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Study of metabolic alterations in inflammatory bowel diseases

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Abstract

Inflammation is a defence mechanism, which constitutes a protective response, following harmful action of physical, chemical and biological agents, whose ultimate goal is the elimination of the initial cause of cellular or tissue damage, as well as the start of the reparative process. Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract of uncertain origin, which includes ulcerative colitis (UC) and Crohn's disease (CD). Tissue and systemic metabolism are particularly affected by the presence of an inflammation process and a consequently immune response. Here, the metabolic changes happening in IBD were studied analysing different type of biological samples coming from IBD affected patients and healthy individuals. Plasma, ileum and colon biopsies were analysed by GC-MS and faeces samples by GC-MS and ^{1}H-NMR. Furthermore, to have a more complete understanding of IBD pathogenesis and persistence, microbiota was characterised in faeces samples. The metabolites content in biopsies, plasma and faeces and the faeces microbiological profiles were significantly different between IBD and healthy subjects. Energetic metabolism was found particularly involved in the disease. In faeces, changes in metabolites showed a good correlation with microbiota profile. Between all the altered metabolites, nicotinic acid was found to decrease in faeces of IBD patients respects to healthy subjects. As in literature, nicotinic acid seems to have an anti-inflammatory effect, an in vitro model of intestinal inflammation was obtained with differentiated Caco-2 cells in which inflammation was induced with LPS and IL-1β. Cells were then co-treated with LPS and IL-1β together with nicotinic acid. The in vitro results showed that nicotinic acid was able to reduce inflammation by reducing IL-8 production. In addition, nicotinic acid had an effect in restoring the levels of several metabolites, which changes were caused by the inflammatory stimuli. Concluding, this study underlines the potential role of metabolomic and inter-omics approaches in understanding the alteration of metabolic pathways involved in IBD. Lastly, it also proved the beneficial effect on nicotinic acid on intestinal inflammation and how it is achieved through metabolism changes.

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IBD Inflammatory Bowel Diseases

CD Crohn Disease

UC Ulcerative Colitis

LPS Lipopolysaccharide

IL-1β Interleukin 1β

NA Nicotinic acid

IL-8 Interleukin 8

GC-MS Gas Chromatography Mass Spectrometry

^{1}H-NMR Proton Nuclear Magnetic Resonance

LC-MS Liquid Chromatography Mass Spectrometry

TOF Time Of Flight

HILIC Hydrophilic Interaction Liquid Chromatography

EI Electron Ionisation

TMAO Trimethylamine N-Oxide

OTU Operational Taxonomic Unit

rRNA Ribosomal Ribonucleic Acid

QC Quality Control

MSTFA N-Methyl-N-(trimethylsilyl)trifluoroacetamide

qPCR Quantitative Polymerase Chain Reaction

QIIME Quantitative Insights Into Microbial Ecology

DMSO Dimethyl Sulfoxyde

BSA Bovine Serum Albumine

PCA Principal Component Analysis

PLS-DA Partial Least Square-Discriminant Analysis

OPLS-DA Orthogonal Partial Least Square-Discriminant Analysis

MVA Multivariate Statistical Analysis

TCA Tricarboxylic Acid Cycle

G-6-P Glucose-6-Phosphate

DC Dendritic cells

ROS Reactive Oxygen Species

I. Introduction

The inflammatory response

Inflammation is defined as a response triggered by stimuli and harmful conditions, such as infection and tissue injury (1). The initial response of the body to an infection or to trauma is called the acute inflammatory response. The five cardinal signs of inflammation are heat, pain, redness, swelling, and loss of function (*calor, dolor, rubor, tumor, and functio laesa*). These signs can be explained by increased blood flow, increased cellular metabolism, vasodilatation, release of soluble mediators, extravasation of fluids and cellular influx. Physiologically, several interacting cells and mediators are involved in the acute response. Phagocytic cells that engulf and kill bacteria, signaling proteins that can up- or downregulate inflammation (pro- and anti-inflammatory cytokines), effector molecules and coagulation mediators, all play a role in this process. Quite often, the inflammatory reaction can itself cause tissue damage, and at a systemic level, it can cause hypotension and hypoperfusion of major organs. These consequences also contribute to exacerbate inflammation (2). In some cases, the inflammatory process, which in normal conditions is self-limiting, becomes continuous and chronic inflammatory diseases develop subsequently (3). While the cellular and molecular events that trigger the acute inflammatory response are well studied and characterised, the events that lead to localized chronic inflammation, particularly in chronic infections and autoimmune diseases, are not completely understood. Moreover, very little is known about the causes and mechanisms of systemic chronic inflammation (4). Chronic inflammatory diseases are the most important cause of death in the world. The World Health Organization ranks chronic diseases as the greatest threat to human health. Worldwide, 3 of 5 people die due to chronic inflammatory diseases like stroke, chronic respiratory diseases, heart disorders, cancer, obesity, and diabetes (5).

Most of the features of acute inflammation persist as the inflammation becomes chronic, including expansion of blood vessels, increase in blood flow, capillary permeability and migration of neutrophils into the inflamed tissue through the capillary wall (Figure 1).

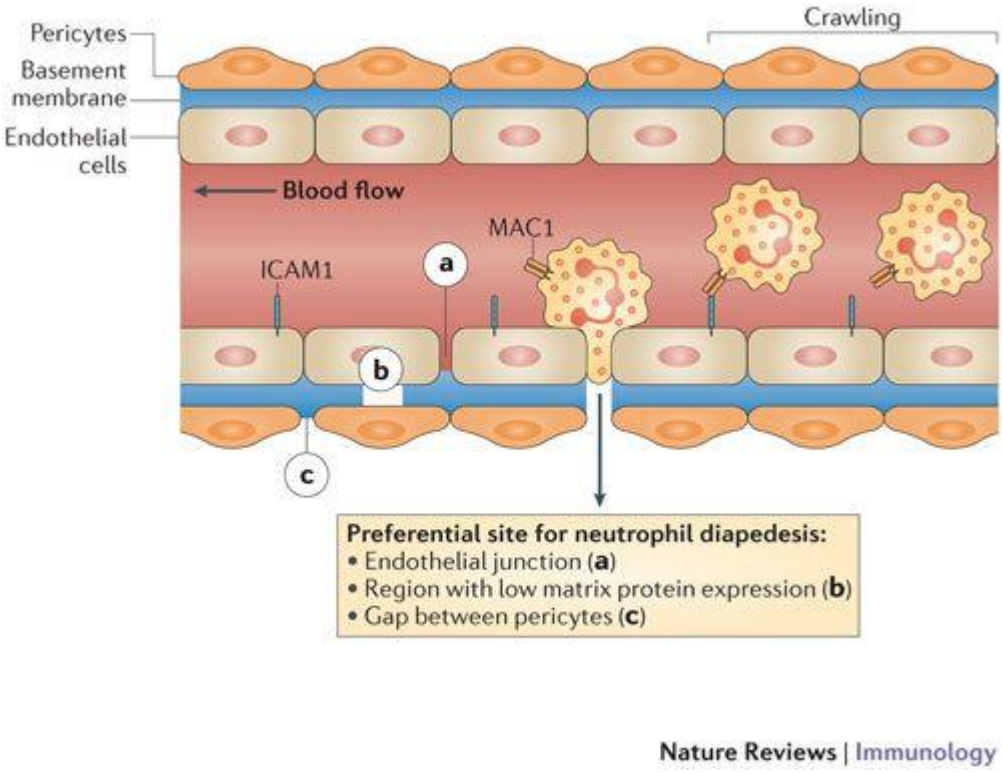

Figure 1. Migrating neutrophils actively choose sites of transmigration (6).

By the way, the composition of the white blood cells changes rapidly, and short-lived neutrophils are soon replaced by macrophages and lymphocytes (7,8). The infiltration of mononuclear cells including macrophages, lymphocytes and plasma cells, tissue destruction by products of the inflammatory cells, and repair involving angiogenesis and fibrosis are the main histological characteristics. Moreover, macrophages, lymphocytes, and plasma cells in the tissue site produce inflammatory cytokines, growth factors, enzymes and contribute to the progression of tissue damage and secondary repair including fibrosis and granuloma formation. There are distinct mechanisms to make inflammatory response long lasting and maintained chronically. They include:

- Conversion of acute inflammation to immune inflammation by acquired immunity
- Amplification and continuation of inflammatory processes by positive feedback mechanism or suppression of negative feedback mechanism

- Progression of inflammation by a chain of changes in active cell populations at the inflammatory site
- Tissue remodelling
- Epigenetic changes associated with the above processes to sustain inflammation (9).

Inflammatory bowel diseases

Intestinal epithelial cells are continuously exposed to a variety of exogenous stresses, including food-derived molecules, environmental chemicals, and intestinal bacteria. To cope with these external stimuli and to reinforce and restore the intestinal barrier, epithelial and immune cells are activated, thereby producing cytokines and other bioactive compounds. However, these protective responses may at the same time, induce inflammation as well. In other words, the intestinal epithelium is an inflammatory tissue by nature, always maintaining a moderate inflammatory state. This kind of inflammation in normal intestines is mild and controllable and is called “controlled inflammation” (10). However, if inflammatory reactions immoderately proceed because of excessive stress or the formation of a vicious cycle, disruption of the epithelial tissues and dysfunction of the intestines will occur. Typical and severe examples of such uncontrollable inflammation is inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC) (11).

IBD represents a set of chronic disorders, of still not completely known aetiology, which affects one or more parts of the intestine. Despite the different distribution of lesions and the peculiar clinical-pathological characteristics, allow differentiation between the two pathologies, in a minority of cases (10-15%) it is not possible to distinguish one condition from the other. In this case, it is referred as indeterminate colitis (12).

IBD appears to be more frequent in Industrialized Countries (North America and Northern Europe) where the incidence rates begin to stabilize in recent years, after a phase of progressive increase in the last fifty years. The annual incidence of ulcerative colitis varies from 0-19.2 cases/100,000 people/year in North America and 0.6-24.3 cases/100,000 people/year in Europe, corresponding to a prevalence of 37.5-248.6 cases/100,000 and 4.9-505 cases/100,000, respectively. The incidence of Crohn's disease is similar (0-20.2 cases/100,000 people/year in North America, 0.3-12.7cases/100,000 people/year in Europe). On the other hand, incidence and prevalence rates continue to increase in countries that, until a few years ago, were considered at a low incidence for

these diseases (Southern Europe, Asia and developing countries). IBD also has a higher frequency among Caucasian populations than in African or Eastern populations, with a higher incidence (3 to 6 times) among Jews. CD is slightly more common in females (M:F = 1:1.2) and occurs earlier (mean age 26 years) than ulcerative colitis (M:F = 1.2:1; mean age 34 years) (13,14).

Both CD and UC are characterised by chronic recurrent inflammation of the gastrointestinal tract. Clinical symptoms may include diarrhoea, rectal bleeding, abdominal pain, weight loss, fever, fatigue, and/or anaemia. IBD can also cause lethal conditions such as perforation, rectal bleeding that does not respond to treatment and toxic megacolon. The increase in mortality was demonstrated for the CD, but not for the UC. Patients also have an increased risk of developing colon cancer after 8-10 years of diagnosis, although overall survival has improved. In fact, in 1950, only 80% of patients survived a period of 10 years, while today patients have a normal life expectancy (15).

IBD is characterized by periods of remission and periods of recurrence. The time between exacerbations may be weeks or years, but some patients still have persistent symptoms (16).

CD affects the entire thickness of the intestinal wall. It can involve indeed any part of the gastrointestinal tract from the mouth to the anus, even if the most affected areas are the most distal part of the small intestine (ileum) and the proximal part of the large intestine (colon) (Figure 2). Unlike ulcerative colitis, between an area of active inflammation and the other, there may be areas of intestine not involved in inflammation. Serious complications may include narrowing of parts of the intestine (stenosis), abnormal tunnels that connect organs (fistulas) and cracks of the anal skin (fissures) (17).

UC is characterized by diffuse inflammation of the colonic mucosa, the innermost layer that is in direct contact with the faecal flow. The disease always originates in the rectum and can be extended as a continuous inflammation throughout the length of the colon (Figure 2) (18). However, some patients develop inflammation in the ileum (19). IBD treatment options are quite limited due to its complicated and unknown aetiology and are focused on relief of symptoms and maintenance in

clinical remission rather than on a cure (20). This lack of appropriate treatment accounts for the high burden of IBD. Commonly recommended standard therapies for IBD patients are corticosteroids, amino-salicylates, and immunosuppressive agents. In addition, biological agents such as infliximab and tacrolimus have been used as part of the regime for UC since 1998 and 2006, respectively. This current treatment is short term and relieves symptomatic complications, but is accompanied by serious side effects, including loss of immune tolerance and drug resistance (21). The low therapeutic efficacy and high adverse effects of modern therapy are impetus enough to seek an alternative effective therapeutic strategy such as prebiotics, probiotics, and symbiotic, as either complementary or alternative medicines to treat IBD and other similar diseases (22).

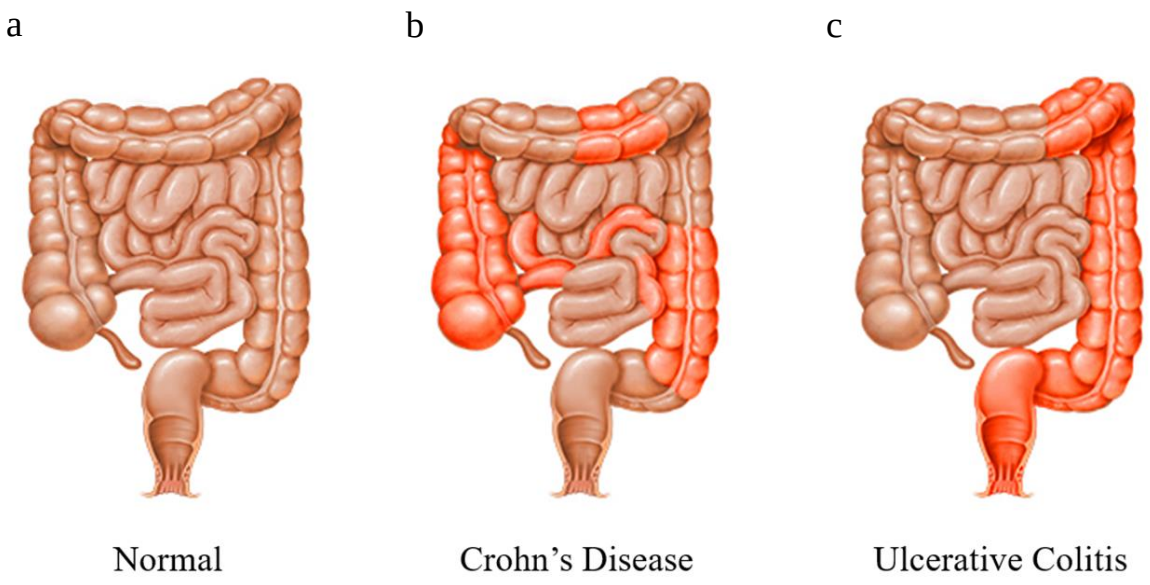

Figure 2. Macroscopical localization of inflammation in IBD: a) normal intestine, b) Crohn's Disease and c) Ulcerative colitis.

The most widespread hypothesis on the pathogenesis of IBD is the development of an excessive aggressiveness of the acquired immune response (T cells) to a subset of commensal enteric bacteria in genetically hosts sensitive, and to which is added the influence of environmental factors that together cause the onset or the reactivation of the disease. The etiopathogenesis of IBD is therefore multifactorial, deriving from internal factors (genetic and immunological) and external to the host

(environmental) such as improved hygiene, diet, smoking and infectious agents (Figure 3). The involvement of gut microbiota in IBD pathogenesis has recently been highlighted in the research. The human gut microbial flora is a dynamic and diverse community of commensal bacteria, fungi, and viruses; among them, bacteria, of which there are over 1000 different species, constitute the major part. The co-existence of the gut microbiota and host reveals the crucial role of the gut microbial flora in host health, and maintenance of the gut microbiota equilibrium is highly important to the host gut and overall systemic physiology.

Any change in the steady state of the gut microbiota structural composition that can alter the microbial equilibrium is termed "dysbiosis" and is associated with a variety of gut pathologies and intestinal inflammation. Dysbiosis is considered a matter of causation for deterioration of the homeostatic relationship between the microbes and host (22). Considering the preponderance of gram-negative bacteria in the gastrointestinal tract, the role of endotoxin (lipopolysaccharide; LPS) in the activation of immunity and complications of the disease in patients with IBD is an important fact to take in consideration. Lipopolysaccharide (LPS) is the most abundant component within the cell wall of Gram-negative bacteria. It can stimulate the release of interleukin 8 (IL-8) and other inflammatory cytokines in various cell types, leading to an acute inflammatory response towards pathogens. These responses are initiated by the activation of the Tod-like receptors signaling through adaptor proteins and include induction of gene expression via the activation of the NF-κB and AP-1 signal transduction pathways (23). Indeed, systemic exposure to LPS has been linked to clinical disease activity in adults with IBD (24).

As mentioned above, IBD is in part due to a dysregulated or an inappropriate immune reaction, which has been thought to be against the microflora of the gut. Upon activation of the immune system, cytokines and chemokines are produced and trigger a cascade of downstream reactions revealing their significant role in the pro- and anti-inflammatory processes in the inflamed gut mucosa.

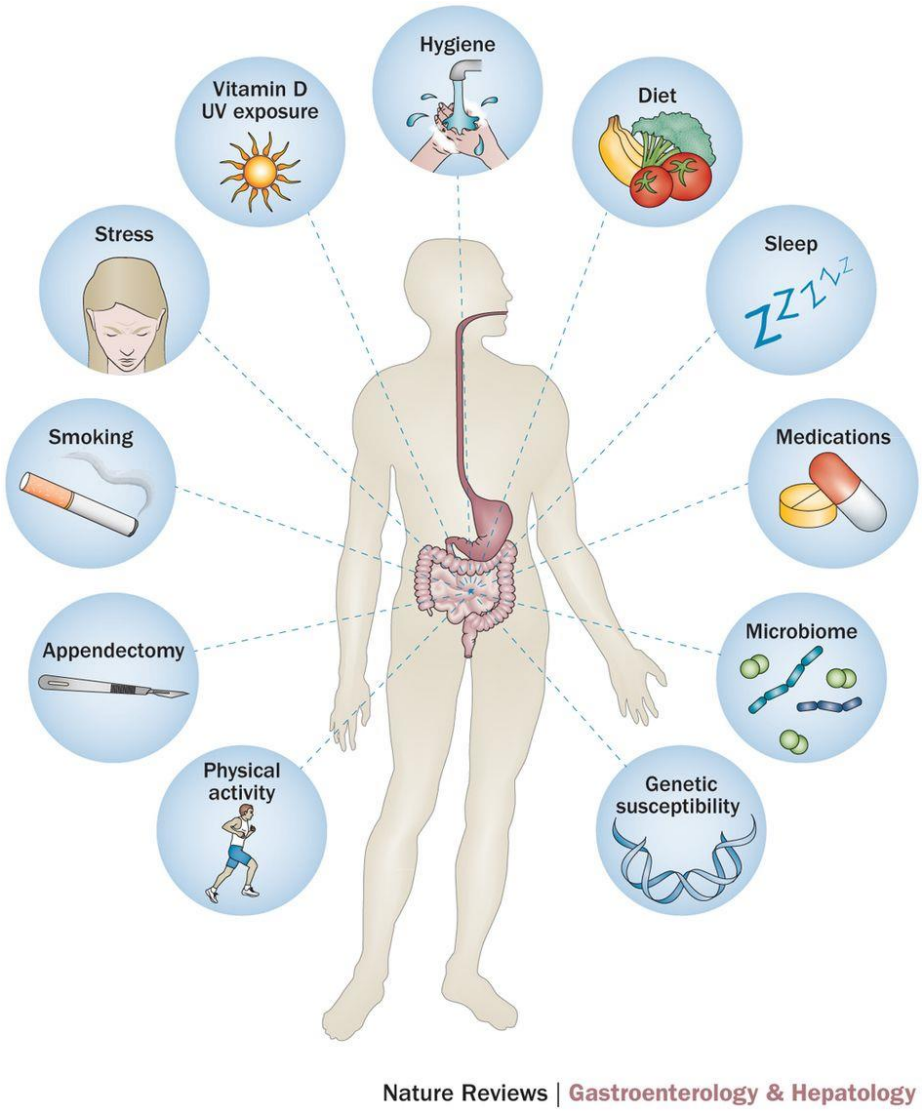

Figure 3. The interaction between genetics, immunology, environment and microbiome (14).

These cytokines are increasingly being defined as important molecules in the pathogenesis of IBD as well as putative and known targets for the therapy of IBD. In different murine models of mucosal inflammation, it has been initially noted that the inflammation is due to either an excessive Th1 or Th2 cell response, with the former characterized by increased interleukins production. Different cytokines are secreted during this phase, such as TNF-α, INF-γ, IL-1, IL-6, IL-4, IL-5, IL10 and TGF-β. Among all these, interleukin 1 (IL-1) seems to be one of the most important cytokines produced. IL-1 is part of a cytokine superfamily, which is composed of 11 members, including IL-1α and il-1β, which are both produced by monocytes, macrophages and endothelial cells. Stimulation of IL-1ra

secretion has been shown to be activated by IL-1 itself, therefore forming a negative feedback loop. IL-1 has different functions, some of which are mediated indirectly by the induction of the synthesis of other mediators including adrenocorticotropic hormone, prostaglandin E2, IL-6 and IL-8. The main biological activity of IL-1 is the stimulation of T-helper cells, which are induced to secrete IL-2 and to express IL-2 receptors. IL-1 can also act on B-cells, promoting their proliferation and the synthesis of immunoglobulins. Moreover, IL-1 stimulates the proliferation and activation of other immune cells such as NK-cells, fibroblasts and thymocytes. The production of IL-1 can be induced by other cytokines including TNF-α, IFN-γ, or and by bacterial endotoxins and viruses. It also promotes the adhesion of neutrophils, monocytes, T-cells, and B-cells by increasing the expression of adhesion molecules such as intercellular adhesion molecule-1 and endothelial leukocyte adhesion molecule all of which can contribute to the pathogenesis of IBD. IL-1 is also a strong chemoattractant for leukocytes as demonstrated by the local accumulation of neutrophils at the site of injection of tissue with IL-1. It has been demonstrated that in intestinal lesions in patients with both CD and UC, IL-1 levels are elevated (25).

Inflammation and metabolism

Tissue metabolism is really affected by the presence of an inflammation process and a consequently immune response. The majority of the changes that happen during these processes are quite known, but some still need to be fully elucidated. These changes include local exhaustion of nutrients, increased oxygen consumption and generation of large quantities of reactive nitrogen and oxygen intermediate. These changes in tissue metabolism are, at least in part, due to the massive recruitment of inflammatory cell types, particularly myeloid cells such as neutrophils and monocytes. By contrast, adaptive immune responses are characterized high rates of local T and B cell proliferation and have significantly different metabolism requests (26). Moreover, the inflammatory response leads to mobilization of energy storages, causing the release of fatty acids by lipolysis, the release and degradation of glucose by glycolysis, and its release from glycogenolysis, and gluconeogenesis in the liver and the release of amino acids from muscle proteolysis; amino acids may be used in the liver as substrates for gluconeogenesis. Different cytokines seem to have a direct effect on metabolism; TNF and IL-1β, that ate responsible of the activation of lipolysis, inhibition of gluconeogenesis, and increase of vascular permeability to fluids and solutes, or IL-6 that induces changes in hepatic protein synthesis (4). TNF and IL-1β suppress the expression of GLUT2 and glucokinase in pancreatic β-cells, thus making them less sensitive to the blood glucose level. Consequently, β-cells produce less insulin given the same amount of plasma glucose. Between metabolites, also lactate seems to be affected by inflammation. Indeed it is one of the bridge-molecules between the liver and the muscle in energy source mobilization (27,28). Muscle wasting is one of the main consequences of this deployment of storages and it is worsened by the excess cortisol that impairs protein synthesis. This creates a negative nitrogen balance, which is the difference between nitrogen intake and loss. All this culminates in uncontrolled catabolism and resistance to anabolic substances, such as insulin, giving rise to hyperglycaemia (26).

The influence on metabolism carried on by the inflammatory process is particularly important when talking about the gastrointestinal tract. Indeed, this epithelium is composed of a very dynamic barrier that is complexly regulated to both accommodate the transport of nutrients and fluids and then select and exclude any antigens from the luminal interface. For all these reasons, the intestinal mucosa shows a unique metabolic profile that is governed by different stimuli, which include, for example, changes in the enteric microflora composition and in intestinal perfusion, which may be present even in steady-state conditions. It is also noteworthy that this metabolic profile is altered in conditions of active inflammation, such as those associated with inflammatory bowel disease (IBD) and has become an area of significant interest for research. The work of the last decade has revealed that the pathways regulated by hypoxia are strongly associated with barrier function in IBD and may contribute to the resolution of ongoing mucosal inflammation (29).

Metabolomics

Metabolomics is a comprehensive analysis of metabolites, in different biological samples. This analytical approach involves the study of intermediates and products of metabolism, such as carbohydrates, fatty acids, amino acids, nucleotides, organic acids, vitamins, antioxidants and many other classes of compounds (30). Metabolome, the complete set of metabolites of a sample, can be characterized at all levels of biological complexity: organisms, tissues, cells or cell compartments (31). Metabolomics allows studying the metabolic fingerprint and provides an integrated view of the organism (32). The metabolic information strongly depends on the physiological or the pathological state of organism, tissue or cells. The described metabolic phenotype might provide an insight into the biological status and it might be used as information about the organism (33). Considering that metabolites are the final product of the genic expression and the protein activity, among the other "omics" technologies, metabolomics is the research platform most closely related to the phenotype (34). Several metabolomics studies have been conducted with the aim to discriminate between different physiological and/or pathological conditions, based on their different metabolomes, making metabolomics approach useful to the discovery of biomarkers and the identification of the biochemical pathways involved in biological processes (35). The metabolomics analytical workflow can be divided into different crucial steps, from the sample collection to the metabolomic profile and data analysis (Figure 4). Sample collection is a critical step, which may determine the success of metabolomics experiments. There is a wide variety of biological samples that can be used for metabolomics studies, and the sample state (in term of typology, collection technique, sex, ages, diet) may affect the analysis, introducing variations or bias into the experiments (36). Furthermore, the sample preparation might remove the complexity or problems from the collection step. Indeed, the extraction processes may "clean" the sample and increase the specificity and sensitivity of the method.

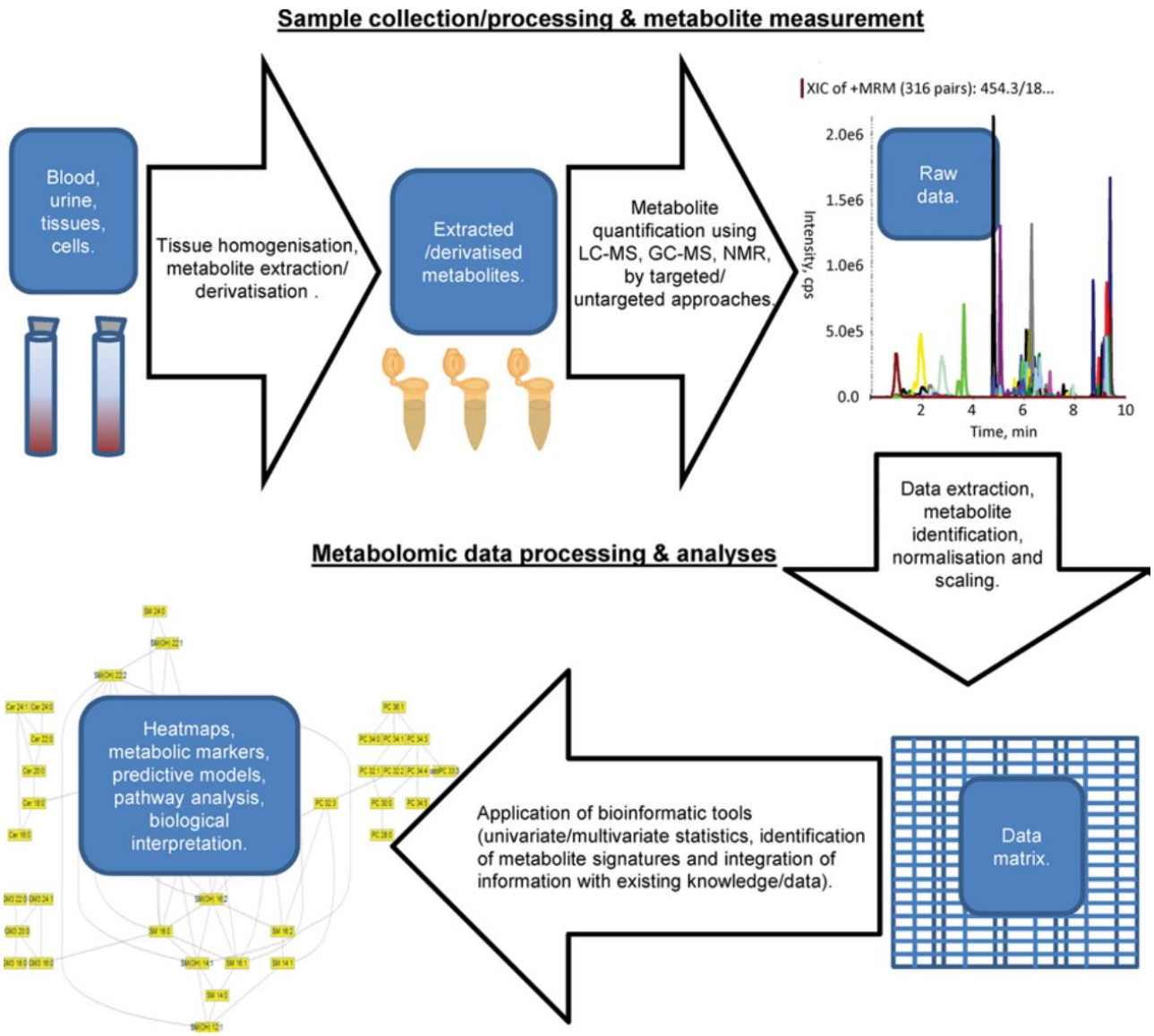

Figure 4. Typical workflow of metabolomic studies (37)**.**

The instrumental approach used to detect the metabolites within samples can be divided as well as either targeted or untargeted. The first approach, defines number and type of metabolites, maximizing the sensitivity; the second approach scans a wide range of metabolites inside the biological samples (31). For the instrumental analysis, different analytical techniques can be used, and the choice depends both on the type of the sample and on the type of required information (38). The analytical platforms typically involve either nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS), which usually is coupled with separation technologies, such as gas-

chromatography (GC-MS), liquid chromatography (LC-MS) and capillary electrophoresis (CE-MS) (39). Mass spectrometry employs a range of different mass analysers depending on the type of experiments, such as single quadrupole, triple quadrupole, time-of-flight mass spectrometer and ion traps. Among all the separation techniques, GC-MS and LC-MS are the most widely used in metabolomics approach, while recently CE-MS is getting more attention in this field (40). GC-MS allows the analysis of metabolites in the mass range of 30-1000m/z and its applications frequently rely on sample derivatization, which enhances the volatility of the metabolites and ensure the chemical separation (41,42). GC-MS presents different advantages, such as high separation efficiency and highly reproducible performance. A universal electron ionization (EI) is applied and it provides a standardized mass spectral fingerprint (43). One of the disadvantages is that some metabolites, after the required derivatization, are not very stable and they can be degraded during injection and separation (44,45). Moreover, GC-MS allows the analysis of volatile metabolites (volatilomics) produced by all living organisms (plants, animals, etc.), the analyses of those volatiles can offer, amongst others, the possibility of rapid diagnoses of patients using non-invasive techniques (46). In term of the type of MS, detectors for GC-MS analysis single quadrupole or a time-of-flight (TOF) are the most used. On the other hand, LC-MS has the advantage of requiring minimal sample preparation, without derivatization before the analysis. It is highly applicable to the analysis of a wide range of semi-polar compounds and it is highly sensitive. The traditional reverse-phase chromatography is used in the separation of non-polar and slightly polar molecules (47) whereas HILIC (Hydrophilic Interaction Liquid Chromatography) mode is becoming the most common technique for strongly retained species and polar metabolites (48). Several MS detectors can be used in LC-MS analysis, ranging from ultra-high resolution MS, such as Fourier Transform Ion Cyclotron resonance (FT-ICR) or Orbitrap FT, and high resolution MS (TOF) to low resolution MS (ion traps, triple quads) (49). One of the disadvantages of LC-MS is the ion suppression, as the ionization processes may depend on the presence of matrix compounds, and this effect is closely depending on the type of ionization (Electrospray Ionisation or Atmospheric Pressure Chemical Ionization). If ion

suppression is not assessed and corrected in an analytical method, the sensitivity of the LC–MS method can be seriously undermined, and the targeted analyte may be undetected even when using very sensitive instrumentation (50). Metabolite detection employs several options including single (MS) or tandem (MS/MS) mass analysers, which show different sensitivity and resolution performances (51). The MS/MS mode, which is characterized by an additional MS/MS fragmentation, offers more information about the identification and the structure of the metabolites. These methodological approaches allow the analysis of a huge number of metabolites in different types of samples, representing a powerful tool for the exploration of the metabolic mechanisms underneath the pathogenesis of diseases.

II. Aim of the study

The aim of this work is the evaluation of changes in metabolic processes occurring in the intestinal inflammation process. Particularly the attention was focused on inflammatory bowel diseases, IBD. As IBD are nowadays of mostly unknown aetiology, the possibility to detect changes in metabolism and to understand the meaning of these changes represents an intriguing perspective for the study and the better understanding of the development and persistence of IBD.

To achieve this goal, it was planned to analyse biological samples from IBD affected patients and compare them with healthy controls. To have a complete scenario of the metabolic changes happening during these pathological conditions the analysis was made on different types of samples: faeces, plasma and biopsies. Plasma and biopsy samples were analysed by GC-MS, while faeces sample with both GC-MS and ^{1}H-NMR. Moreover, a combined metabolomics and metagenomics approach was applied to faeces samples to understand the correlation between the metabolic changes and the different microbiota composition in IBD patients. For microbiota analysis, 16S rRNA gene sequencing data were produced from each bio-specimen by the Illumina Hi-Seq platform to reveal the gut microflora composition.

Finally, the results obtained with the human samples pointed out the attention particularly on the decreased levels of nicotinic acid found in the faeces of both UC and CD patients compared to healthy subjects. Since in the literature there is evidence that nicotinic acid seems to exercise an anti-inflammatory effect on other pathologies and cell lines, it was decided to study its effects on enterocytes. To study the nicotinic acid effect on intestinal inflammation, an in vitro model was built using differentiated Caco-2 cells in which inflammation was induced with the two major players in intestinal inflammation, LPS and IL-1β.

III. Materials and methods

Human study

Patients and healthy subjects

Patients were recruited at the Department of Gastroenterology of the University Hospital of Monserrato (CA), Italy. The ethics committee approved the project and each patient signed informed consent. The diagnosis of each patient admitted to the study, UC (n = 82) and CD (n = 50), was confirmed by endoscopic, histological and radiographic data. Disease activity was verified by well-established criteria, including endoscopic grading according to the CDEIS and Rutgeerts scores for CD patients, and the Mayo score for UC patients (52). Healthy volunteers (C) (n = 51) were recruited locally. The data of the healthy and sick volunteers enrolled in this study are summarized in Table 1 (Table 1). The exclusion criteria were age over 80 or under 20, recent use of antibiotics or probiotics and pregnancy. Each participant was provided with a sample collection kit with instructions. From each subject stool and plasma samples were collected, subsequently delivered to the laboratory within 3 hours. Only IBD patients underwent colonoscopy for disease assessment and had biopsies taken from endoscopically inflamed and normal non-inflamed tissues.

Groups		**Healthy**	**Crohn Disease**	**Ulcerative Colitis**
Age		40.7±13	48.8±13	47.3±12
Sex	*Male*	31	24	44
	Female	20	26	38
Therapy	*Mesalazine/Salazopyrin/Steroids*	-	8	54
	Azatioprine	-	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

Table 1. Summary of clinical and pathological data of the studied subjects.

Faeces samples

Frozen faeces (300mg) were extracted with 1 mL of cold methanol/water (80:20) containing Succinic acid-2,2,3,3-d4 as internal standard (Sigma-Aldrich, St. Louis, MO, USA) and then vortexed. The internal standard was used to estimate extraction recovery and efficiency. After 30 minutes incubation, samples were centrifuged at 16873 x g, for 10 minutes at 4°C. The supernatant (300µL)

were dried under vacuum with speed-vac over-night and were derivatised with 50µL of a solution of methoxamine in pyridine (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). After 1 h at 70°C, 100µL of N-Methyl-N-(trimethylsilyl)-trifluoroacetamide, MSTFA, (Sigma-Aldrich, St. Louis, MO, USA) were added and left at room temperature for one hour. Successively, samples were resuspended with 400µL of hexane (Sigma-Aldrich, St. Louis, MO, USA) and filtered with Acrodisc Syringe Filters with 0.45 mm PTFE Membrane (SIGMA, St. Louis, MO, USA). A pool of all samples was created and used as quality control (QC). The QC was injected every 10 samples to check the batch effect and no batch effect was evidenced in this analysis. For NMR analysis, dried hydrophilic faecal extracts were re-dissolved with 650µL 100mM KH2PO4/D2O buffer pH 7.2 (99,8%, Cambridge Isotope Laboratories Inc, Andover, USA) and added with 50µL of internal standard solution 5mM (sodium 3-trimethylsilyl-propionate-2,2,3,3,-d4, TSP, 98 atom % D, Sigma-Aldrich, Milan, Italy). An aliquot of 650µL was transferred to 5mm NMR tubes.

Plasma samples

The plasma was obtained from whole blood samples collected in tubes with EDTA from fasting IBD patients and healthy subjects and subsequently centrifuged at 775 x g for 5-10 minutes. The obtained plasma was stored at -80°C until use. Plasma samples were centrifuged at 1800 x g for 10 minutes at 4°C and 400µL of supernatant were extracted with a modified Folch method. Briefly, 600µL of methanol containing Succinic acid-2,2,3,3-d4 as internal standard (Sigma-Aldrich, St. Louis, MO, USA), 600µL of chloroform and 175µL of Milli-Q water were added to 400µL of each plasma sample. The internal standard was used to estimate extraction recovery and efficiency. After centrifugation at 1800 x g for 30 minutes at 4°C, the hydrophilic and lipophilic phases were separated. For GC-MS analysis, 150 µL of hydrophilic phase were brought to dryness by speed-vac (Eppendorf, NY, USA) O.N. and were derivatized with 50µL of a solution of methoxamine in pyridine (10mg/mL)

(Sigma-Aldrich, St. Louis, MO, USA). After 1 h at 70°C, 100µL of MSTFA (Sigma-Aldrich, St. Louis, MO, USA) were added. After 1 h at room temperature, the samples were resuspended with 150µL of hexane (Sigma-Aldrich, St. Louis, MO, USA) and filtered with syringes with Acrodisc filters with 0.45 mm PTFE membrane (SIGMA, St. Louis, MO, USA). A pool of all samples was created and used as quality control (QC). The QC was injected every 10 samples to check the batch effect and no batch effect was evidenced in this analysis.

Biopsies samples

Samples were extracted with 1 mL of cold methanol/water (80:20) containing Succinic acid-2,2,3,3-d4 as internal standard (Sigma-Aldrich, St. Louis, MO, USA) and then vortexed. The internal standard was used to estimate extraction recovery and efficiency. After 30 minutes, samples were centrifuged at 345 x g for 5 minutes at 4°C. The supernatant (700µL) was dried under vacuum with speed-vac O.N. Dried samples were derivatized with 50µL of a solution of methoxamine in pyridine (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). After 1 h at 70°C, 100µL of N- MSTFA (Sigma-Aldrich, St. Louis, MO, USA) were added and left at room temperature for one hour. Successively, samples were resuspended with 50µL of hexane (Sigma-Aldrich, St. Louis, MO, USA). A pool of all samples was created and used as quality control (QC). The QC was injected every 10 samples to check the batch effect and no batch effect was evidenced in this analysis.

Gut microbiota analysis

DNA extraction from thawed faecal samples was performed using the QIAamp DNA stool MiniKit according to the instructions of the manufacturer (Qiagen), with minor modifications. Quantitative PCR (qPCR) was performed using degenerate primers encompassing the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene. Barcoded amplicon libraries for the analysis on the Illumina MiSeq platform were generated using degenerate primers targeting the V3 and V4 hypervariable region of the bacterial 16S rRNA gene and Nextera XT index kit (Illumina). Analysis of the data

generated on the Miseq System was carried out using the BaseSpace 16S Metagenomics App (Illumina), whereas operational taxonomic unit (OTU) mapping to the Greengenes database (V.13.8) was performed using the Quantitative Insights Into Microbial Ecology (QIIME) platform (V.1.8.0) (53). Sequences containing ambiguous or low-quality bases were filtered out using QIIME filter. Remaining sequences were assigned to each sample according to the unique barcodes. Alpha diversity was estimated using Shannon index metric.

In vitro study

Cell culture

Caco-2 cells (ECACC Salisbury, Wiltshire UK) were cultured in Dulbecco's Modified Eagle's Medium Low Glucose with L-Glutamine with Sodium Pyruvate (Euroclone, Milan, Italy) supplemented with 10% heat-inactivated bovine serum, 100 U/ml penicillin, and 100 mg/mL streptomycin (Euroclone, Milan, Italy), in monolayers at 37 °C in a humidified atmosphere of 5% CO2, replacing the medium twice a week.

MTT viability test

The MTT assay was assessed on Caco-2 cells in order to evaluate any toxic activity of the tested compounds. Cells were seeded in 96-well plates (5×10^4 viable cells/mL in 100µL), exposed to various concentrations of the compounds alone (LPS, IL-1β and NA) and incubated for 48h. After incubation, the medium was removed and 100µL of MTT solution (2.5 mg/mL in fresh medium) were added and left for 2h at 37 °C. The medium was then aspirated, 100µL of dimethyl sulfoxide (DMSO) were added in each well and the absorbance was read at 570 nm by using a microplate reader (Infinite 200, Tecan, Salzburg, Austria).

Cell culture treatment

For experimental studies, Caco-2 cells were plated in Petri dishes and used 15–17 days post seeding when fully differentiated. To induce the inflammation process, cells were treated with two different compounds: LPS (Sigma-Aldrich, Milan, Italy) and IL-1β (Merck, Rome, Italy), 50 µg/mL and 25 ng/mL respectively and incubated for 48h. To check the effect of Nicotinic Acid (NA) (Sigma-

Aldrich, Milan, Italy) on inflammation, cells were pre-treated for 24h with NA at two different concentrations (100 and 200 µg/mL) and then treated with both NA plus LPS and NA plus IL-1β for 48h. At the end of the exposure time, cells and respectively media were collected.

Evaluation of IL-8 protein levels

An aliquot of 150µL of cell culture medium was collected from the Petri dishes and used for ELISA detection. Levels of IL-8 were quantified using the Human IL-8 ELISA kit (CliniScinces, Nanterre, France) and following the manufacturer's instructions. Sample absorbance values were read at 450 nm in a microplate reader (Infinite 200, Tecan, Salzburg, Austria).

Cell culture samples preparation for metabolomics analysis

In order to perform metabolomics analysis, the growth medium was removed from the Petri dishes and aliquoted (500µl) in Eppendorf tubes to be extracted as the cells. Cells were washed with 3mL of physiological solution (150 mM NaCl), and then extracted with 1.2mL of cold methanol/water (80:20) containing Succinic acid-2,2,3,3-d4 as internal standard (Sigma-Aldrich, St. Louis, MO, USA) and were shaken for 15 minutes at low temperature (4°C). The internal standard was used to estimate extraction recovery and efficiency. Then cells were harvested by scraping and transferred in Eppendorf tubes. To ensure the complete lysis of the cells, the extraction was combined with 10 minutes of ultrasonic treatment at a controlled temperature (4°C). The growth medium was extracted as described above. Briefly, 500µl of medium were aliquoted in Eppendorf tubes and centrifuged at 4500 rpm, 10 minutes, 4°C, to remove death-floating cells. Then 1.2mL of cold methanol/water with the internal standard (80:20) was added to the supernatant of the medium and the extraction procedure followed as described for the cells. Cell suspensions and growth medium were centrifuged at 4500 rpm for 30 minutes at 4°C. For GC-MS analysis, 400uL of supernatant was aliquoted and dried in an Eppendorf™ Concentrator Plus overnight. Dried pellets were derivatised with 50µL of a solution of

methoxamine in pyridine (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). After 1h at 70°C, 50µL of MSTFA (Sigma-Aldrich, St. Louis, MO, USA) were added and left at room temperature for one hour. Successively, 50µL of hexane were added and samples were transferred in a vial for the GC-MS analysis. A pool of all samples was created and used as quality control (QC). The QC was injected every 10 samples to check the batch effect and no batch effect was evidenced in this analysis. For ^{1}H-NMR analysis, 700µL of supernatant was aliquoted and dried in an Eppendorf™ Concentrator Plus overnight. Dried hydrophilic cells and medium extracts were re-dissolved in 690µL of potassium phosphate buffer in D2O (100mM, pH 7.4) and 10µL of TSP (sodium 3-trimethylsilyl-propionate-2,2,3,3,-d4) as chemical shift reference (δ 0.0) (98 atom % D, Sigma-Aldrich, Milan). An aliquot of 650µL was analysed by 1H-NMR.

Protein extraction and quantification

Total proteins were extracted with the Bradford assay (Bradford 1976), with some modifications. Cell pellets were added with 500µl CellLytic M lysis buffer (Sigma-Aldrich, Milan, Italy) for protein extraction, added with mammalian protease and phosphatase inhibitor cocktail (1:100 v/v). Cells were scraped on ice and lysates were incubated for 15 min on ice before centrifugation at 12500 g at 4°C for 7 minutes and then stored at -20°C prior quantification. The calibration curve was generated using bovine serum albumin (BSA) in CellLytic M reagent, at different standard concentrations (0.1mg/ml-5mg/ml). Bradford Reagent solution, 500µl, was incubated with either 10µl of standard or protein sample at room temperature for 5 minutes. The absorbance was measured at 595nm using a microplate reader (Infinite 200, Tecan, Salzburg, Austria) at a controlled temperature of 37°C. The equation of the line of the standard curve was used to calculate the protein concentration of the cell samples. The total protein content together with the total area of chromatograms and spectra were used to normalise the metabolites measurements of each cell and medium sample.

Gas chromatography-mass spectrometry analysis

One microliter of derivatised sample was injected splitless into a 7890A gas chromatograph coupled with a 5975C Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m ×0.25 mm ID, fused silica capillary column, with a 0.25 μM TG-5MS stationary phase (Thermo Fisher Scientific, Waltham, MA, USA). The injector and transfer line temperatures were at 250°C and 280°C, respectively. The gas flow rate through the column was 1 ml/min. For faeces, biopsies and cell culture analysis, the column initial temperature was kept at 60 °C for 3 min, then increased to 140°C at 7°C/min, held at 140°C for 4 min, increased to 300°C at 5°C/min and kept for 1 min and EI spectra were acquired at 70eV energy. For plasma samples analysis, the column initial temperature was kept at 50 °C for 3 min, then increased from 50°C to 250°C at 10°C/min and held at 250°C for 12 minutes and EI spectra were acquired at 70eV energy. Identification of metabolites was performed using the standard NIST 08 and GMD mass spectra libraries and, when available, by comparison with authentic standards. Peak detection and deconvolution, filtering and normalization were performed using a dedicated pipeline on Knime (54).

^{1}H-NMR measurements

^{1}H-NMR measurements of samples were carried out using a Varian UNITY INOVA 500 spectrometer operating at 499.839 MHz for proton and equipped with a 5 mm double resonance probe (Agilent Technologies, CA, USA). ^{1}H-NMR spectra were acquired at 300 K with a spectral width of 6000 Hz, a 90° pulse. For faeces samples, the acquisition time was 2s, the relaxation delay of 2s and free induction decay (FID) was 256. For cells and medium samples, the acquisition time was of 1.5s, the relaxation delay was 2s and for each sample, 512 FID were collected into 64K data points. The residual water signal was suppressed by applying a presaturation technique with low power radiofrequency irradiation for 2s. After Fourier transformation with 0.3 Hz line broadening and a zero-filling to 64 K, ^{1}H-NMR spectra were manually phased and baseline corrected using ACD Lab Processor Academic Edition (Advanced Chemistry Development, 12.01, 2010). Spectral chemical shift referencing on the TSP CH3 signal at 0.00 ppm was performed on all spectra. Metabolites were identified and quantify of each NMR spectra of samples using the Chenomx NMR Suite 7.1 (Chenomx Inc., Edmonton, Alberta, Canada) (55). Chenomx NMR Suite is an integrated set of tools for identifying and quantifying metabolites in NMR spectra. It is equipped with reference libraries that contain numerous pH-sensitive compound models that are identical to the spectra of pure compounds obtained under similar experimental conditions (five second of recycle delay). Essentially, a Lorentzian peak shape model of each reference compound is generated from the database information and superimposed upon the actual spectrum. The linear combination of all modelled metabolites gives rise to the total spectral fit, which can be evaluated with a summation line.

Statistical Analysis

Multivariate statistical analysis

Multivariate statistical data analysis was performed using SIMCA (version 14.0, Umetrics, Umea, Sweden). Raw data from both ^{1}H-NMR and GC-MS techniques were processed and organized in a matrix for the multivariate statistical analysis. To account for variable dilution factors of metabolite concentrations, faeces datasets were normalized with median-fold change normalization, biopsies and plasma datasets to the total area of chromatograms. All imported data were scaled for the multivariate statistical analysis, using Pareto scaling for ^{1}H-NMR data and UV-scaling for GC-MS data. A Principal component analysis (PCA), an unsupervised analysis, coupled with Hotelling t-squared was used to identify the presence of outlier. PCA is characterized by a linear transformation, which preserves as much of the variance in the original data (56). Subsequently, a supervised analysis, Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA), was performed to classify samples and elucidate metabolites able to differentiate the classes (57).

Univariate statistical analysis

GraphPad Prism software (version 7.01, GraphPad Software, Inc., CA, USA) was used to perform the univariate statistical analysis of the data and Spearman correlations between the microbiome and the metabolome. To verify the significance of metabolites U-Mann Whitney and Student t-Test were performed.

IV. Results

Human study

Faeces Metabolomics analysis

Faeces obtained from 132 IBD patients (50 CD and 82 UC) and 51 healthy subjects were analysed with both GC-MS and ^{1}H-NMR. Eighty metabolites were identified with GC-MS and thirty with ^{1}H-NMR. The OPLS-DA score plots obtained with MVA of GC-MS and ^{1}H-NMR are shown in figure 5. It is clearly demonstrated a separation between CD patients and healthy controls (Figure 5a,c) and between UC patients and healthy controls (Figure 5b,d) with both GC-MS and ^{1}H-NMR analysis. The quality of the models was evaluated using the corresponding Partial Least Square Discriminant Analysis (PLS-DA) models using a 7-fold cross-validation and permutation test over 400 times (Table 2). To investigate if we could distinguish between the two pathological conditions, new OPLS-DA models were created (Figure 6a,b). Contrarily, The classification model was unable to separate between CD and UC, indicating an intrinsic similarity in the metabolic profiles of these two diseases (Table 2). Discriminant metabolites were highlighted by the means of an S-plot and a Mann-Whitney test was carried out to find significant differences between the classes. Spectral resonances were assigned to individual metabolites based on data published in the literature (58,59) and using the 500 MHz library from Chenomx NMR suite 7.1 (Chenomx Inc., Edmonton, Alberta, Canada).

Eighteen metabolites were detected to be responsible for the separation of the CD patients from the healthy control group (Figure 7). Of these, nine were significantly increased in CD patients (alanine, beta-alanine, phenylacetic acid, 4-hydroxyphenylacetic acid, glyceric acid, phenylethylamine, putrescine and cadaverine and phenylalanine) and nine metabolites were significantly decreased in CD patients (nicotinic acid, pantothenic acid, 3-methyladipic acid, 5β-coprostanol, 3-hydroxybutyric acid, hydrocinnamic acid, 2-hydroxy-3-methylvaleric acid, citric acid and methylamine).

Regarding the comparison between UC patients and healthy controls, twenty-three metabolites were able to discriminate between the UC and healthy subjects (Figure 8). Nine metabolites were significantly increased in UC patients (beta-alanine, glycine, 4-hydroxyphenylacetic acid, glucose,

cadaverine, 5-aminovaleric acid, trimethylamine-N-oxide (TMAO), tyramine and phenylalanine), while 14 metabolites were significantly decreased in UC patients (nicotinic acid, pantothenic acid, 3-methyladipic acid, pyroglutamic acid, 5β-coprostanol, 3-hydroxybutyric acid, hydrocinnamic acid, linoleic acid, sebacic acid, tricarballylic acid, 2-hydroxy-3-methylvaleric acid, glutamic acid, citric acid and methylamine). Thirteen were in common with the CD patients-healthy controls comparison, precisely: beta-alanine, 5β-coprostanol, cadaverine, methyladipic acid, pantothenic acid, nicotinic acid, 3-hydroxybutyric acid, 2-hydroxy-3-methylvaleric acid, methylamine, phenylalanine, citric acid, hydrocinnamic acid and 4-hydroxyphenylacetic acid. For this reason, with both GC-MS data and ^{1}H-NMR, it was not possible to distinguish between the two pathological conditions (UC and CD) (Table 2). The altered metabolites participate to different metabolic pathways, such as phenylalanine and beta-alanine metabolism, are shown in figure 9 a and b for CD and UC respectively. The effects of the different therapies were checked as confounding factors, but there were no influences of the therapy on the metabolome here detected.

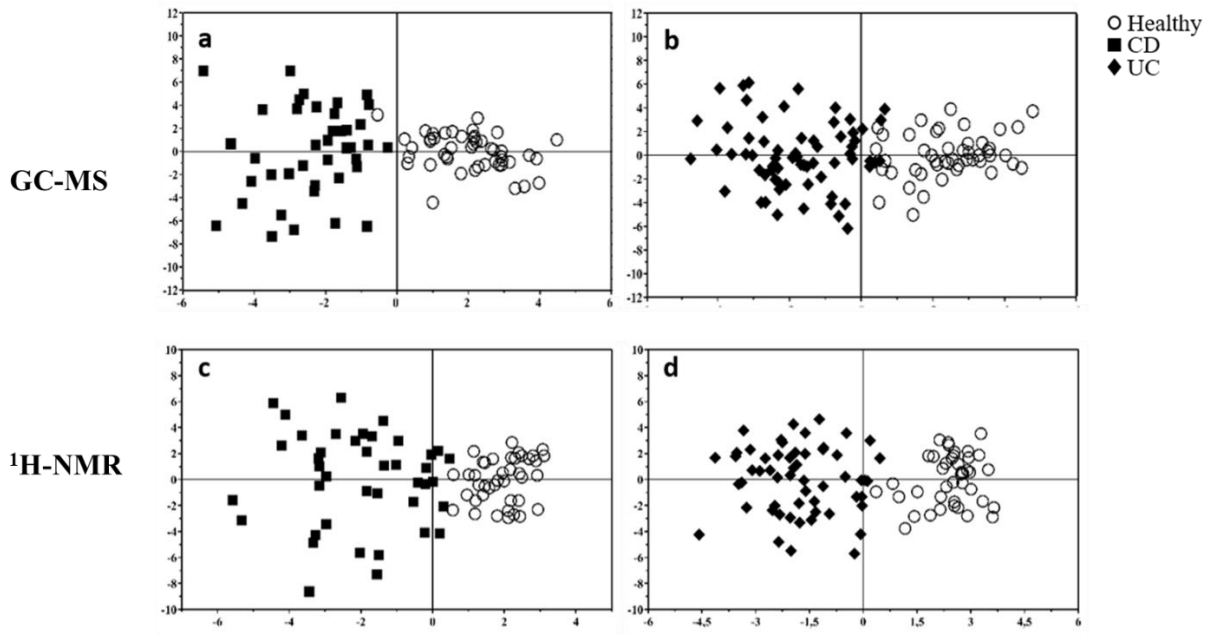

Figure 5. OPLS-DA score plots of faeces samples metabolome analysed by GC-MS (a,b) and ^{1}H-NMR (c,d). In the first column CD vs healthy comparisons are shown while the second column of plots contains UC vs healthy comparisons.

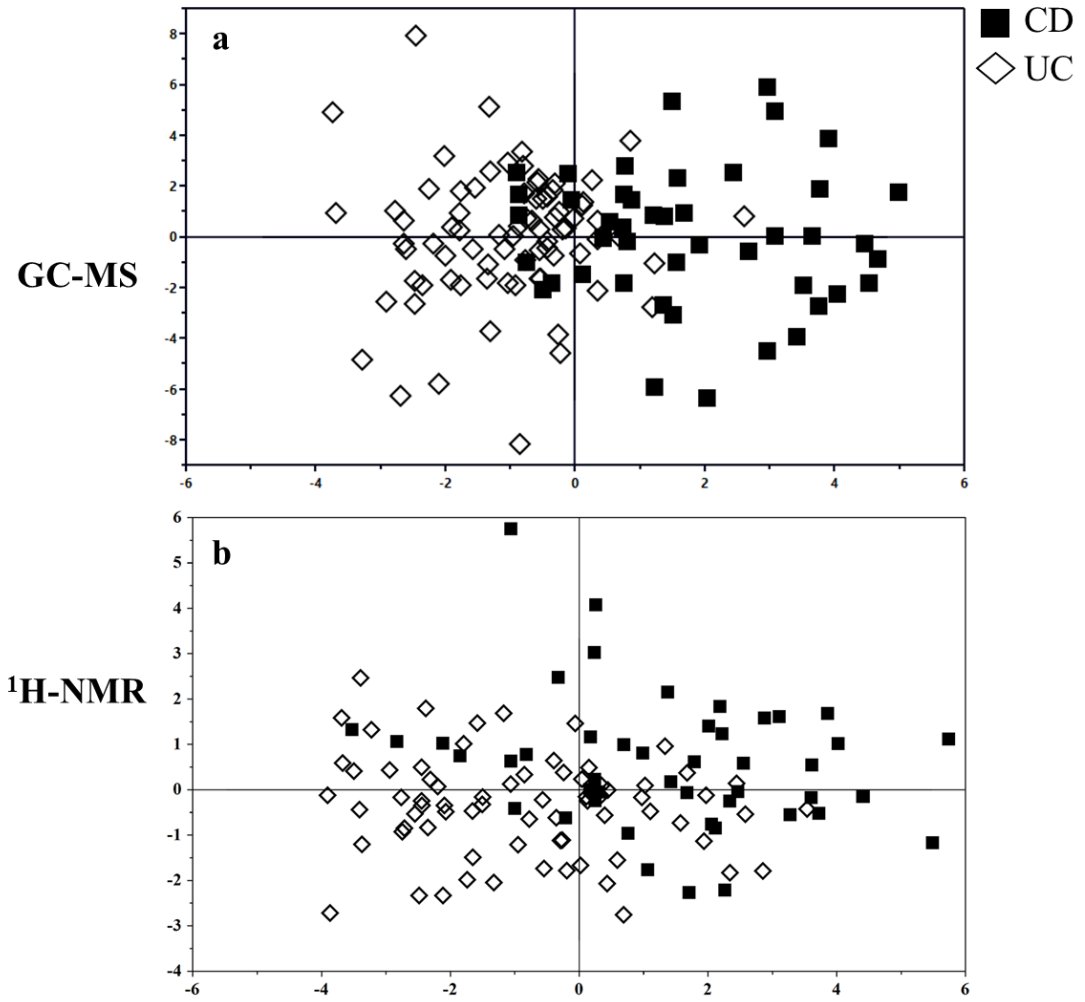

Figure 6. OPLS-DA score plots of faeces samples metabolome analysed by GC-MS (a,b) and ^{1}H-NMR (c,d). (2). CD vs UC comparisons.

	OPLS-DA models				Permutation*	
	GC-MS					
Groups	**Components[a]**	**R_2Xcum[b]**	**R_2Ycum[c]**	**Q_2cum[d]**	**R_2 intercept**	**Q_2 intercept**
Healthy *vs* IBD	1P+1O	0.112	0.663	0.439	0.333	-0.228
Healthy *vs* CD	1P+1O	0.144	0.778	0.519	0.478	-0.215
Healthy *vs* UC	1P+1O	0.119	0.734	0.504	0.408	-0.239
CD *vs* UC	1P+1O	0.105	0.494	0.036	0.419	-0.201
	^{1}H-NMR					
Healthy *vs* IBD	1P+3O	0.495	0.754	0.626	0.188	-0.430
Healthy *vs* CD	1P+1O	0.314	0.730	0.645	0.231	-0.241
Healthy *vs* UC	1P+2O	0.458	0.805	0.688	0.181	-0.327
CD *vs* UC	1P+1O	0.202	0.440	0.140	0.257	-0.235

Table 2. Faeces samples MVA parameters. [a] The number of Predictive and Orthogonal components used to create the statistical models.

[b,c] R_2X and R_2Y indicated the cumulative explained fraction of the variation of the X block and Y block for the extracted components.

[d] Q_2cum values indicated cumulative predicted fraction of the variation of the Y block for the extracted components.

[*] R_2 and Q_2 intercept values are indicative of a reliable model. The Permutation test was evaluated on the corresponding partial least square discriminant analysis (PLS-DA) model.

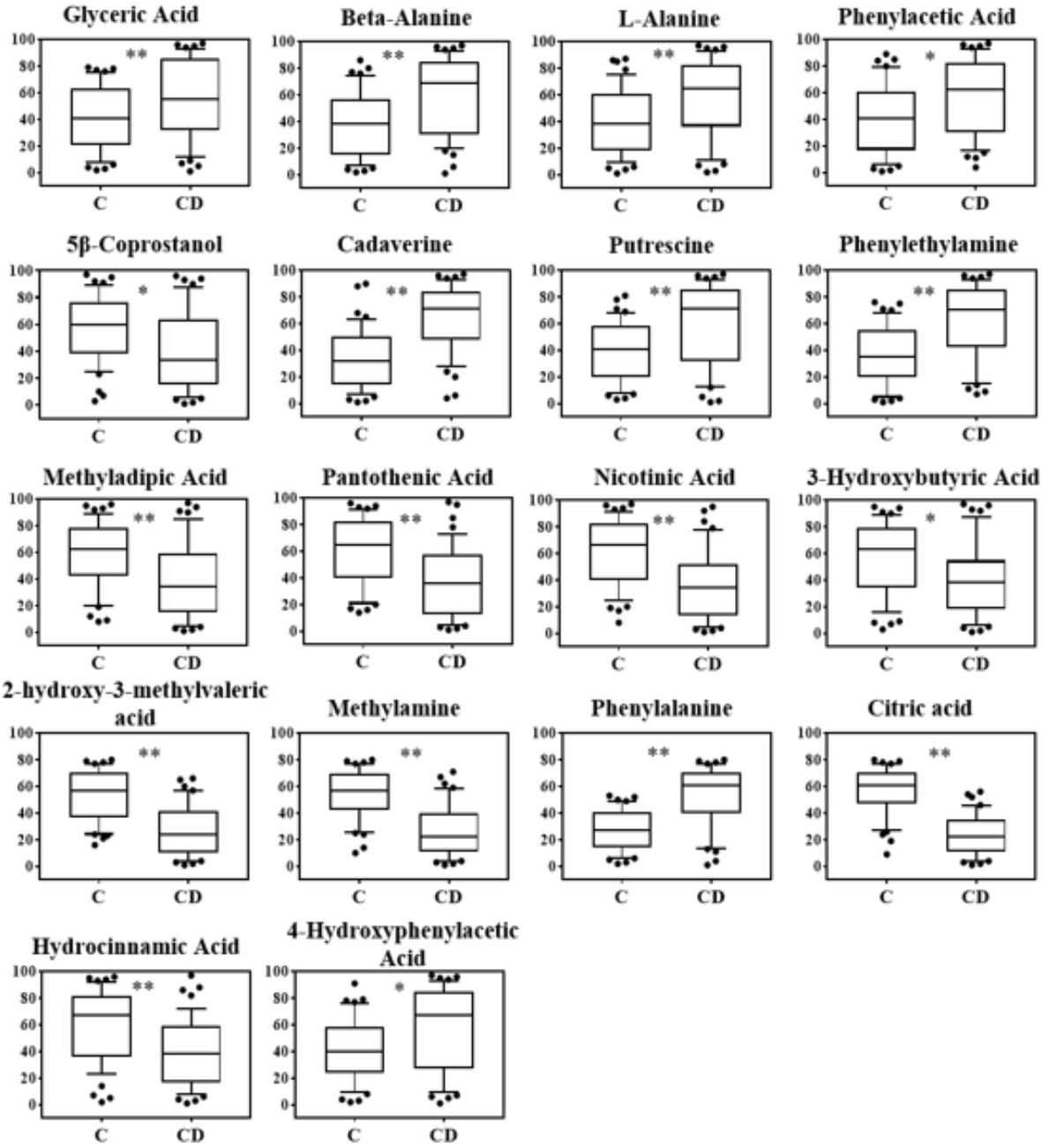

Figure 7. Statistically significant metabolites in CD patients vs healthy subjects (C). Discriminant metabolites obtained with the MVA, underwent to a Mann-Whitney test to determine which metabolites were statistically significantly variated. The resulted metabolites are shown and expressed in the graphs y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted). * and ** indicates levels of significance with $p < 0.05$ and <0.01, respectively.

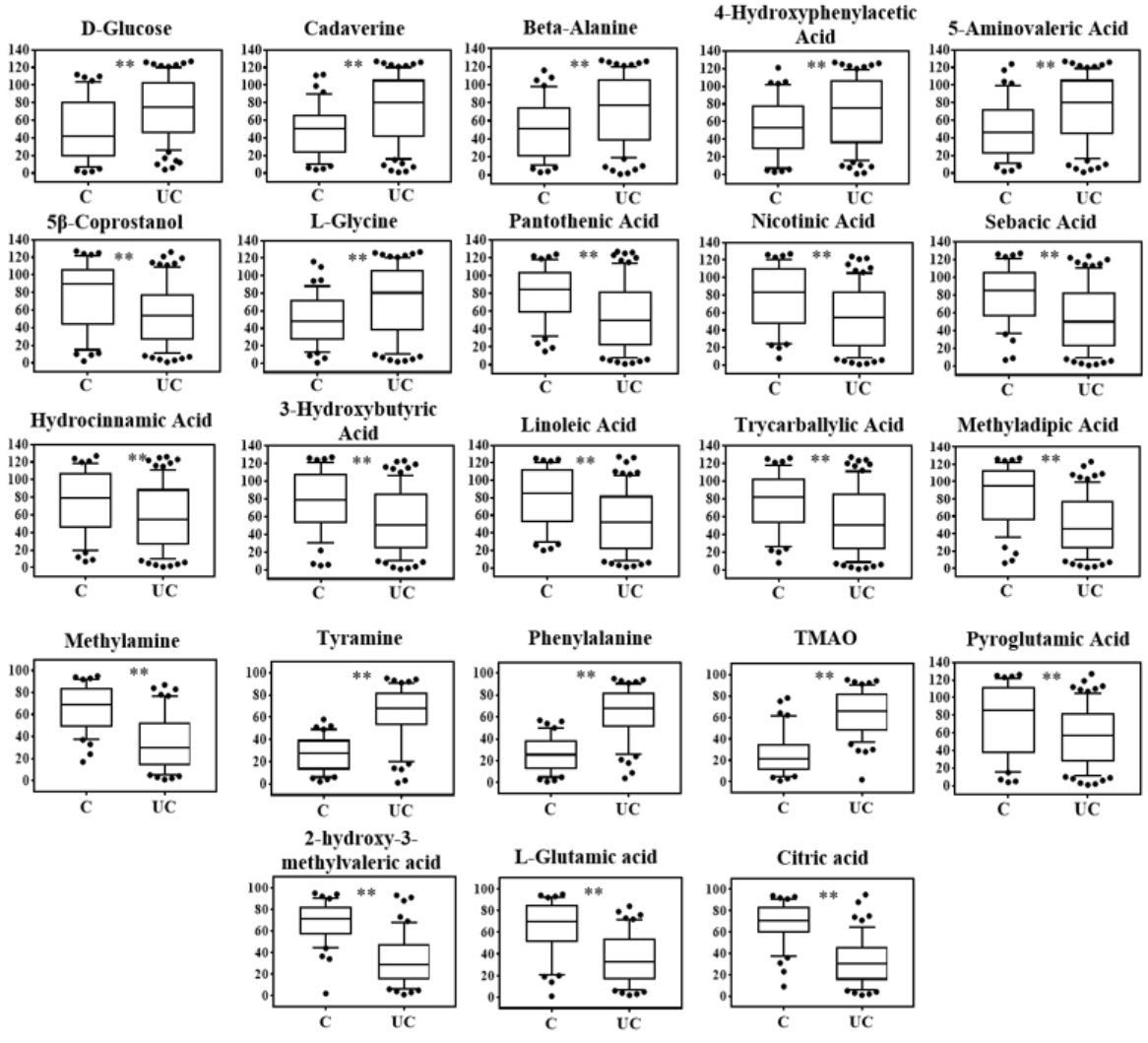

Figure 8. Statistically significant metabolites in UC patients vs healthy subjects (C). Discriminant metabolites obtained with the MVA, underwent to a Mann-Whitney test to determine which metabolites were statistically significantly variated. The resulted metabolites are shown and expressed in the graphs y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted). * and ** indicates levels of significance with $p < 0.05$ and < 0.01, respectively.

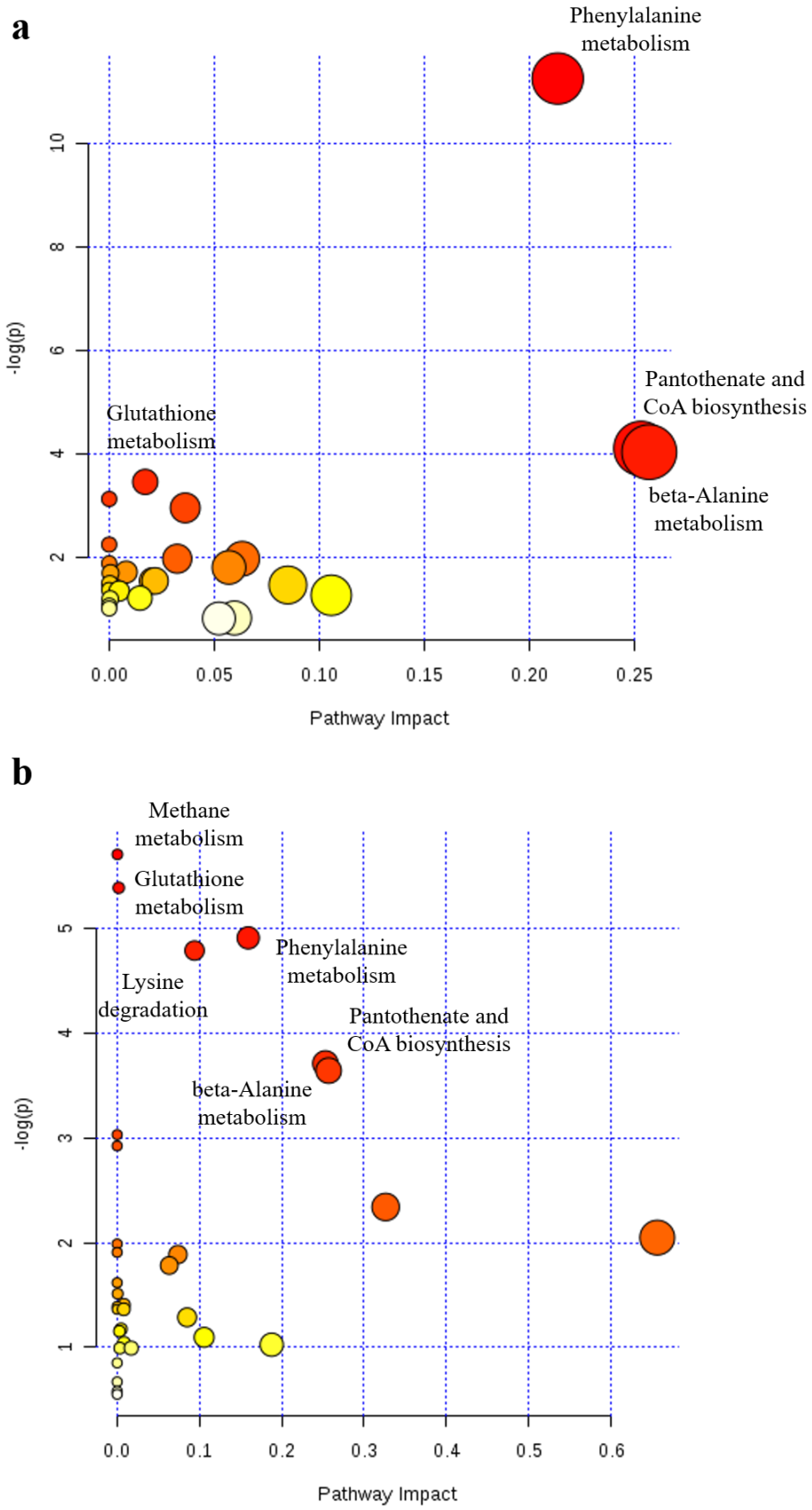

Figure 9. Relevant metabolic pathways involved in CD (a) and UC (b). Plots were obtained using Metaboanalyst

Faeces microbiota analysis

A total of 18,899,323 sequencing reads were obtained from the 183 faecal samples. The numbers of operational taxonomic units (OTU) varied from 4,901 to 672,146. The mean differences in the α-Shannon values between the CTLs, the IBD, and the CD and UC groups were always statistically different (Figure 10). The majority of the OTU sequences were assigned to seven main phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, Cyanobacteria and Fusobacteria. In Figure 10 the relative abundance and frequency of the OTUs at the phylum level are shown for those OTUs that have at least 0.1% abundance. In the IBD group compared to the CTLs group, Firmicutes (46.39% *vs* 38.99%), Proteobacteria (10.49% *vs* 6.54%), Verrucomicrobia (1.90% *vs* 1.02%), and Fusobacteria (0.72% *vs* 0.19%) were significantly increased, whereas Bacteroidetes (33.63% *vs* 47.45%) and Cyanobacteria (0.73% *vs* 0.51%) were decreased. The Firmicutes:Bacteroidetes ratio was 0.82 in the CTLs group, but 1.38 in IBD patients (Fig. 10a).

Only Bacteriodetes (32.83% *vs* 47.45%), Proteobacteria (13.67% vs 6.54%), Verrucomicrobia (3.76% *vs* 1.02%) and Cyanobacteria (0.38% vs. 0.73%) showed the same evidence of variation between CD and healthy subjects, while in UC patients only Proteobacteria did not attain the same significant difference when compared with healthy subjects. Additionally, in this group, Actinobacteria phylum (8.42% *vs* 2.26%) was significantly increased compared to CTLs (Fig. 10b,c).

At the genus level, *Escherichia*, *Faecalibacterium*, *Streptococcus*, *Sutterella* and *Veillonella* were all significantly increased in the IBD group compared to controls, whereas *Bacteroides*, *Flavobacterium*, and *Oscillospira* genera all decreased. In CD patients, only *Escherichia* and *Veillonella* were significantly increased respect to healthy subjects, and, among the genera, *Prevotella* decreased significantly. Interestingly, in this group, the *Faecalibacterium* genus decreased compared with the control group, and contrary to what was observed in IBD as a whole and in the UC group. In UC patients, an increase of the *Faecalibacterium* and decrease of the *Oscillospira* genera, respectively, were not statistically significant (Figure 11).

Finally, in Figure 12, the distribution of the OTUs at the species level is shown for those species that appeared increased and decreased in the CD and UC groups, respectively. In the CD group, 25 species were increased, and 22 species were decreased, respectively, whereas in the UC group, 18 species were increased, and 17 species appeared reduced, respectively. Amongst Proteobacteria, *Escherichia alberti* resulted much more abundant than in controls (4.61% *vs* 0.24% in CD; 3.28% *vs* 0.24% in UC), and, among Bacteroidetes, *Prevotella copri* was the main species reduced (0.02% *vs* 3.91% in CD; 0.68% vs 3.91% in UC) in both patient groups. Neither the diet nor the activity of disease did affect the microbiome composition.

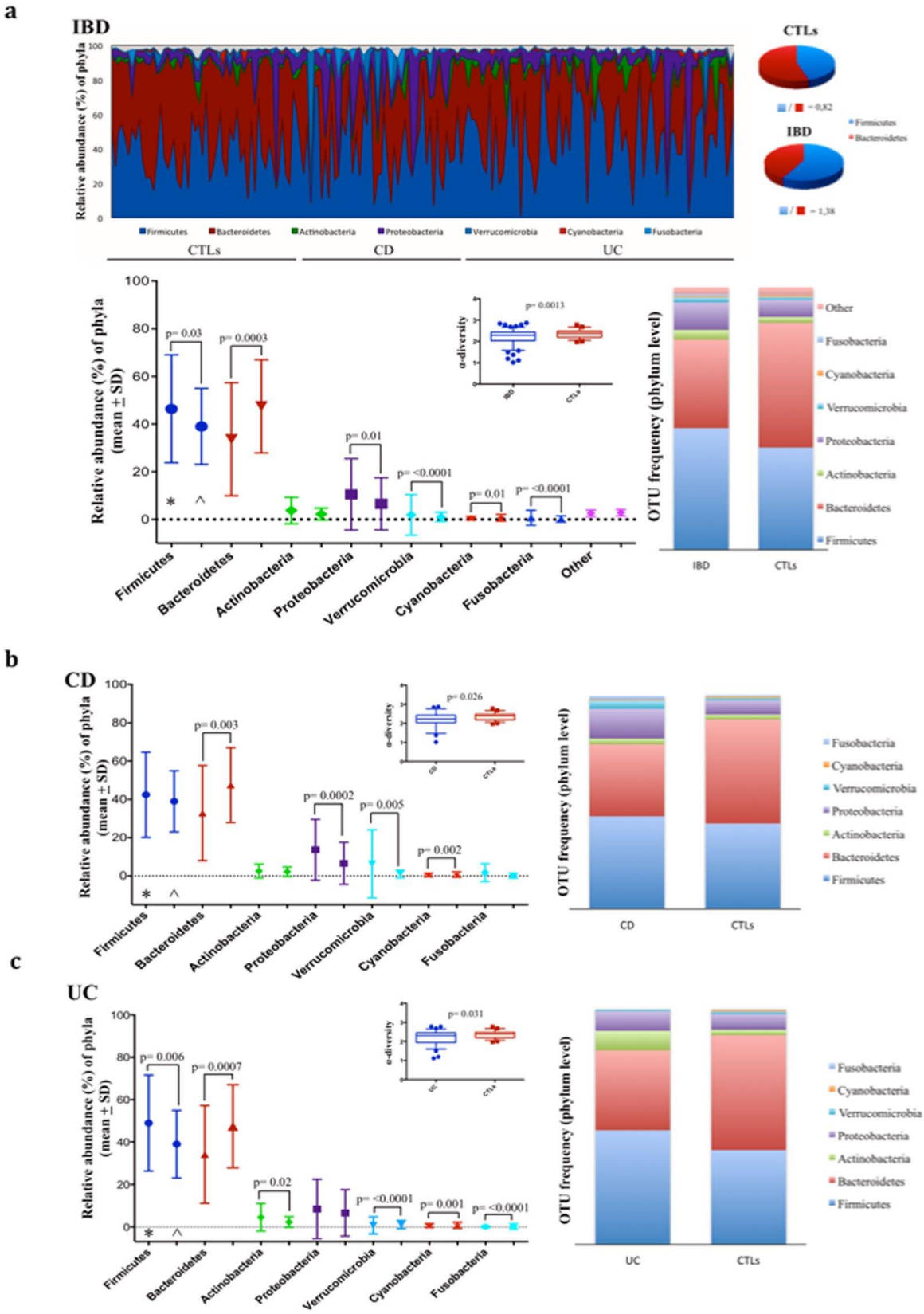

Figure 10. Microbiome taxonomic composition in IBD, CD, UC patients and control subjects (CTLs). Relative abundance at OTU frequency at phylum level, Firmicutes/Bacteroidetes ratio, and α-diversity are shown. The data are filtered by a frequency higher than 0.1% (a). Relative abundance of phyla and OTU frequency are shown in CD (*) (b) and UC (*) (c) patients compared to controls (CTLs, ^). Significant differences with p <0.05 are shown. *= patients; ^= controls.

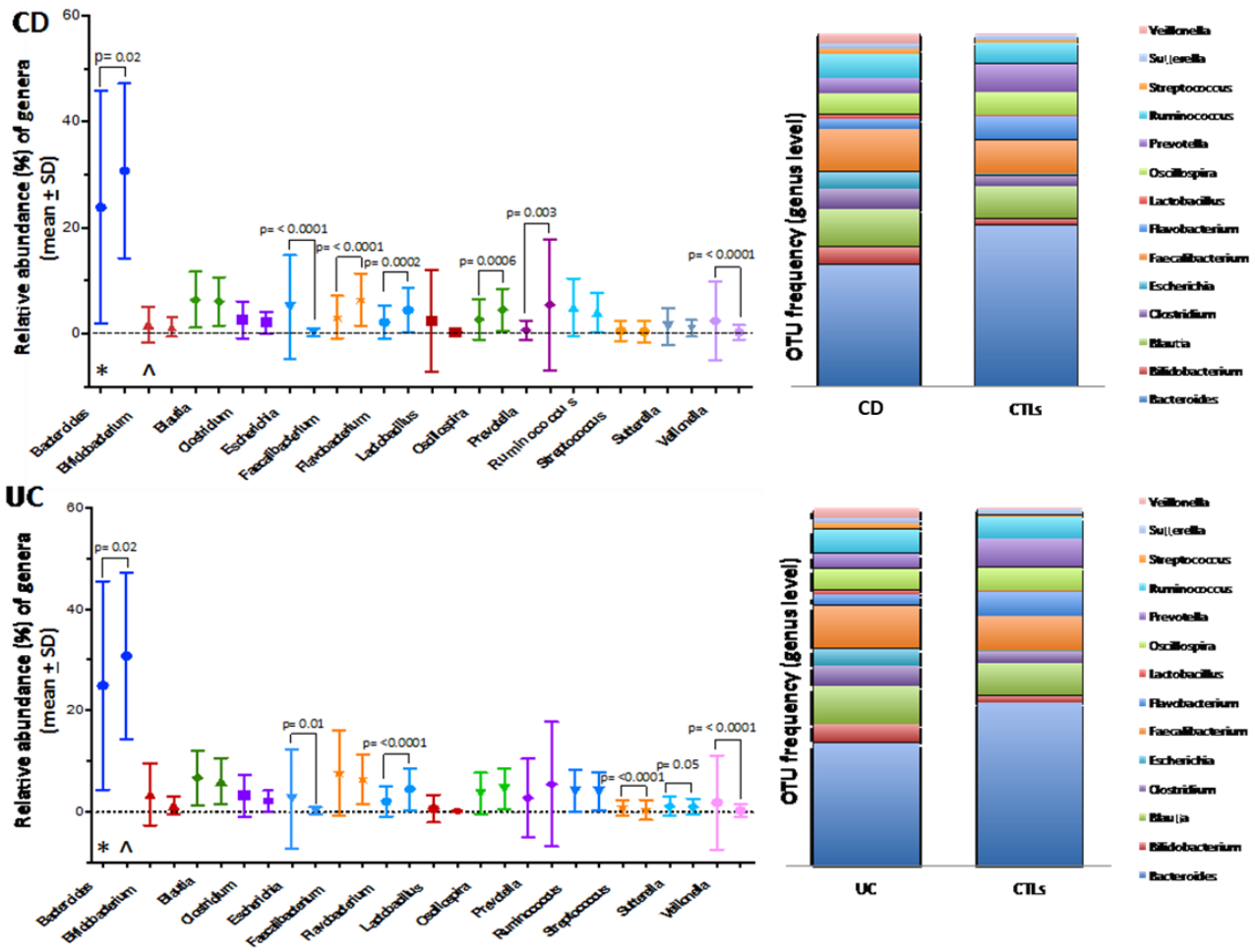

Figure 11. Microbiome taxonomic composition at genus level in CD, UC and controls subjects. Relative abundance of genera and OTU frequency are shown in CD (*) and UC (*) patients compared to controls (CTLs). Significant differences with p <0.05 are shown.

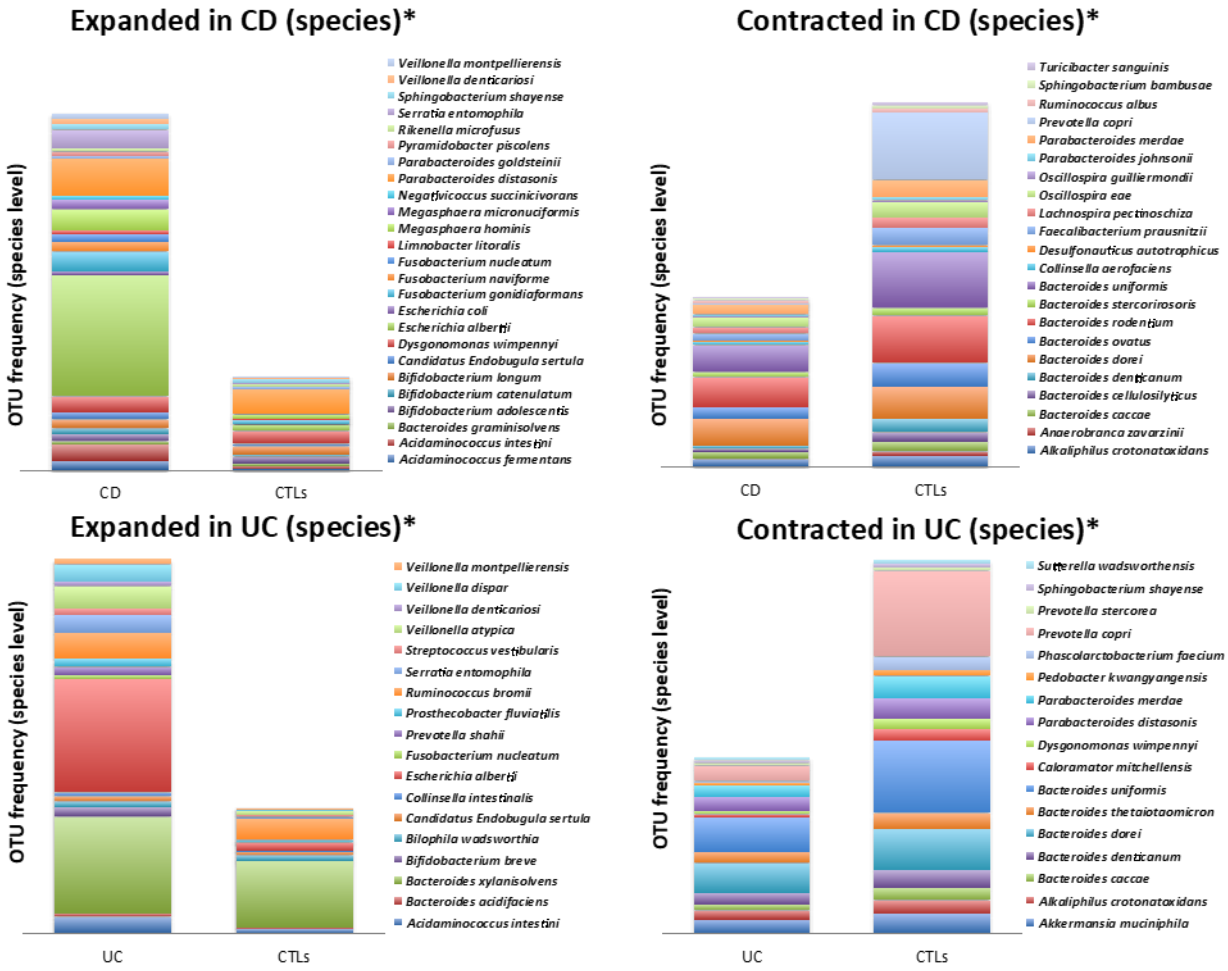

Figure 12. Relative abundance of species in CD e UC patients compared to controls subjects. OTU frequency of species higher than 0.01% are indicated as expanded or contracted in patients groups and controls subjects. * indicates that only levels of significance with $p < 0.05$ for the species indicated are shown.

Correlation between metabolome and microbiome

The observation was restricted to the microbial genera whose relative abundance resulted in being statistically different among the different groups of subjects and to metabolites that were characterized by the highest discriminative power among the patients and healthy controls.

The Spearman correlation analysis revealed a strong association between five bacterial genera with 10 discriminant metabolites in CD patients (Figure 13a and more in details 14a). The most correlated genus was *Oscillospira*, particularly with hydrocinnamic acid, 3-methyladipic acid, 5β-coprostanol, citric acid, methylamine and 2-hydroxy-3-methylvaleric acid (all positive correlations), while the genus was negatively correlated with cadaverine and putrescine. In addition, the *Faecalibacterium* genus was negatively correlated with the two biogenic amines, cadaverine and putrescine, while only nicotinic acid was positively correlated. The *Escherichia* genus was negatively correlated with nicotinic acid and citric acid and positively correlated with cadaverine. Lastly, a positive correlation was detected between the *Flavobacterium* genus and 5β-coprostanol and between the *Veillonella* genus and cadaverine.

The correlation between metabolites and microbiota was less strong for UC than for CD patients (Figure 13 b and more in details 14b). Only three bacterial genera were correlated with six metabolites. Particularly, a strong positive correlation was observed between *Flavobacterium* genus and 3-methyladipic acid, 2-hydroxy-3-methylvaleric acid, citric acid and methylamine, this genus was negatively correlated with trimethylamine-N-oxide (TMAO). UC patients also presented correlations between *Oscillospira* genus and 3-methyladipic acid, 2-hydroxy-3-methylvaleric acid and citric acid, and between *Veillonella* genus and citric acid.

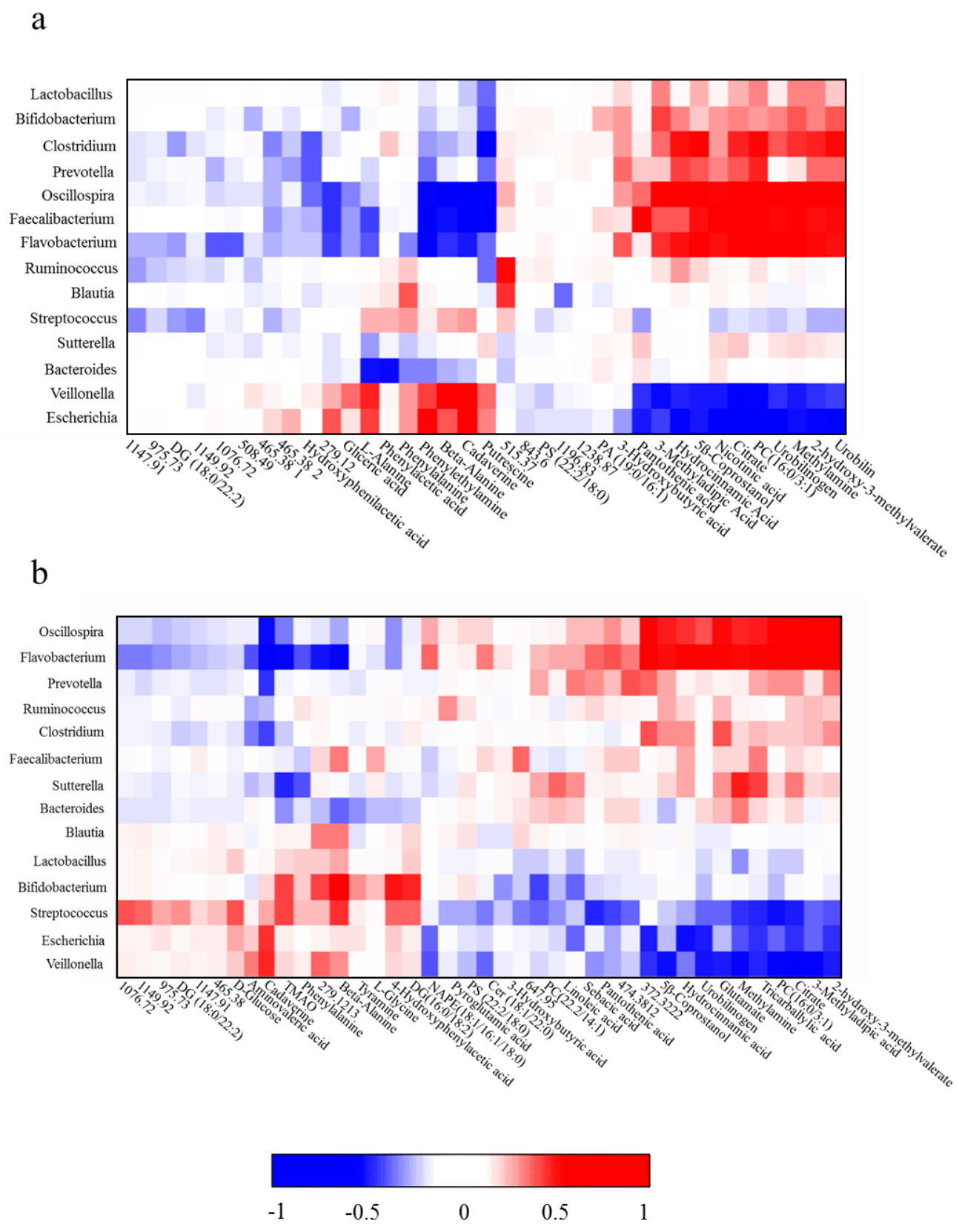

Figure 13. Inter-omic Spearman's rank correlation between metabolites and bacterial genera. Spearman correlation between statistically different metabolites and bacterial genera was calculated both for CD (**a**) and UC (**b**). All calculated correlations are shown.

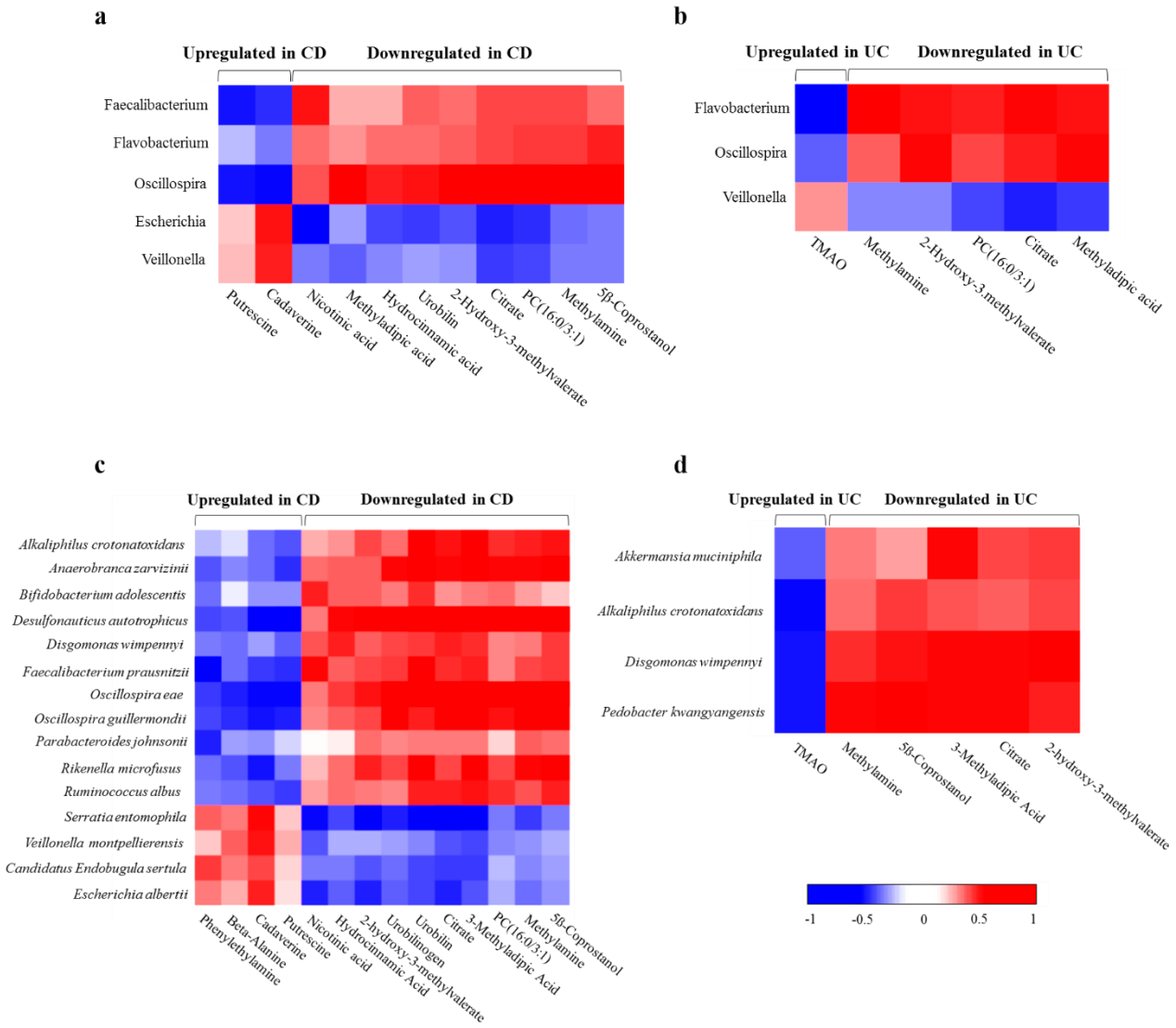

Figure 14. Inter-omic Spearman rank correlation between metabolites and bacterial genera and species. Spearman correlation between statistically different metabolites and bacterial genera was calculated both for CD (a) and UC (b). Spearman correlation between statistically different metabolites and bacterial species was calculated both for CD (c) and UC (d). Correlations with an r coefficient >0.5 are shown.

Fifteen bacterial species showed a good correlation with 14 discriminant metabolites in Crohn disease patients (Figure 14c). Seven species, *Faecalibacterium prausnitzii, Oscillospira eae, Oscillospira guillermondii, Anaerobranca zavarzinii, Veillonella montpellierensis, Ruminococcus albus* and *Alkaliphilus crotonatoxidans* belonging to the Firmicutes phylum, four, *Desulphonauticus Autotrophicus, Serratia entomophila, Escherichia albertii and Candidatus Endobugula sertula* to the Proteobacteria phylum, three, *Dysgonomonas wimpennyi*, *Rikenella microfusu*s and *Parabacteroides*

johnsonii to the Bacteroidetes phylum and finally only one, *Bifidobacterium adolescentis* to the Actinobacteria phylum. In particular, a strong positive correlation was seen between *Oscillospira eae* and 5β-coprostanol, 3-methyladipic acid, citric acid, methylamine and 2-hydroxy-3-methylvaleric acid; between *Oscillospira guillermondii* and 5β-coprostanol and methylamine; and finally, between *Desulphonauticus autotrophicus* and 5β-coprostanol, 3-methyladipic acid, citric acid and methylamine. On the other hand, a strong negative correlation between *Faecalibacterium prausnitzii* and phenylethylamine and between *Desulphonauticus autotrophicus* and putrescine and cadaverine was observed.

Evaluation of the metabolome-species relationship in UC patients showed good correlations between *Pedobacter kwangyangensisvs* and *Dysgonomonas wimpennyi* and 3-methyladipic acid, 5ß-Coprostanol, 2-hydroxy-3-methylvaleric acid, citric acid and methylamine, while only 3-methyladipic acid was positively correlated with *Akkermansia muciniphila species*. Large negative correlations were detected between TMAO and *Pedobacter kwangyangensisvs, Dysgonomonas wimpennyi* and *Alkaliphilus crotonatoxidans* species (Figure 14d).

Plasma analysis

A total of 39 metabolites were identified in plasma samples from CD patients, UC patients and healthy controls using GC-MS. These metabolites included organic acids, amino acids, fatty acids and sugars. An unsupervised PCA and all the supervised OPLS-DAs were performed as previously described for faeces analysis. The comparison between Crohn disease patients and controls showed good statistical performance (OPLS-DA score plot is shown in Figure 15a). Similar results were obtained also between ulcerative colitis and healthy patients and the resulting score plot is shown in Figure 15b. All statistical parameters are resumed in Table 3. To investigate if we were able to distinguish between the two pathological conditions, an OPLS-DA model was then created (Figure 16a). As already observed for faeces analysis, the classification model was unable to separate between CD and UC, confirming the intrinsic similarity in metabolic profiles (Table 3). Since the metabolomic analysis. did not allow to separate between CD and UC conditions, the two groups were merged into a unique class (Figure 16b). The score plot resulting from the OPLS-da analysis is shown in figure 16b and the statistical parameters are reported in table 3. Discriminant metabolites were highlighted by the means of an S-plot and a Mann-Whitney U-test with Holm- Bonferroni correction was carried out to find significant differences between IBD and healthy patients (Figure 17). As seen in the figure, there was a decrease in the levels of ornithine, proline, threonine, urea and 1,5-anydro-d-sorbitol while levels of 2-hydroxybutyric acid, 3-hydroxybutyric acid, citric acid, lactic acid, lysine and uric acid were significantly increased. The metabolic pathways in which the statistically different metabolites are involved are shown in figure 18. The effects of the different therapies were checked as confounding factors, but there were no influences of the therapy on the metabolome here detected.

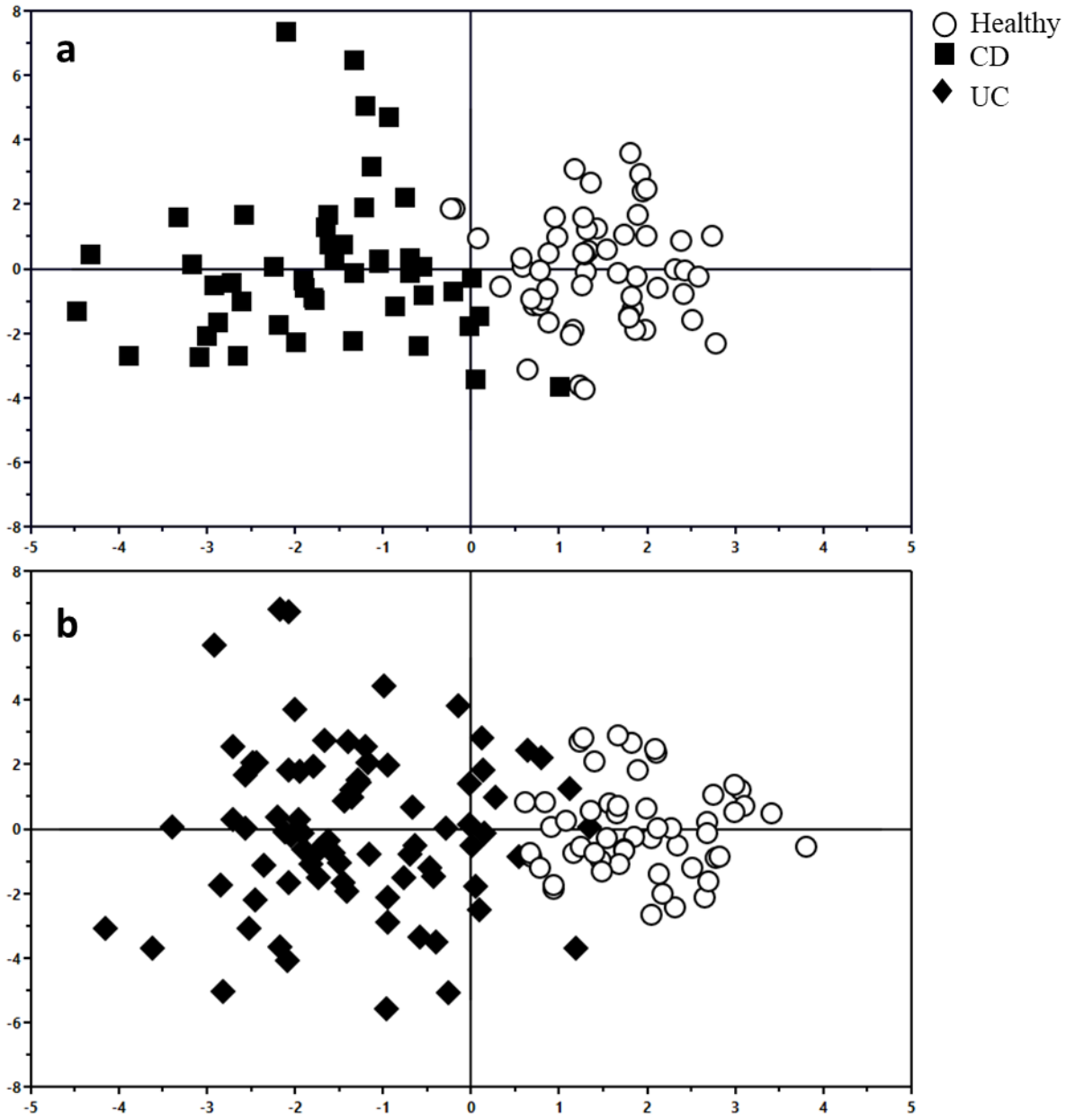

Figure 15. OPLS-da score plot of plasma samples. CD vs healthy comparison (a), UC vs healthy comparison (b) and IBD vs healthy comparison (c).

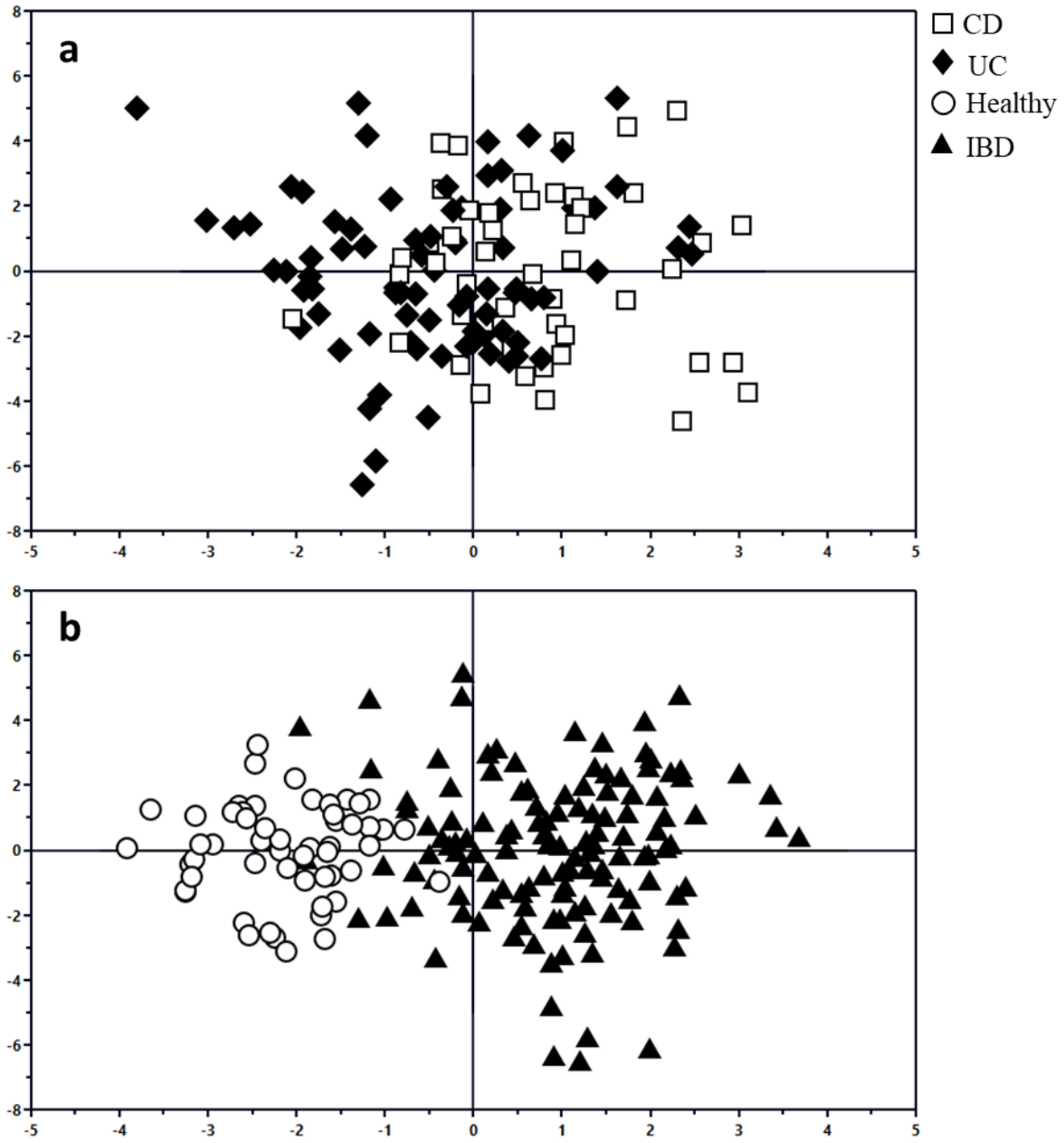

Figure 16. OPLS-da score plot of plasma samples (2). CD vs UC comparison (a), and IBD vs healthy comparison (b).

	OPLS-DA models				Permutation*	
	GC-MS					
Groups	**Components[a]**	**R_2Xcum[b]**	**R_2Ycum[c]**	**Q_2cum[d]**	**R_2 intercept**	**Q_2 intercept**
Healthy *vs* IBD	1P+1O	0.195	0.632	0.563	0.102	-0.220
Healthy *vs* CD	1P+1O	0.192	0.723	0.609	0.180	-0.248
Healthy *vs* UC	1P+1O	0.214	0.709	0.610	0.138	-0.239
CD *vs* UC	1P+1O	0.202	0.188	-0.224	0.184	-0.178

Table 3. Plasma samples MVA parameters. [a] The number of Predictive and Orthogonal components used to create the statistical models.
[b,c] R_2X and R_2Y indicated the cumulative explained fraction of the variation of the X block and Y block for the extracted components.
[d] Q_2cum values indicated cumulative predicted fraction of the variation of the Y block for the extracted components.
* R_2 and Q_2 intercept values are indicative of a reliable model. The Permutation test was evaluated on the corresponding partial least square discriminant analysis (PLS-DA) model.

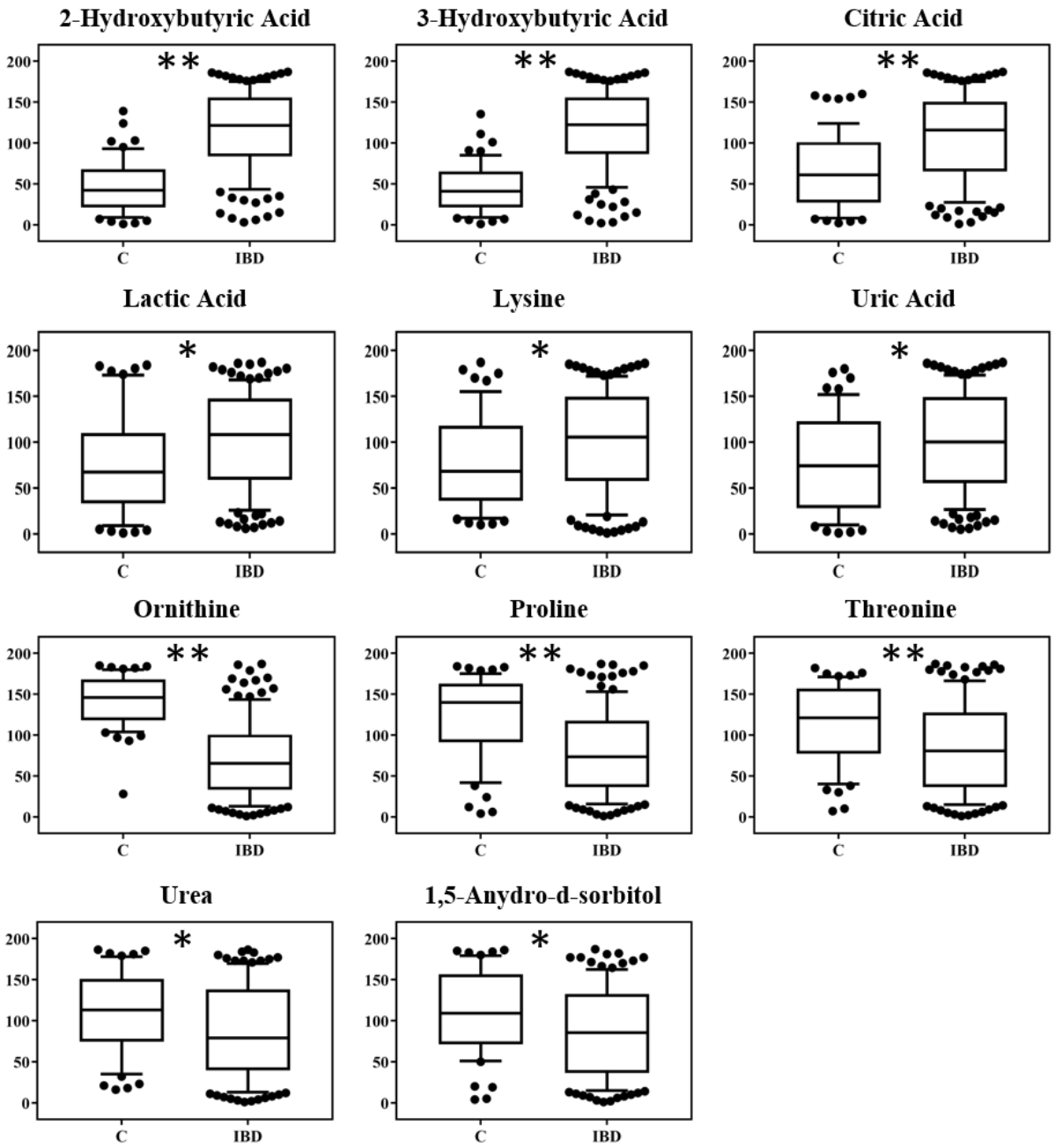

Figure 17. Statistically significant metabolites in IBD vs healthy plasma samples (C) comparison. Discriminant metabolites obtained with the MVA, underwent to a Mann-Whitney U-test with Holm-Bonferroni correction to determine which metabolites were statistically significantly variated. The resulted metabolites obtained are shown and expressed in the graphs y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted). * and ** indicates levels of significance with $p < 0.05$ and < 0.01, respectively.

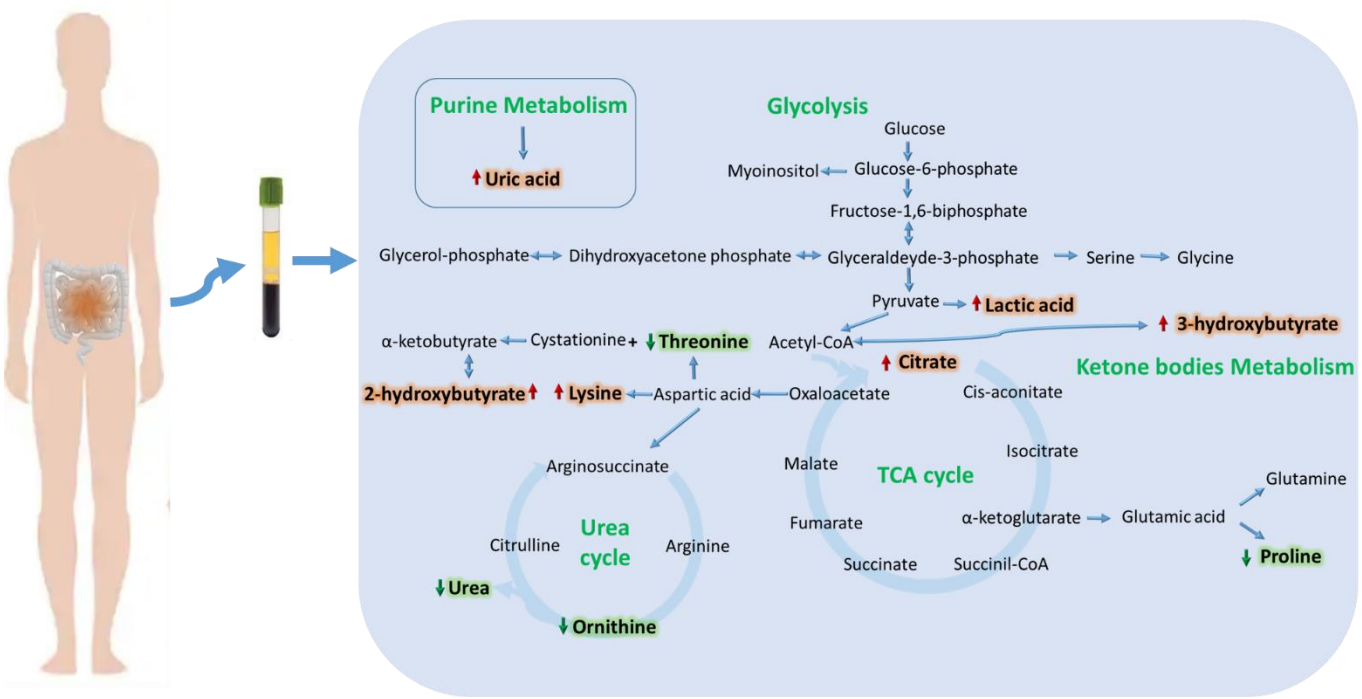

Figure 18. Relevant metabolic pathways involved in IBD, obtained from the analysis of metabolites in plasma samples. Increased and decreased metabolites are highlighted in red and green, respectively.

Biopsies analysis

Fifty metabolites were identified in biopsies samples from CD and UC patients colon and ileum biopsies using GC-MS. Peaks were identified and attributed to metabolites that included organic acids, amino acids, fatty acids and sugars. An unsupervised PCA and all the supervised OPLS-DAs were performed as previously described for faeces and plasma analysis. At first, a comparison between inflamed and normal colon biopsies from UC patients was made (Figure 19a) and the model diagnostics indicate a good predictive performance (Table 4). The same analysis was carried out for inflamed and normal colon biopsies (Figure 19b) and inflamed and normal ileum biopsies (Figure 19c) in CD patients. Both MVA analysis showed good predictive performance (Table 4). For CD patients, the OPLS-DA was used also to check the impact of inflammation on the different metabolism between colon and ileum. A comparison between healthy ileum and colon and between affected ileum and colon was made (Figure 19d and e, respectively) and the statistical parameter are shown in table 4. As made for faeces and plasma, to investigate if it was possible to distinguish between the two pathological conditions, an OPLS-DA model was created comparing UC affected colon biopsies and CD affected colon biopsies. The classification model was unable to separate between CD and UC, indicating an intrinsic similarity in metabolic profiles (Figure 19f and Table 4). As already observed for plasma and faeces, it was not possible to clearly separate CD from UC patients, they were considered as a unique class. The score plot resulting from the OPLS-da analysis between healthy and IBD affected colon and ileum are shown in figure 19g and h, respectively, and the statistical parameters are reported in table 4. Discriminant metabolites coming from these two last analyses were highlighted by the means of an S-plot and a Mann-Whitney U-test with Holm-Bonferroni correction was carried out to find significant differences (Figure 20 and 21). As showed in figure 20, in the comparison between healthy colon and colon affected by IBD, levels of aspartic acid, glutamic acid, glutamine, glycine and ornithine were significantly upraised, while fumaric acid, lactic acid, myo-inositol, glycerol-phosphate and oleic acid were found decreased. Moreover, looking

at the ileum samples (figure 21) aspartic acid, oleic acid and linoleic acid were low while eicosanoic acid and maltose were increased.

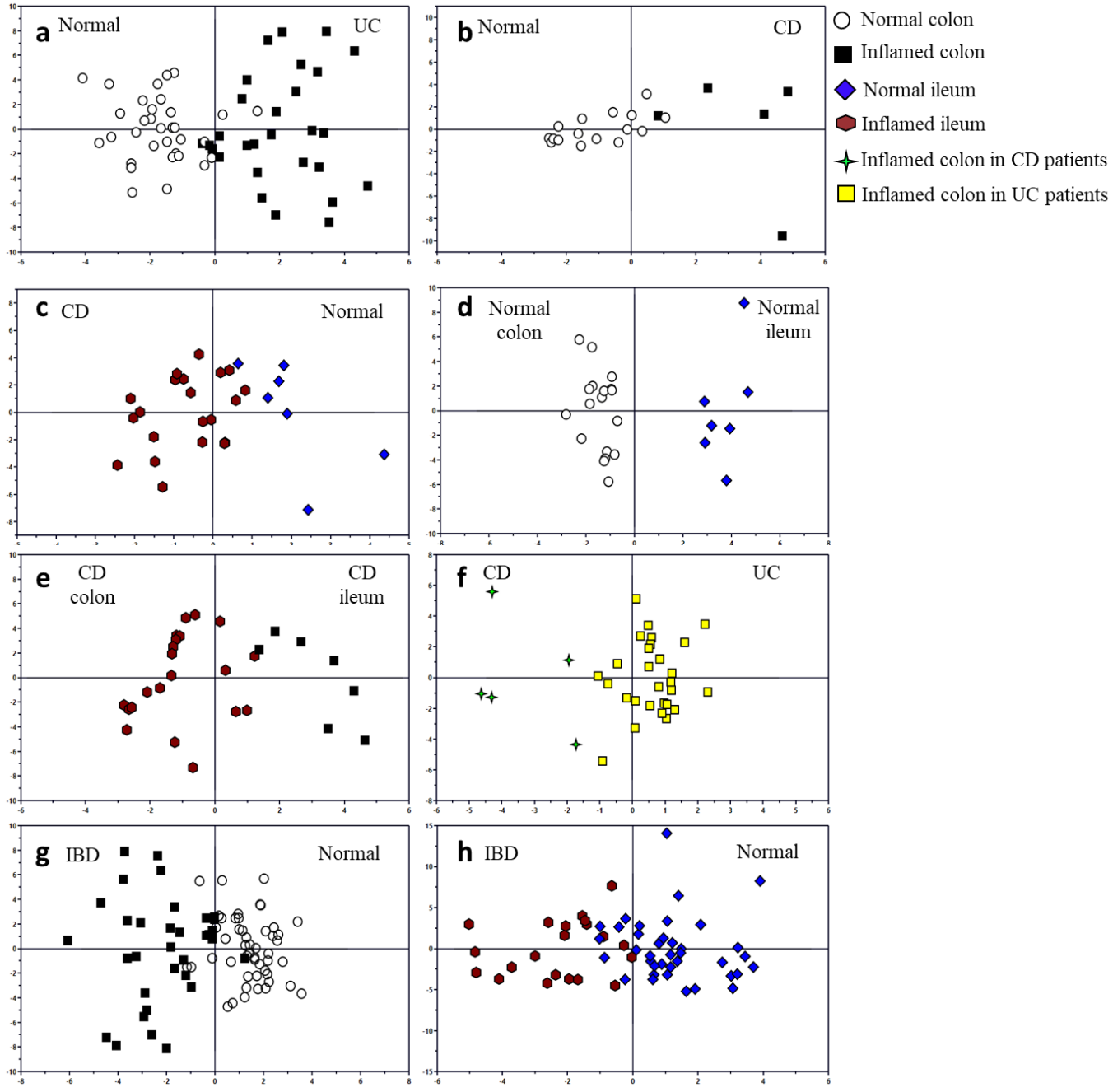

Figure 19. OPLS-da score plot of biopsy samples. Healthy colon vs affected colon in UC (a), healthy colon vs affected colon in CD (b), healthy ileum vs affected ileum in CD (c), healthy ileum vs healthy colon in CD (d), affected ileum vs affected colon in CD (e), CD affected colon vs UC affected colon (f), healthy colon vs affected colon in IBD (g) and healthy ileum vs affected ileum in IBD (h).

	OPLS-DA models				Permutation*	
	GC-MS					
Groups	**Components[a]**	**R_2Xcum[b]**	**R_2Ycum[c]**	**Q_2cum[d]**	**R_2 intercept**	**Q_2 intercept**
Healthy colon *vs* affected colon in UC	1P+1O	0.257	0.688	0.508	0.391	-0.217
Healthy colon *vs* affected colon in CD	1P+1O	0.448	0.693	0.314	0.514	-0.136
Healthy ileum vs affected ileum in CD	1P+1O	0.476	0.588	0.239	0.431	-0.115
Healthy ileum vs healthy colon in CD	1P+1O	0.272	0.937	0.558	0.658	-0.218
Affected ileum vs affected colon in CD	1P+1O	0.247	0.711	-0.310	0.683	-0.119
CD affected colon vs UC affected colon	1P+1O	0.138	0.719	-0.406	0.570	-0.0732
Healthy colon vs affected colon in IBD	1P+1O	0.233	0.641	0.489	0.325	-0.215
Healthy ileum vs affected ileum in IBD	1P+1O	0.262	0.603	0.202	0.423	-0.318

Table 4. Biopsies samples MVA parameters. [a] The number of Predictive and Orthogonal components used to create the statistical models.
[b,c] R_2X and R_2Y indicated the cumulative explained fraction of the variation of the X block and Y block for the extracted components.
[d] Q_2cum values indicated cumulative predicted fraction of the variation of the Y block for the extracted components.
[*] R_2 and Q_2 intercept values are indicative of a reliable model. The Permutation test was evaluated on the corresponding partial least square discriminant analysis (PLS-DA) model.

In figure 22 are shown the metabolic pathways in which the statistically different metabolites in the colon are involved. The effects of the different therapies were checked as confounding factors, but there were no influences of the therapy on the metabolome here detected.

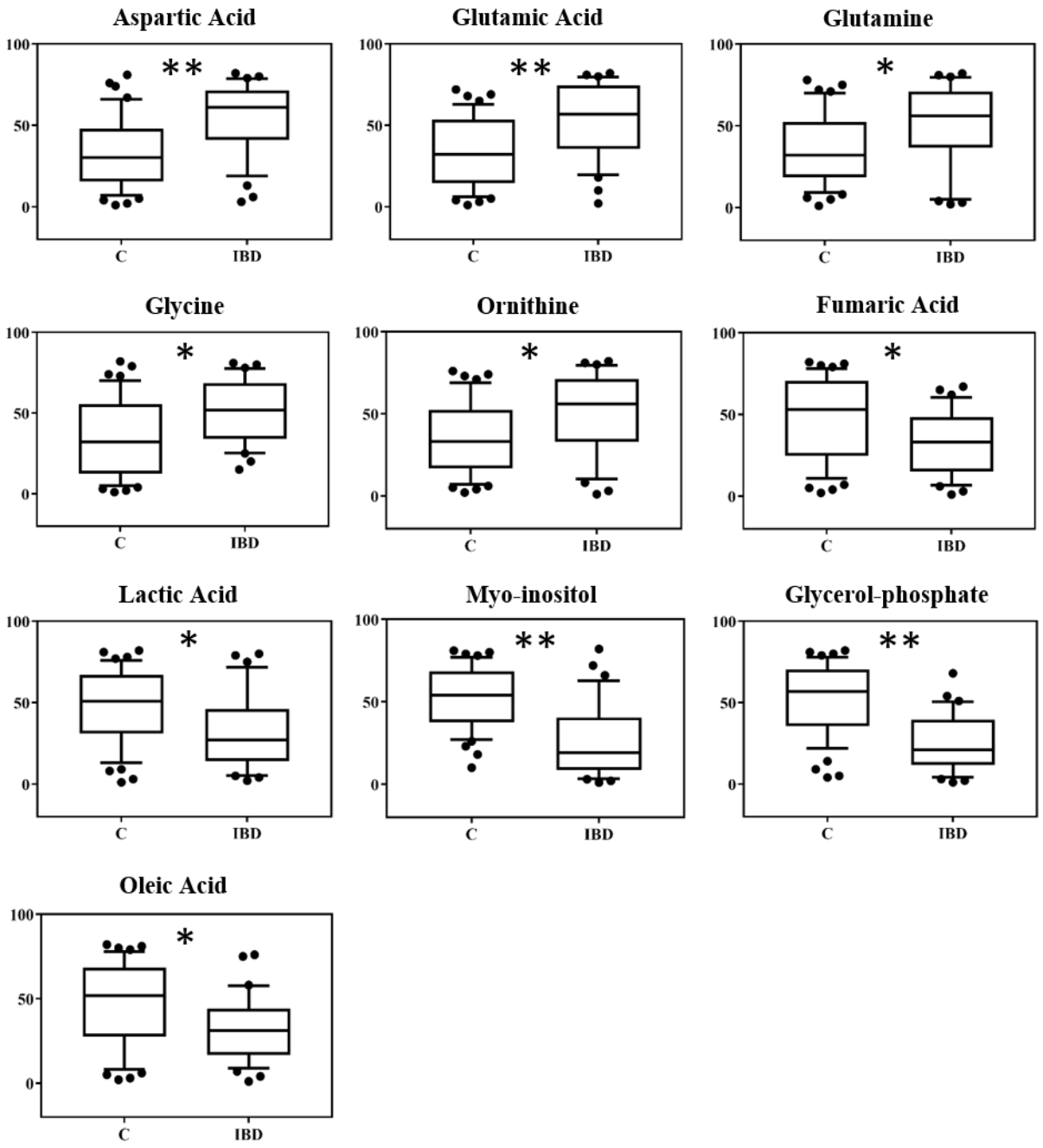

Figure 20. Statistically significant metabolites in colon affected by IBD vs healthy colon (C) comparison. Discriminant metabolites obtained with the MVA, underwent to a Mann-Whitney U-test with Holm-Bonferroni correction to determine which metabolites were statistically significantly variated. The resulted metabolites obtained are shown and expressed in the graphs y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted). * and ** indicates levels of significance with $p < 0.05$ and < 0.01, respectively.

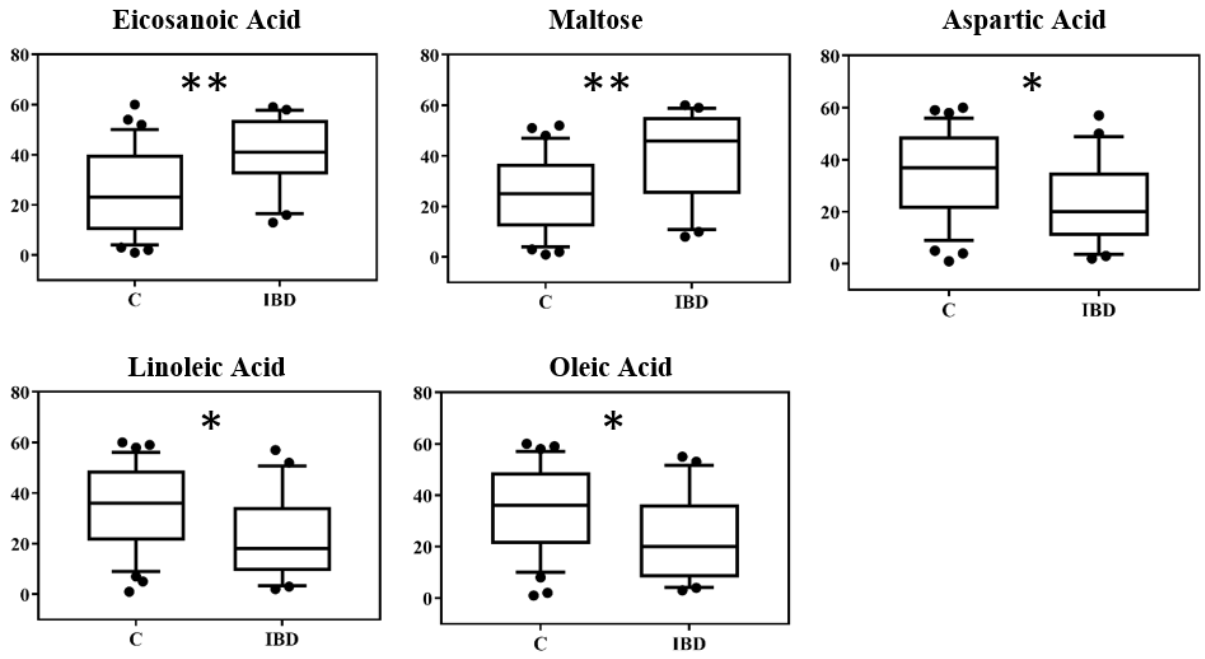

Figure 21. Statistically significant metabolites in ileum affected by IBD vs healthy ileum (C) comparison. Discriminant metabolites obtained with the MVA, underwent to a Mann-Whitney U-test with Holm-Bonferroni correction to determine which metabolites were statistically significantly variated. The resulted metabolites obtained are shown and expressed in the graphs y axis as ranks (data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted). * and ** indicates levels of significance with $p < 0.05$ and < 0.01, respectively.

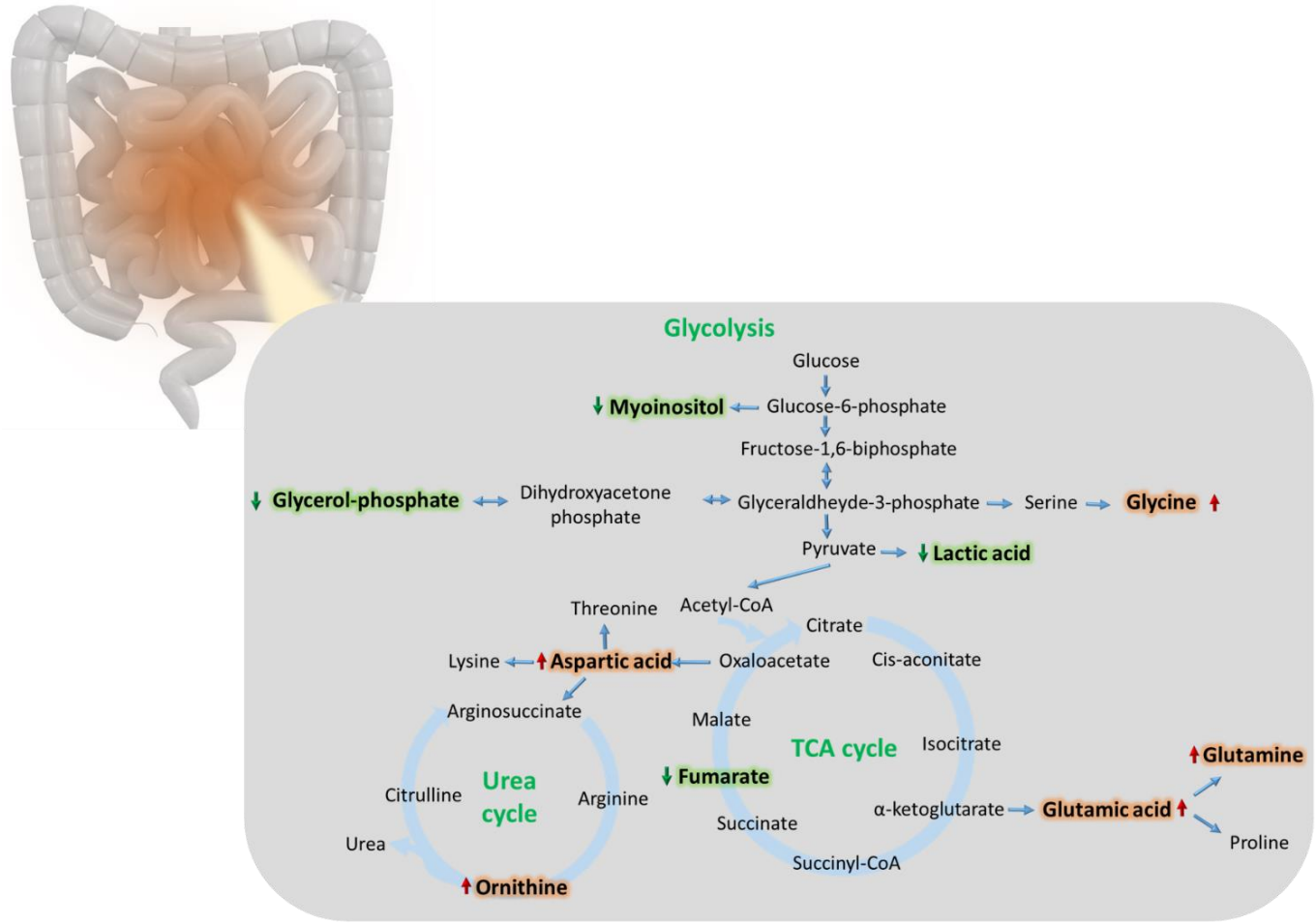

Figure 22. Relevant metabolic pathways involved in IBD, obtained from the analysis of metabolites in colon biopsies samples. Increased and decreased metabolites are highlighted in red and green, respectively.

In vitro study

Evaluation of cell viability

Among all the altered metabolites, nicotinic acid was found to decrease in faeces of IBD patients respects to healthy subjects. As in literature, nicotinic acid seems to have an anti-inflammatory effect, an in vitro model of intestinal inflammation was obtained with differentiated Caco-2 cells in which inflammation was induced with LPS and IL-1β. To investigate the effect of LPS, IL-1β and NA on differentiated Caco-2 cell monolayers, experiments were initially carried out to assess cell viability after treatment and to choose a non-toxic concentration for each compound. As reported in figure 23, cell viability remained unchanged in the presence of the tested compounds. LPS was tested in the concentration range 10–100μg/mL, IL-1b in a concentration of 10 and 25ng/mL and NA in the concentration range 100-300μg/mL. The cell viability was tested after 48h of treatment for each compound.

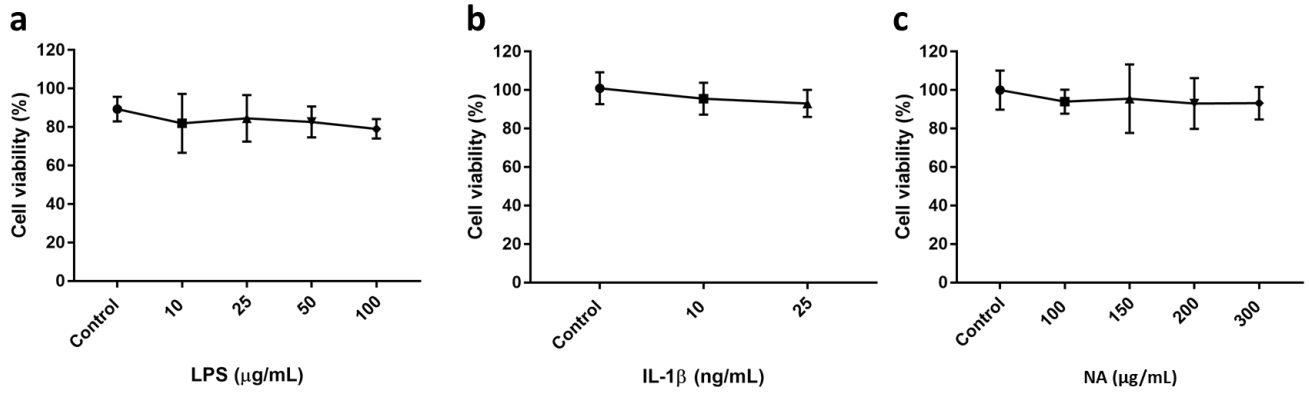

Figure 23. Effects of LPS (a), IL-1β (b) and NA (c) on Caco-2 cell viability. The effects on the cell viability are after 48h of incubation of each compound. Data, expressed as % of control, are presented as means ± Standard Deviation. Statistical analysis was performed by Student t-Test.

Evaluation of IL-8 production

As IL-8 is one of the earliest reported chemokines produced by intestinal epithelial cells is the IL-8, to estimate the inflammatory effect of LPS and IL-1β and the effect of NA on inflammation, an ELISA assay was carried out to measure the levels of IL-8 released after treatments with each compound. In the first experiment, cells were treated with LPS (50µ/mL) and LPS in combination with NA (100µg/mL) (Figure 24a) and with IL-1β (25ng/mL) and IL-1β plus NA (100µg/mL) (Figure 24b). As showed in the figure, the levels of IL-8 were significantly up-regulated after treatment with both LPS and IL-1β, while NA at the concentration of 100 µg/mL does not seem to induce any statistically significant effect on IL-8 production when compared with LPS and IL-1β alone.

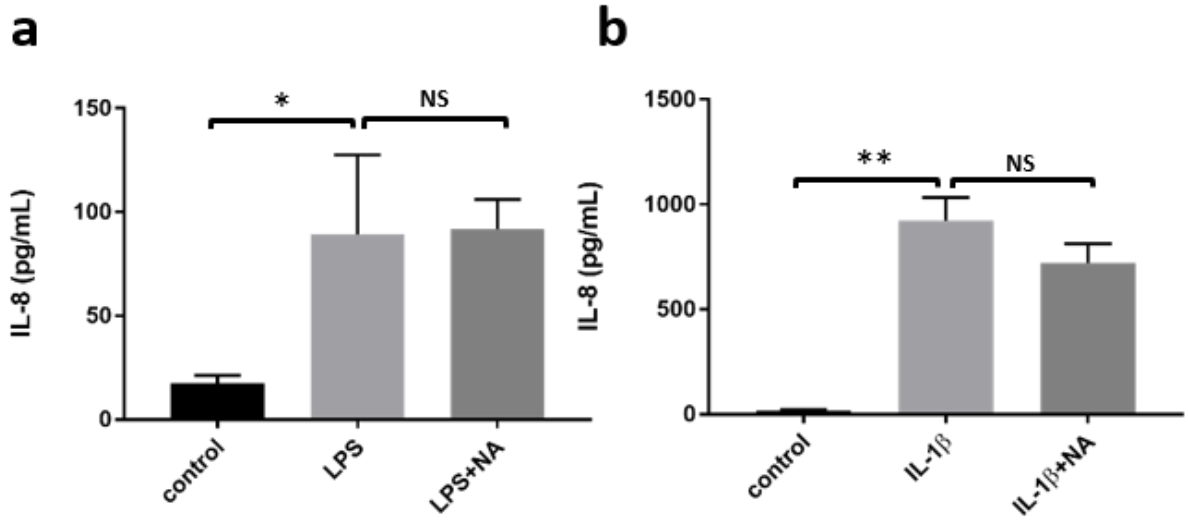

Figure 24. IL-8 measurement. Detection of IL-8 levels in Caco-2 cells medium (a) after treatment with LPS (50µg/mL) and LPS (50µg/mL)+NA (100µg/mL) and (b) after treatment with IL-1β (25ng/mL) and IL-1β (25ng/mL)+NA (100µg/mL). Statistical analysis was performed by Student t-Test. Results were considered significant when *P<0.05 and **P<0.01. Non-significant results are indicated as NS.

A second experiment was conducted with an increased concentration of NA, 200µg/mL. As shown in figure 25, cells were treated with both LPS (50µg/mL) (Figure 25a) and LPS plus NA (200µg/mL) and with IL-1β (25ng/mL) and IL-1β with NA (200µg/mL) (figure 25b). Once again, LPS and IL-1β

had an increasing effect on IL-8 production, but NA at the higher concentration seems to have an effect in reducing the inflammatory effect by decreasing IL-8 levels released by the cells. All these results together are shown in figure 25c.

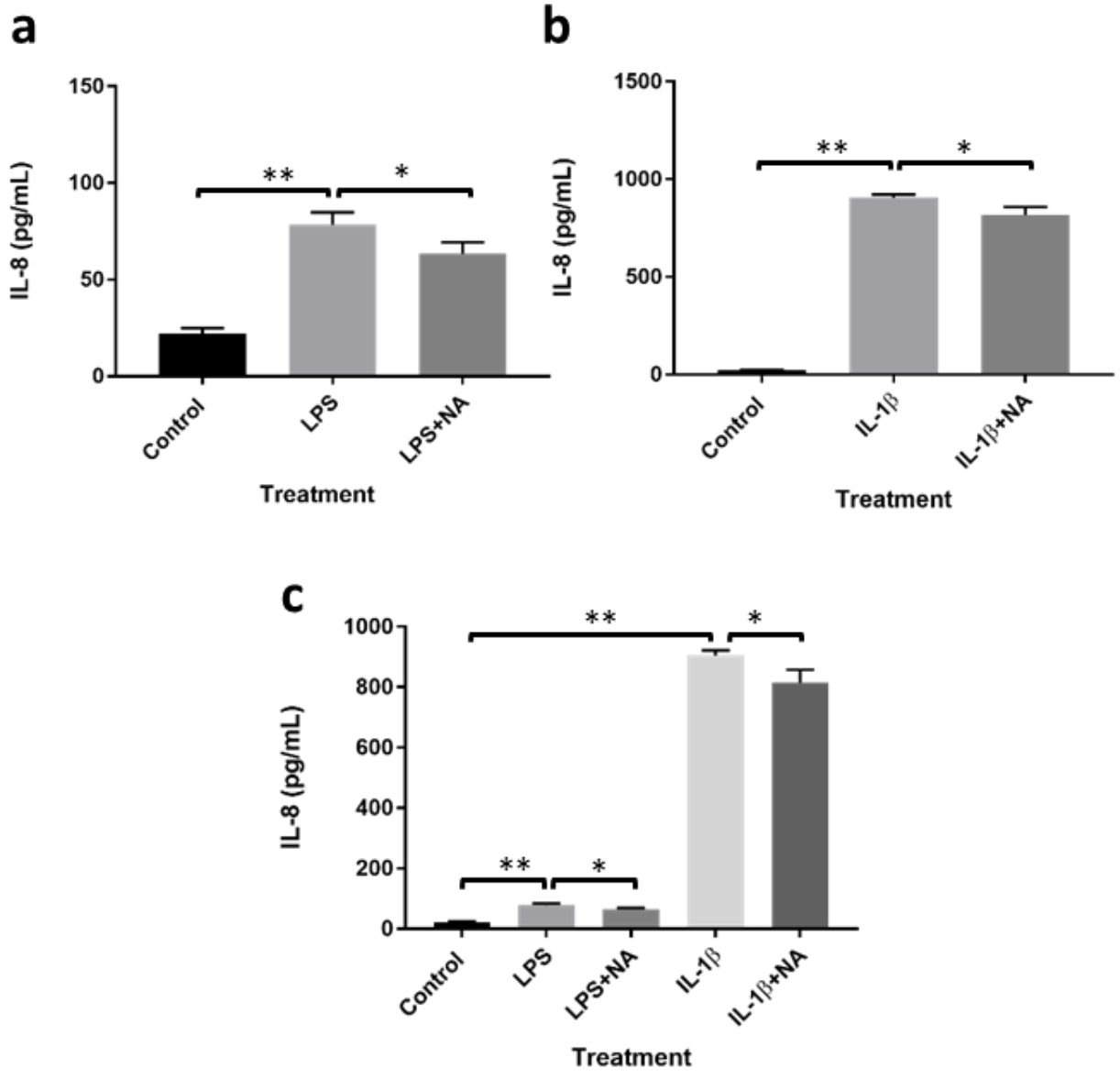

Figure 25. IL-8 measurement (2). Detection of IL-8 levels in Caco-2 cells medium (a) after treatment with LPS (50µg/mL) and LPS (50µg/mL)+NA (200µg/mL) and (b) after treatment with IL-1β (25ng/mL) and IL-1β (25ng/mL)+NA (200µg/mL). Statistical analysis was performed by Student t-Test. Results were considered significant when *P<0.05 and **P<0.01. In figure (c) is shown the totality of the results found in figure (a) and (b).

Metabolomics analysis

To investigate the metabolic changes in Caco-2 cells in which an inflammatory response was induced, pre-treated and not with NA, the metabolomic profile was characterized by ^{1}H-NMR and GC-MS analysis of cellular and media extracts. Looking only at the changes induced by inflammatory stimuli, the results showed that, IL-1β had a major effect on metabolism compared to the LPS effect (Figure 26). Indeed, after 48h IL-1β treatment, there was, at the intracellular level, an increase of acetic acid, glutamic acid, citric acid and malic acid and a decrease of 2-hydroxybutyric acid, 3-hydroxybutyric acid, alanine, fructose, fumaric acid, glutamine, threonine, isoleucine, lactic acid, ornithine, serine and glycerophosphocholine compared to the controls. After LPS treatment there was an increment of malic acid levels again, and a decrease of 2-hydroxyburyric acid, fumaric acid, serine and glycerophosphocholine, which were decreased as well after IL-1β treatment, and we also observed a decrement of pyruvic acid levels in LPS treated cells compared to controls (Figure 26). The levels of some of these metabolites were also found changed in the medium. Precisely, alanine, glutamine, lactic acid and uric acid were increased in the medium after IL-1β treatment, showing an inverse behaviour compared to the intracellular levels, while glycine, ornithine and glucose levels were decreased. As well as IL-1β, LPS induced an increase of glutamine, plus an increase of serine and cholesterol levels and a decrease of glucose in the medium (Figure 27). The treatment with NA alone also changed the levels of detected metabolites levels when compared with controls. Indeed, after NA treatment there was an increase of ATP, glycerolphosphate, lactic acid and glycerophosphocholine and a decrease of fumaric acid, glycine, pyruvic acid and serine at the intracellular level (Figure 26). While at the extracellular level, there were increased levels of serine and tyrosine and lower levels of alanine (Figure 27).

Then, focusing the attention on the effects of NA on the metabolism changes induced by inflammation, it can be seen that after the treatment with both IL-1β and NA the levels of some metabolites that were as well significantly changed after the treatment with only IL-1β, are again

altered but with an inverse trend. For example, glutamine, isoleucine, ornithine and glycerophosphocholine were upregulated in the comparison between IL-1β and IL-1β+NA treatment, while glutamic acid was downregulated (Figure 26). On the other hand, after IL-1β+NA treatment the levels of alanine and 3-hydroxybutyric acid are even more decreased compared to the IL-1b treatment alone. There were also changes in levels of ATP, creatine, glycine and proline, all increased after the treatment while the levels of creatine phosphate were found decreased. The comparison between LPS and LPS+NA treatment showed an inverse trend of the levels of glycerophosphocholine, that was found increased and an increase of ATP, glycerolphosphate and proline levels and a decrease of 3-hydroxybutyric acid and alanine, as well as for IL-1β (Figure 24). At the extracellular level, it was seen a decrease of alanine and glutamine and an increase of ornithine and tyrosine in IL-1β+NA treated samples compared to IL-1β samples and decreased levels of serine, alanine and glycine and an increased level of ornithine in LPS+NA treated samples compared to LPS alone (Figure 27).

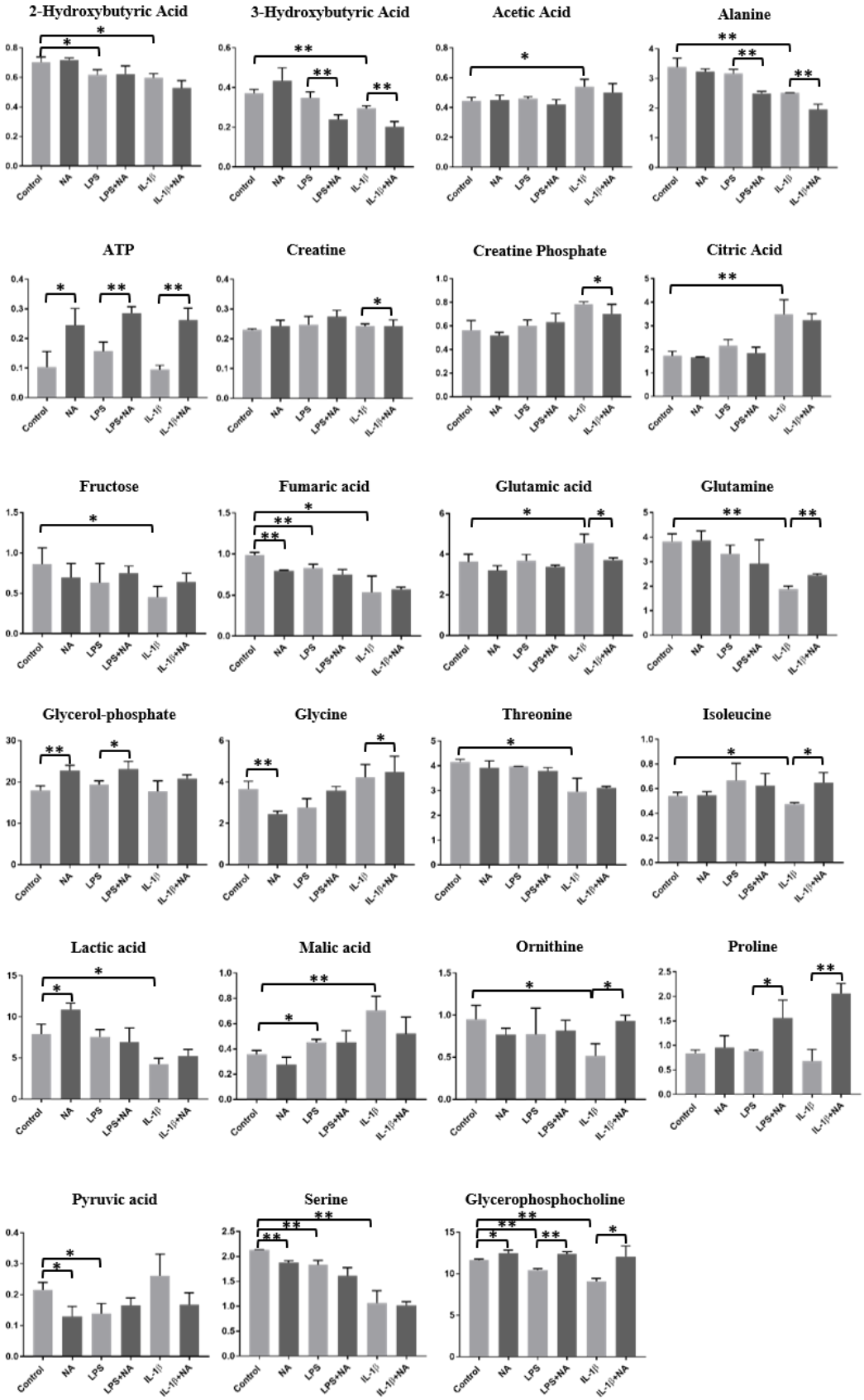

Figure 26. Intracellular metabolites measured in Caco-2 cell line by ^{1}H-NMR and GC-MS. In the y axis is indicated the peak areas of the metabolites (n=3) normalized for total protein content and total area. Statistical analysis was performed by Student T-test. Results were considered significant when * P<0.05, ** P<0.01.

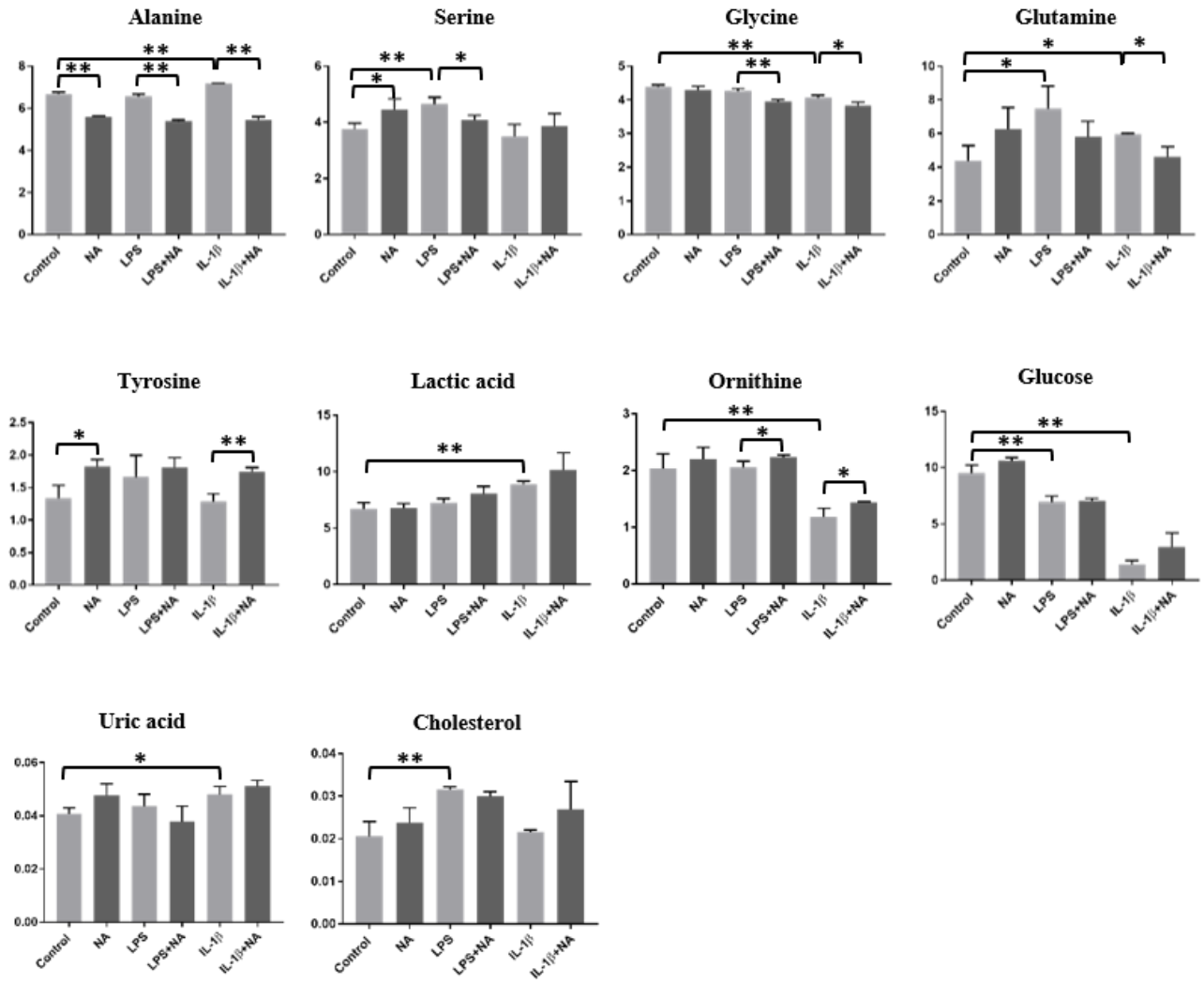

Figure 27. Extracellular metabolites measured in Caco-2 cell line by ^{1}H-NMR and GC-MS. In the y axis is indicated the peak areas of the metabolites (n=3) normalized for total protein content and total area. Statistical analysis was performed by Student T-test. Results were considered significant when * P<0.05, ** P<0.01.

The results revealed that the metabolites that were found statistically altered after the induction of inflammation and after induction of inflammation plus NA treatment in differentiated Caco-2 cells were associated with different energetic pathways. These included glycolysis, tricarboxylic acid (TCA) cycle, urea cycle, ketone bodies metabolism and lipid metabolism at both intracellular and extracellular level (Figure 28 and 29).

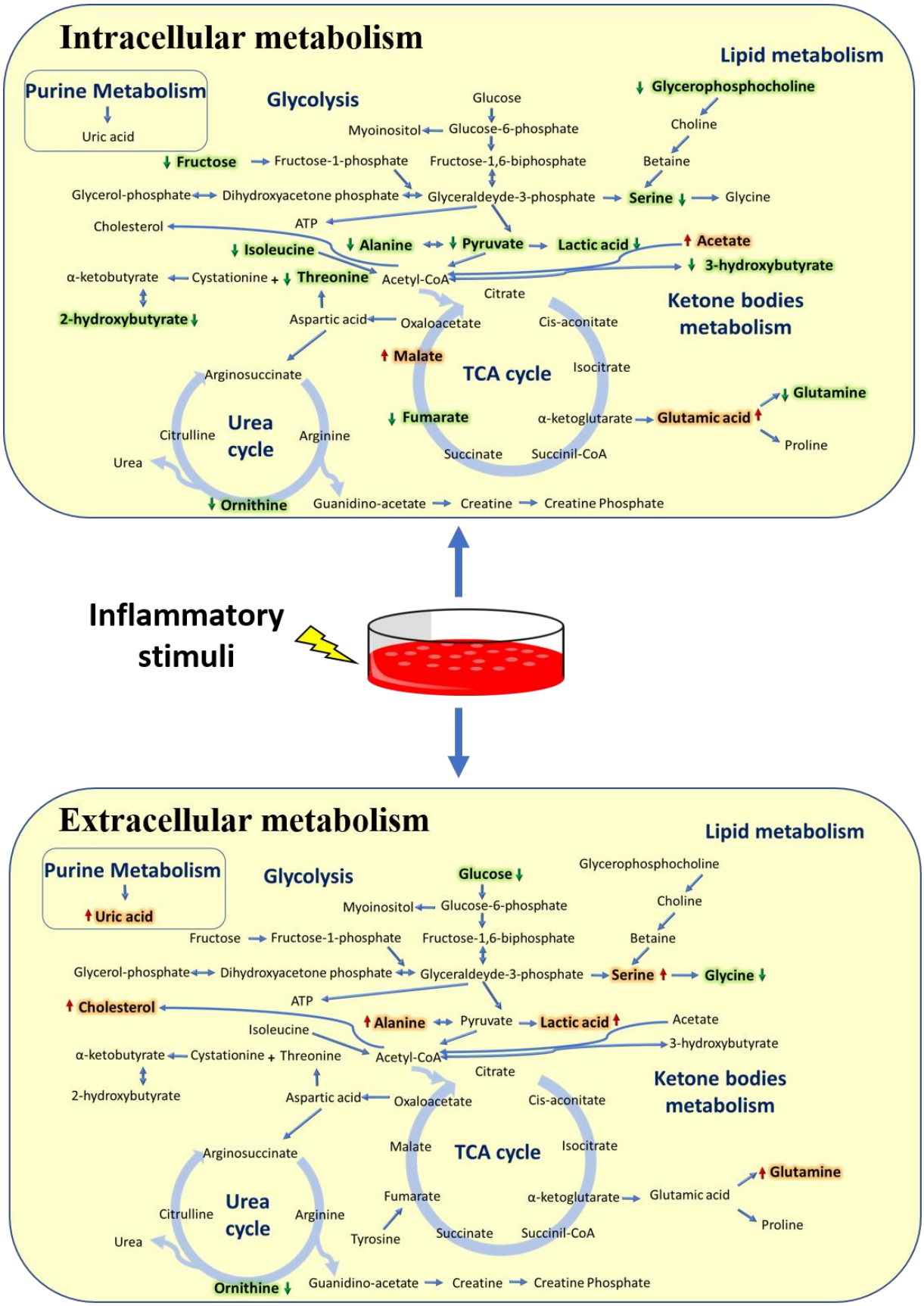

Figure 28. Significantly altered metabolic pathways after treatment with pro-inflammatory stimuli in Caco-2 cells. Increased and decreased metabolites are highlighted in red and green, respectively.

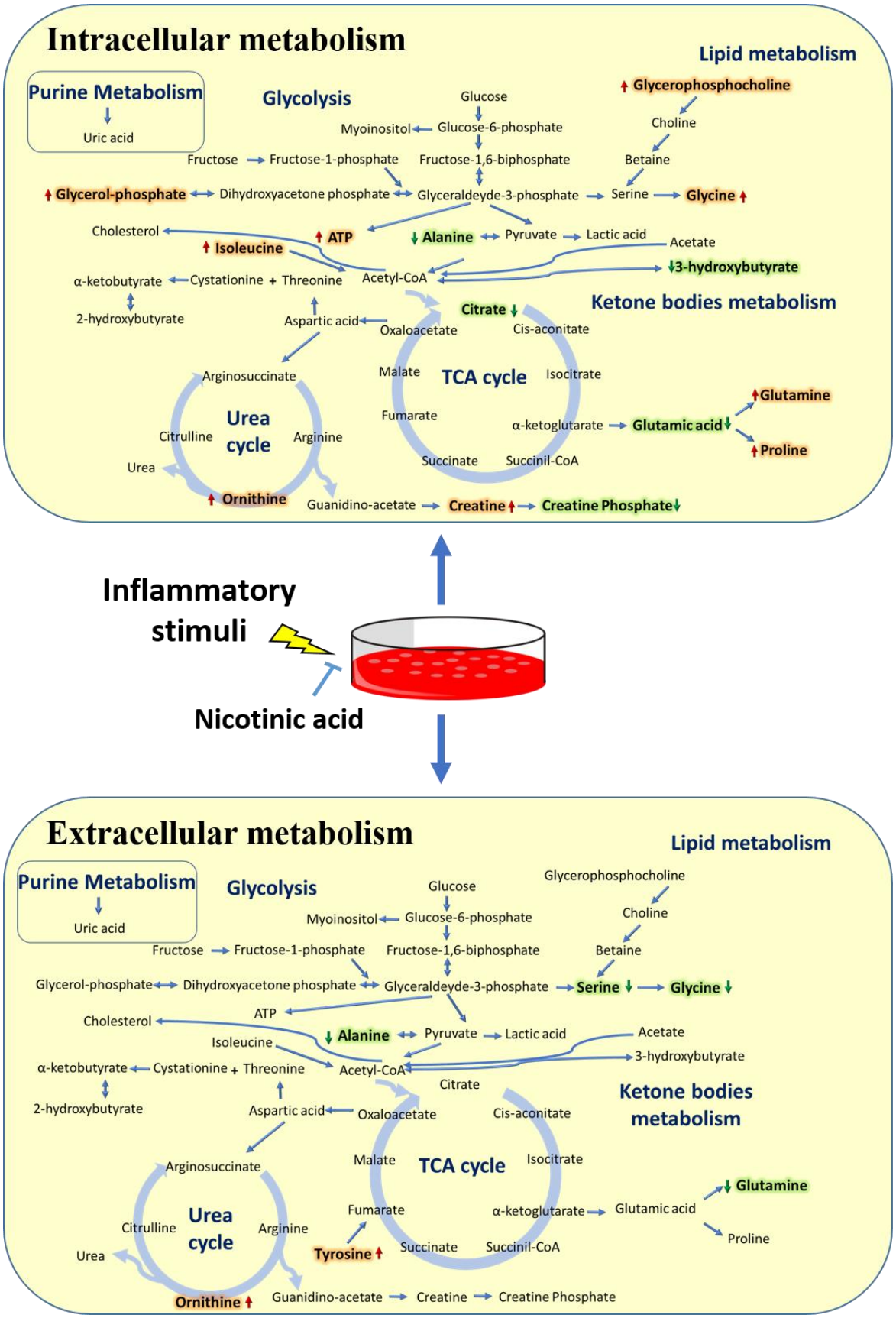

Figure 29. Significantly altered metabolic pathways after treatment with pro-inflammatory stimuli in combination with NA in Caco-2 cells. Increased and decreased metabolites are highlighted in red and green, respectively.

V. Discussion

Human study

Evaluation of the gut-microbiome metabolome axis in faeces

In this study, it has been applied a dual-omics approach in order to improve the current understanding of IBD pathogenesis and the host-microbial interactions, through the evaluation of metabolomic profile and its correlation with the microbiota composition. The analysis of faeces samples showed the presence of an alteration in both metabolism and intestinal flora in patients affected by IBD.

It has been established that the gut microbiota is dysregulated in IBD, which leads to the modification of the bacterial metabolic activity (60). In the clinical management of IBD, there are significant, inherent challenges in elucidating whether dysbiosis contributes to disease pathogenesis or whether, by contrast, it could be a secondary change associated with the inflammatory process, which may be driven by host genetics and environmental factors, such as diet. Whereas it has been previously reported a decrease in Firmicutes and Actinobacteria abundance in IBD, along with high levels of Proteobacteria compared to healthy subjects (61), the above-reported results did not show this in our patient cohort apart from the increase in Proteobacteria. In the present cohort, it was demonstrated, instead, increased levels of Firmicutes and Actinobacteria in IBD. The bacterial complexity was significantly lower in IBD, CD and UC patients compared to control subjects, as previously reported. Taken together, these data may reflect at least in part the results obtained from the metabolomics analysis, which revealed characteristic clustering profiles among healthy subjects and IBD patient categories. Two biogenic amines, cadaverine and putrescine, were found significantly increased in CD patients compared to healthy subjects. Polyamines are formed mainly by the decarboxylation of amino acids or by transamination of aldehydes or ketones and are produced both by the host and by intestinal flora bacteria. While their specific role is still largely unknown, polyamines are involved in numerous physiological processes such as the preservation of membrane integrity and nucleic acids metabolism. Furthermore, they have a crucial role in the regulation of gene transcription and

translation (62). Several studies demonstrated how polyamines are necessary for the division of epithelial cells (63–66). In fact, the normal growth of the intestinal mucosa depends on the polyamines availability within the cell division crypts, and such substrates could be synthesized endogenously or absorbed at the lumen level. Elevated levels of polyamines seem to have a toxic effect and are associated with several diseases. It is believed that at the basis of this toxicity there is the oxidative stress caused by polyamines catabolism (67). In the present study, the Spearman correlation analysis demonstrated that the increase of the two polyamines was negatively correlated with the amount of two bacterial genera belonging to the Firmicutes phylum, Faecalibacterium and Oscillospira. Moreover, cadaverine levels were also directly associated with the abundance of another Firmicutes genus, Veillonella, and the Proteobacterium Escherichia. Interestingly, Oscillospira and Faecalibacterium genera seem to have a protective action against inflammation (68). The increase of glyceric acid in the stool of patients with CD could be due to a release of triacylglycerols associated with the colon mucosa (69). The high levels of amino acids, such as alanine, beta-alanine and phenylalanine in the faeces of CD patients might result at least in part from malabsorption due to inflammation of colonic mucosa in IBD patients or reflect an increase of aminoacid-producing bacteria. Two group B vitamins, nicotinic acid and pantothenic acid were significantly decreased in faeces of CD patients (70). Some studies report that pantothenic acid may have a protective effect against oxidative stress in mammalian tissues (71). Furthermore, it seems that nicotinic acid might also exert a beneficial effect on the mucosa of the colon, reducing inflammation (72). The decreased levels of nicotinic acid in patients with CD may be due to a decrease in bacteria producing NA. Decreased levels of this vitamin were directly correlated with the reduced amount of the Faecalibacterium bacteria, particularly, with the decreased abundance of Faecalibacterium prausnitzii. It has already been demonstrated that Faecalibacterium has a protective role against inflammation of the colon mucosa (73). Faecalibacterium prausnitzii abundance was also inversely correlated with phenylethylamine. This amino acid is biosynthesized from the amino acid L-phenylalanine by enzymatic decarboxylation via the enzyme aromatic L-amino acid decarboxylase

(74). In addition to its presence in mammals, phenethylamine is found in many other organisms and foods, such as chocolate, especially after microbial fermentation. The concentration of phenylethylamine was increased in faeces of CD patients, suggesting that the depletion of Faecalibacterium prausnitzii could lead to an increase of some amine, which in turn could play a role in the pathogenesis of an inflammatory process (75).

The amount of methylamine was decreased in the aqueous faecal water extracts of patients with CD. This compound is derived from intestinal degradation of food components such as choline and carnitine by microbiota. The depletion of this microbiota-related metabolite correlated with the decrease of Oscillospira amount in faeces, confirming the perturbation of the microbial homeostasis in patients with CD (76).

In this work, 5β-coprostanol was found to be decreased in faeces of CD patients. 5β-coprostanol is produced from the catabolism of cholesterol by gut microbiota (77). Again, the reduced amount of this metabolite correlated with the Oscillospira and Flavobacterium decrease in faeces of CD patients. The low abundance of Oscillospira genus bacteria correlated also to the reduced levels of hydrocinnamic acid, 3-methyladipic acid, citric acid and 2-hydroxy-3-methylvaleric acid, confirming again the importance of this bacterium in the gut metabolism and wellness.

In addition to the previously described metabolites, faecal extracts derived from UC patients showed increases in tyramine, TMAO, glycine, glucose, 5-aminovaleric acid, and decreases of glutamic acid, pyroglutamic acid, linoleic acid, sebacic acid and trycarballylic acid. Furthermore, in UC patients there were decreases of 3-methyladipic acid, 2-hydroxy-3-methylvaleric acid, citric acid, and methylamine. The reduced levels of all these metabolites strongly correlated with the lower abundance of the Flavobacterium genus, belonging to the Bacteroidetes phylum. This genus was also negatively correlated with TMAO levels. TMAO is generated by anaerobic bacteria through the digestion of dietary phosphatidylcholine and carnitine in a microbial-mammalian co-metabolic pathway. One previous study has found that TMAO to induce inflammatory gene expression in both

of these relevant cell types as well as endothelial cell adhesion of leukocytes. (78). 3-Methyladipic acid, 2-hydroxy-3-methylvaleric acid and citric acid levels were also positively correlated to Oscillospira abundance, suggesting a putative anti-inflammatory effect of Oscillospira in UC. As previously described in the results, glucose was increased in faeces of UC patients. Glucose serves as an energy source for normal intestinal mucosa and its use is reduced during malnutrition and starvation, a common symptom observed in UC (79). High levels of glucose thus indicate the inability of the colonic mucosal cells to use it for energy requirements. All these data are reported in a published work (80).

Metabolic alteration in plasma and biopsies from IBD patients

Beyond faeces analysis, also plasma and biopsies samples deriving from the same population group were analysed. The untargeted metabolomics approach constitutes one of the most frequently applied methods in metabolomics studies. It aims to measure the comprehensive metabolomics profiles of various biological samples. The main goal of the application of the untargeted approach in biomedical studies is to discover novel biological markers as well as to gain new insights into mechanisms underlying the pathophysiology of human diseases (81).

This study showed that thanks to the metabolites profile obtained by the GC-MS analysis of plasma samples of patients, it was possible to distinguish both CD and UC patients from healthy patients. As showed in the MVA indeed, both OPLS-DA were reported excellent statistical parameters, indicating that the models are robust and with good predictive power. This result is confirmed also by the analysis of the patient's biopsies. OPLS-DA of comparisons between healthy colon and affected colon showed a good separation for both UC and CD. Moreover, this result is also confirmed by the comparison between healthy and affected ileum from CD patients. Talking about CD, the attention was focused on understanding the effect of the acquisition of an inflammatory metabolism on ileum

and colon normal metabolism. To check this effect, a first OPLS-DA analysis was carried out between healthy ileum and healthy colon. As seen in figure 19d and table 4, there is a clear separation between the two tissues indicating the different metabolic profile of each tissue. However, when both ileum and colon are affected by inflammation, the differences between the two tissues seem to disappear as they are pooled together by a common inflammatory metabolism (Figure 19e and table 4).

Meanwhile, the OLPS-DA aiming to differentiate between CD and UC did not show good parameters in plasma. The same outcome derives from biopsies analysis when comparing CD and UC affected colon. This result, confirming the same situation already seen in faeces samples, and underline the intrinsic similarity between these two pathological conditions. As a matter of fact, in some cases, it is not possible to have a differentiation between the two pathologies (82). Due to their similar metabolic profile, in plasma and biopsies of CD and UC patients were analysed together and referred as IBD. The OPLS-DA between healthy and IBD showed again good statistical parameters both in plasma and colon and ileum biopsies (Figure 16b and 19g and h). Furthermore, the analysis was focused on understanding which metabolites were responsible for this separation. From the univariate statistical analysis, it was seen that two metabolites were found altered in both plasma and colon biopsies: lactic acid and ornithine. Particularly, lactic acid was increased in IBD plasma and decreased in IBD colon. Lactate, the final product of anaerobic glycolysis, is produced in high amounts by innate immune cells during inflammatory activation. Extracellular lactate induces metabolic reprogramming in innate immune cells, as evidenced by reduced glycolytic and increased oxidative rates of monocytes immediately after exposure to lactate (83). Moreover, these results are in accordance with the recent finding that suggested that increased lactate level in blood was possibly due to highly expressed lactate transporter, monocarboxylate transporter 4 (MCT4), expression caused by inflammation in intestinal mucosal epithelial tissue (84).

On the other hand, ornithine was found decreased in IBD plasma and increased in IBD colon. Ornithine is a non-proteinogenic amino acid that plays a role in the urea cycle (85). Urea level, as

well as ornithine, was lower in plasma of patients with IBD, indicating an involvement of urea cycle in the inflammation-driven metabolic shift.

The results also showed that in IBD patient's plasma there was an increased level of 2-hydroxybutyric acid and decreased levels of threonine. 2-Hydroxybutyric acid (alpha-hydroxybutyrate) is an organic acid derived from alpha-ketobutyrate. Alpha-ketobutyrate is produced by amino acid catabolism such as threonine anabolism and is metabolized to propionyl-CoA and carbon dioxide. 2-Hydroxybutyric acid has been shown to be an early marker for both insulin resistance and impaired glucose regulation. The underlying biochemical mechanisms may involve increased lipid oxidation and oxidative stress (86). Many chronic diseases linked with higher production of ROS result in oxidative stress and protein oxidations. Many authors have documented the relationship between oxidative stress and inflammation. Evidence indicated that oxidative stress plays a pathogenic role in chronic inflammatory diseases (87).

While in faeces samples 3-hydroxybutyric acid was found to be decreased in IBD patients, in plasma 3-hydroxybutyric levels were increased in patients with IBD. 3-hydroxybutyric acid (or beta-hydroxybutyrate) is a ketone body. Apart from serving as energy fuels for extrahepatic tissues like brain, heart, or skeletal muscle, ketone bodies play pivotal roles as signaling mediators, drivers of protein post-translational modification, and modulators of inflammation and oxidative stress. High levels of 3-hydroxybutyric acid could be related to the increased energetic pathway already described in inflammation (88). In addition, citric acid was increased in IBD plasma and decreased in IBD faeces. Citric acid together with fumaric acid, decreased in colon affected by IBD, are part of the TCA cycle. Activated dendritic cells (DCs) and macrophages have an altered TCA cycle, one consequence of which is the accumulation of both citrate and succinate. Citrate is exported from the mitochondria via the mitochondrial citrate- carrier. Cytosolic metabolism of citrate to acetyl-coenzyme A (acetyl-CoA) is important for both fatty-acid synthesis and protein acetylation, both of which have been linked to macrophage and DC activation. Citrate-derived itaconate has a direct

antibacterial effect and has been shown to act as an anti-inflammatory agent, inhibiting succinate dehydrogenase (89). These findings identify the TCA cycle as an important pathway involved in the inflammatory process.

In plasma of IBD patients altered levels of lysine, uric acid and proline were also found. The first two were increased while proline was decreased. Uric acid is the oxidative product of hypoxanthine and xanthine through the action of the enzyme xanthine oxidase and it takes part in the purine metabolism. It has been shown that an impaired purine metabolic pathway is associated with excessive inflammation and inappropriate resolution in numerous human inflammatory diseases, including inflammatory bowel disease (IBD), ischemia, diabetes and cancer (90,91).

Proline, together with aspartic acid, that was increased in IBD affected colon, also participates in immune responses that may maintain intestinal health and protect against animal and human diseases (92). Moreover, proline is also derived from glutamine and glutamic acid metabolism. In the present study, both glutamine and glutamic acid were increased in IBD colon biopsies and glutamic acid decreased in IBD faeces. In intestinal epithelial cells, glutamine is quantitatively the most important fuel. It is metabolized to glutamate, which undergoes transamination, so the metabolites of this reaction are oxidized in the TCA cycle to generate pyruvate. Newer research has shown that glutamine is the main energy substrate of enterocytes. It is required also by cell division, as glutamine supplies of half of the nitrogen requirement for purine and pyrimidine synthesis. The entire gastrointestinal tract extracts around 20 % of circulating post-absorptive glutamine and over 90% of the glutamine extracted by the small intestine are metabolized by mucosal cells (93). The measurements of intestinal glutamine metabolism also showed that glutamine is the precursor for several important metabolic pathways, especially those leading to the synthesis of ornithine, citrulline, proline, and arginine (94). Glutamine dipeptides induce mucosa proliferation of the ileum and colon in human. Several lines of evidence indicate that glutamine has an anti-inflammatory property by influencing several inflammatory signaling pathways, including the nuclear factor κB (NF-κB) and signal transducer and

activator of transcription (STAT) pathways (95). Higher levels of glutamine and glutamic acid found in IBD colon could be then a consequence of the protective host reaction to inflammation. Aspartic acid metabolism also seems to be changed in IBD. The level of aspartic acid was indeed upraised in IBD colon biopsies and lysine was higher in IBD plasma when compared to healthy indicating an impairing of this pathway. It has already been seen that aspartic acid is associated with inhibition of TLR4 and NODs/NF-κB and p38 signaling pathways and concomitant improvement of intestinal integrity under an inflammatory condition in animal studies (96). Looking at colon biopsies, the level of myo-inositol was found to be higher in these patients. Myo-Inositol is synthesized from glucose, via glucose-6-phosphate (G-6-P) in two steps. First, G-6-P is isomerised by an inositol-3-phosphate synthase enzyme to myo-inositol 1-phosphate, which is then dephosphorylated by an inositol monophosphatase enzyme to give free myo-inositol. In humans, myo-inositol is primarily synthesized in the kidneys at a rate of a few grams per day. As it is a component of the plasma membrane, it represents an important molecule in many biological processes including intracellular signaling, intracellular calcium concentration control, and cell membrane potential maintenance. It has been shown that myo-inositol stimulates contractility of the gastrointestinal tract improving intestinal regularity and bloating (97). Finally, glycine was found increased in IBD colon biopsies, reflecting what was seen also in the faeces samples. It has been shown to have anti-inflammatory, immunomodulatory, and direct cytoprotective activity (92).

In vitro study

Once the results of the in vivo study were obtained, the attention was pointed particularly to the minor levels of nicotinic acid found in the faeces of both UC and CD patients compared to healthy patients. Nicotinic acid, also known as niacin or vitamin B3, is a water-soluble vitamin whose derivatives such as NADH, NAD, NAD+, and NADP play essential roles in energy metabolism in the living cell and DNA repair. The designation vitamin B3 also includes the amide form, nicotinamide or niacinamide. Severe lack of niacin causes the deficiency disease pellagra, whereas a mild deficiency slows down the metabolism decreasing cold tolerance. The recommended daily allowance of niacin is 2-12 mg a day for children, 14 mg a day for women, 16 mg a day for men, and 18 mg a day for pregnant or breast-feeding women. Good sources of niacin are liver, meat and meat products, fish, peanuts and whole grains. The liver can synthesize niacin from the essential amino acid tryptophan, but the synthesis is extremely slow and requires vitamin B6. Bacteria in the gut may also perform the conversion but are inefficient (98). Moreover, different studies proved that nicotinic acid has a role in ameliorating the inflammatory process (99,100).

In the present project, an in vitro model was built to study the nicotinic acid effect on intestinal inflammation. Differentiated Caco-2 cells were used and inflammation was induced with the two major players in intestinal inflammation, LPS and IL-1β. As explained before, it is widely accepted that the composition of the intestinal microbiota contributes to the progression and outcome of IBD. LPS is a toxic component of the outer membrane of Gram-negative bacteria and it is a potent initiator of inflammation. Indeed, LPS induces systemic inflammatory injury and various kinds of pathological changes. Particularly, the intestine is exposed to many antigens derived from food, toxins and bacteria, is also one of the target organs of LPS. By inducing inflammatory injury of the intestinal epithelium, LPS can lead to damage of the integrity of the mucosal barrier, which plays an important role against the penetration of a variety of antigens from the gut lumen to the blood stream (101). Furthermore, LPS activates endothelia in the rich capillary network of the small intestine to secrete

high levels of cytokines (102). Among cytokines, IL-1β seems to play a very important role in intestinal inflammation as several clinical studies have reported high levels of IL-1β secretion by colon lamina propria monocytes from patients with active IBD. Moreover, IL-1β levels in the colon correlated with disease activity and high levels of IL-1β were associated with active lesions (103,104), suggesting an important role of this cytokine in promoting localized inflammation. Different genetic lesions associated with IBD development in animal models are associated with increased IL-1β and it has been proved that IL-1β can modulate the function of both immune and nonimmune cells. Indeed, stimulation with IL-1β promotes the activation and effector functions of dendritic cells, macrophages, and neutrophils. Moreover, IL-1β can induce neutrophilia and promote neutrophil migration. IL-1β promotes T cell activation and survival and has recently been shown to act in concert with other proinflammatory cytokines to promote the differentiation of CD4+ Th17 cells (105). In this study, LPS and IL-1β were considered singularly in order to evaluate their individual effect on metabolism and the inflammatory effect was evaluated measuring the production of IL-8. Indeed, one of the earliest reported chemokines produced by intestinal epithelial cells is the IL-8. This cytokine, as a member of the CXC chemokines family, is not only a strong chemoattractant for neutrophils, monocytes, etc. but also triggers the secretion of superoxide anions and lysosomal enzymes in neutrophils, thus contributing to the tissue damage during inflammation (106). IL-8 mRNA expression in the inflamed mucosa is shown to be significantly higher than the level in non-inflamed mucosa of IBD patients or in the normal mucosa of non-IBD patients (25).

The results obtained in the present study showed that IL-8 levels were strongly upregulated in Caco-2 cells when treated with both LPS and IL-1β. It is also clear that the production of IL-8 is much higher after IL-1β compared to LPS treatment, indicating that the effect of IL-1β is much stronger than the LPS effect in this in vitro model (Figure 25). These results are in accordance with the data reported in the literature, in which it was seen that the production of IL-8 is up regulated by IL-1β and LPS in human HT-29 colonic epithelial cells (107,108). Once the inflammation state was

obtained, samples were analysed to investigate the effect of inflammation on metabolism in this in-vitro intestinal model. It is evident that both LPS and IL-1β had the common effect of decreasing the levels of 2-hydroxybutyric acid, fumaric acid, serine and glycero-phosphocholine and increasing malic acid at the intracellular level and increasing glutamine and decreasing glucose in the extracellular medium (Figure 26). Moreover, IL-1β induced also an increase of acetic acid, citric acid and glutamic acid and a decrease of 3-hydroxybutyric acid, alanine, fructose, glutamine, threonine, isoleucine, lactic acid and ornithine in Caco-2 cells. Otherwise, alanine, lactic acid and glutamine were found to increase in the medium of IL-1β treated cells (Figure 27). As the levels of these metabolites were low inside the cells this could mean that these metabolites could have been released outside the cells. Uric acid as well was found increased in IL-1β treated cells medium. The levels of glycine, ornithine and glucose were decreased in the medium of the same treated cells. Only in the LPS treated cell, there was a decrease of pyruvic acid at the intracellular level and an increase of serine and cholesterol at the extracellular level (Figure 27). The obtained results are in accordance with what was seen in the clinical samples from IBD patients. Comparing this data with the data coming from the in vivo study previously discussed it is clear as several metabolites are altered the same way in both in vivo and in vitro model. Indeed, looking at the colon biopsies metabolites, there was an increase of glutamic acid and a decrease of fumaric and lactic acid. The same alterations were found in the Caco-2 cell model. On the other hand, glutamine that in colon biopsies were up regulated was decreased in Caco-2 cells. The same state was highlighted comparing altered metabolites in plasma samples and extracellular metabolites in the in vitro model. Lactic acid and uric acid were found to increase in both plasma and cell medium in fact, while ornithine levels were downregulated. Higher levels of alanine were pointed out in both patients faeces and Caco-2 cells medium. Taken together these results showed that it was possible to reproduce some of metabolic alterations that were highlighted in the samples coming from IBD patients were shared also in the Caco-2 in vitro model of inflammation. This means that this model is reliable and trustworthy to study inflammation mechanism. NA treatment was able to significantly reduce the level of IL-8 produced in both LPS

and IL-1β treated cells (Figure 25). These results mean that nicotinic acid seems to have an effect in reducing inflammation in the enterocytes and that this effect is dose depending. This data is in accordance with what was found in the literature. In fact, as mentioned above, anti-inflammatory effects of nicotinic acid were described in other in vitro and in vivo models (109). Moreover, one of the purposes of the study was to investigate the effect of nicotinic acid on normal and inflammation-related metabolism. As shown in figure 26, nicotinic acid seems to have an effect on differentiated Caco-2 cell metabolism. Indeed, when comparing non-treated and treated cells an increase of ATP, lactic acid, glycerol-phosphate and glycerophosphocholine and a decrease of fumaric acid and pyruvic acid at the intracellular level and an increase of serine and tyrosine and a decrease of alanine at the extracellular side were observed. The increase of the ATP levels and the decreased level of pyruvate may indicate a higher glycolytic metabolism. Therefore, nicotinic acid seems to affect itself the energetic metabolism. These data may help to understand and clarify the effects of nicotinic acid on lipids and glucose metabolism described in the literature (110). As mentioned before, the effects of nicotinic acid on the inflammation metabolism were evaluated as well. Interestingly, from the results obtained in the present study, it was noticed that nicotinic acid treatment seemed to have a restoring effect on different metabolites. For example, the level of glutamic acid, which was downregulated by IL-1β treatment, was then increased comparing it with IL-1β plus nicotinic acid treated cells. On the other hand, levels of glutamine, isoleucine, ornithine and glycerophosphocholine, which were upregulated by IL-1β, were decreased in the comparison between IL-1β and IL-1β plus nicotinic acid treated cells. This same trend was confirmed at the extracellular level, where the levels of alanine and glutamine that were increased by IL-1β were then decreased thanks to nicotinic acid effect and ornithine level that was downregulated by IL-1β was increased by nicotinic acid. Looking at the effect of nicotinic acid on LPS induced inflammation, it is visible again the restoring effect of nicotinic acid as it was able to increase the level of glycerophosphocholine, which was decreased by LPS treatment and to decrease the level of serine, which was increased by LPS.

VI. Conclusion

In conclusion, we can say that metabolomics is a tool that allows us to advance our understanding of the pathogenesis, diagnosis and treatment of chronic inflammatory bowel diseases. Using this approach, altered metabolic pathways in patients with these diseases could be identified.

The study here presented showed that the analysis of metabolites in plasma, colon biopsies and faecal samples of IBD patients could be used to discriminate between healthy and affected subjects. This analysis was also able to confirm the peculiar metabolic profile of plasma, colon tissue and faeces of IBD patients, in accordance with literature data, suggesting that this overlapping of results may be very important in the future for the determination of new biomarkers for these intestinal diseases. Unfortunately, as reported above, our analysis was not able to discriminate between patients with CD and patients with UC, confirming the high similarity between these two intestinal pathologies. Moreover, this study demonstrates that the metabolic profile could be a powerful tool to identify intestinal inflammation, which can be useful both in the management of IBD, indicating possible therapeutic targets and in clinical studies that explore the pathogenesis of diseases. The actual clinical utility of the metabolic changes identified by this work will have to be established in future studies on a higher number of subjects.

In addition, it was demonstrated the correlation between metabolic changes and microbiota alterations and how this can allow a greater understanding of gastrointestinal pathophysiological processes and clarify the importance of alteration of the intestinal bacterial flora due to the loss of health of our intestine.

Lastly, thanks to the in vitro study of the intestinal inflammation it was demonstrated that nicotinic acid seems to have a protective effect against intestinal inflammation, having the effect of reducing the levels of IL-8 production after both treatments with IL-1β and LPS that are the major players in the IBD development. These data also showed that nicotinic acid seems to fulfil its role acting also on metabolism and restoring several metabolites changes caused by the inflammation process. Even

if future studies are needed to better understand the effect of nicotinic acid in the protection from IBD development and progression, our data could be considered as a very interesting starting point.

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