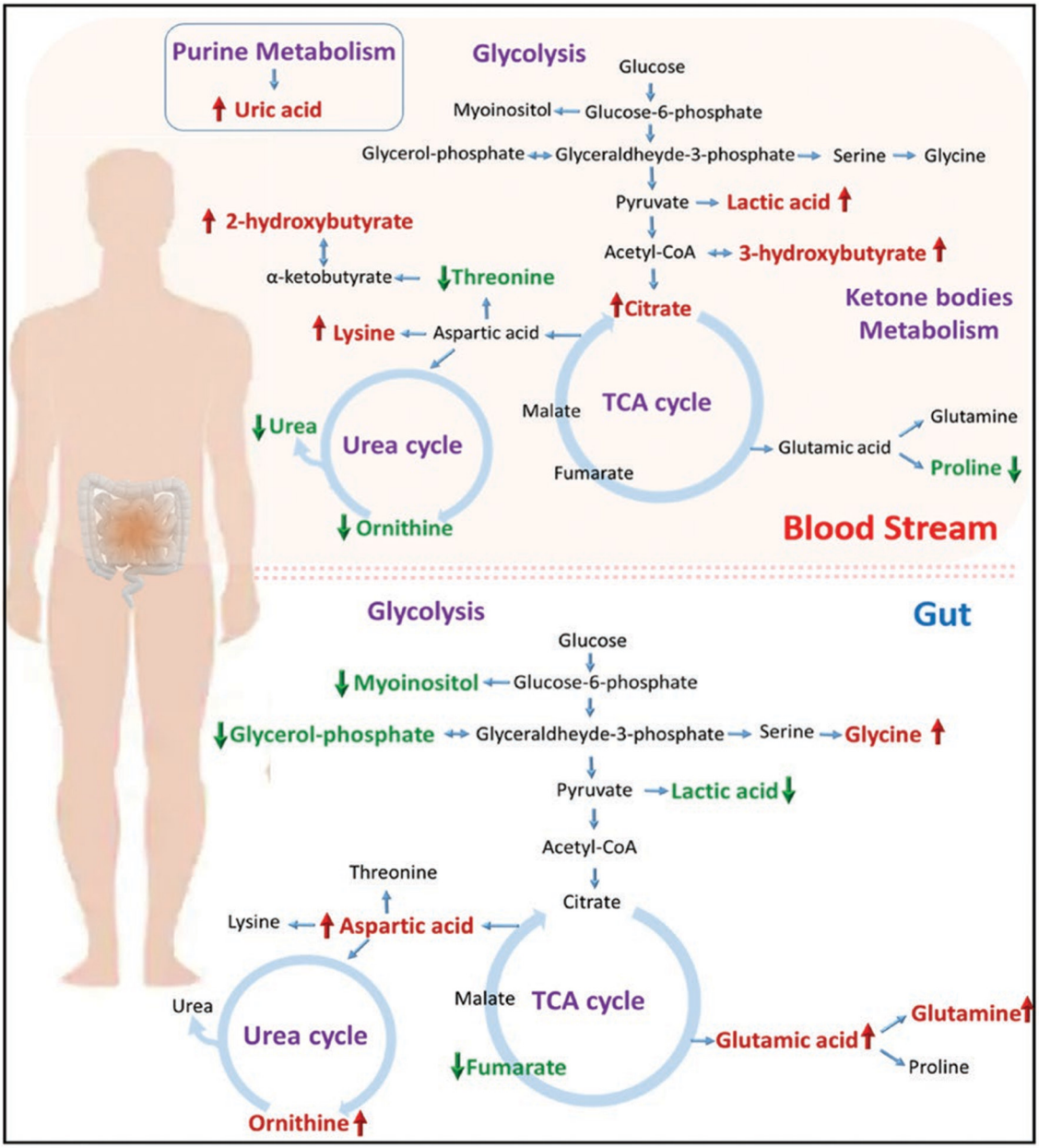

FIGURE 5. Relevant metabolic pathways involved in IBD, obtained from analysis of metabolites in plasma and colon biopsy samples. Increased and decreased metabolites are highlighted in red and green, respectively.

In addition, the levels of 3-hydroxybutyric acid, a ketone body, were higher in the plasma of patients with IBD. Ketone bodies are oxidized in the TCA acid cycle for energy production in different organs such as the brain, heart, and skeletal muscle and play a very important role as modulators of inflammation and oxidative stress. An increase in the concentration of ketone bodies in the plasma could be linked to the increased energetic pathway observed in inflammation.[27]

Furthermore, the metabolomic analysis revealed an increase in the concentration of citric acid in the plasma of patients with IBD and a decrease of fumaric acid in the colon. An altered TCA cycle was also indicated in activated dendritic cells and macrophages, resulting in the accumulation of citrate and succinate. The cytosolic metabolism of citrate to acetyl-coenzyme A is central for both fatty-acid synthesis and protein acetylation. Moreover, the accumulation of citrate and acetyl-coenzyme A was associated with macrophages and other

immune cell activation. Finally, itaconate, derived from citrate, seems to have a direct antibacterial effect and act as an anti-inflammatory agent.[28] These findings suggest that the TCA cycle may play an important role in the inflammatory process. In a recent metabolomics study of patients with UC and with CD, Scoville et al[29] found evidence of changes in the TCA cycle suggesting that the alteration of energy homeostasis represents a specific feature of IBD, in particular in CD.

Untargeted liquid chromatography-mass spectrometry metabolomic profiling in CD has confirmed significant differences with control patients.[30] Those authors also observed some differences between patients with active and inactive CD (eg, levels of ergothionenine, docosahexaenoic acid, butyrylcarnitine). In the present study, these molecules were not detected. In our study, the plasma profile of IBD was characterized by an increase in lysine and uric acid levels and a decrease in proline. Uric acid is involved in the metabolism of purines and is produced by the catabolism of hypoxanthine and xanthine by the enzyme xanthine oxidase. Several studies have shown that an alteration in purine metabolism is correlated with a persistent inflammatory process in numerous diseases, such as IBD, ischemia, diabetes, and cancer.[31, 32] The metabolomics profile of biopsies of the colon affected by IBD has revealed an increase in proline and aspartic acid, metabolites involved in immune responses.[33] In particular, proline is produced by the metabolism of glutamine and glutamic acid. An increase in both glutamine and glutamic acid levels has been detected in IBD colon biopsies. In intestinal epithelial cells, glutamine is an important energy source. It is required by dividing cells, because it supplies most of the nitrogen requirements for purine and pyrimidine synthesis.[34] The literature has shown that glutamine can influence various inflammatory signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription (STAT), reducing the state of inflammation.[35] Thus, elevated glutamine and glutamic acid levels in the IBD colon can be the consequence of a defense mechanism during an inflammatory state. In our study, IBD colon biopsies were also characterized by an increase in the concentration of aspartic acid, whereas lysine was higher in the plasma profile of patients with IBD than in healthy patients, indicating an impairment of this pathway. Studies conducted on animal models have highlighted the role of aspartic acid in inhibiting signaling pathways such as toll-like receptor 4, nucleotide-binding oligomerization domains/NF-κB, and p38, resulting in an improvement in inflammation and intestinal integrity.[36]

Moreover, myo-inositol levels were higher in colon biopsies of our patients with IBD. Myo-inositol is considered an important molecule in various biological processes, such as controlling the concentration of extracellular calcium and maintaining the potential of the cell membrane. It has been shown that myo-inositol affects the contractility of the gastrointestinal tract, improving intestinal regularity and distending.[37] Finally, the increase in glycine levels in IBD colon biopsies reflects observations about the fecal samples noted in previous research.[10] An overview of the metabolic changes and interplays observed in the plasma and intestinal biopsies of patients with IBD is shown in Fig. 5.

CONCLUSIONS

Metabolomics can be a useful tool to improve our knowledge regarding the pathogenesis, diagnosis, and treatment of chronic IBD. Through the study of the metabolomic profile of both plasma and colon biopsies of patients with IBD, it was possible to identify the altered metabolic pathways. Moreover, it was possible to distinguish the metabolic profile of patients with IBD from that of control patients. Our study identified a characteristic composition of metabolites in the plasma and intestinal tissue of patients with IBD, confirming the potential of metabolomics to identify new biomarkers of this condition. Our results also showed a strong overlap between the metabolomic profile of patients with CD and that of patients with UC, indicating a strong correspondence between these 2 pathologies. Finally, our investigation showed that a better understanding of the metabolic profile of IBD can be useful in disease management by indicating possible therapeutic targets and in defining the pathogenesis. It is important to underline that this work was conducted considering the relative percentage composition of the metabolites, not absolute concentrations. Moreover, the actual clinical utility of the metabolic changes will need to be confirmed in future studies in a larger patient cohort.

ACKNOWLEDGMENTS

The institutional Ethics Committee (University Hospital of Cagliari, Italy) approved the study, written consent was obtained from all participants, and the study was conducted according to the Declaration of Helsinki (AOU Cagliari, Italy. Prot. NP/2014/3504; Prot. PG/2014/11480).

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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