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INFLAMMATORY BOWEL DISEASE STUDY: A METABOLOMICS APPROACH

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PREFACE

This dissertation entitled “Inflammatory bowel disease study: a metabolomics approach” is a result of three academic years of PhD in Molecular and Translational Medicine, from 2014 to 2017. This project covers the part of a wide project made in collaboration with the Gastroenterology Unit of Cagliari and the Department of Biomedical Science. The aim of this project was to investigate the metabolomics profiles of plasma and faecal samples trying to identify possible biomarkers for the early diagnosis of Inflammatory Bowel Disease (IBD) and to find correlation between the microbiota and the metabolome of stools of patients affected by IBD. To whom it may concerns, during my PhD, I followed the metabolomics analysis of faecal and plasma samples setting up new analytical methods for the extraction and the mass spectrometry analysis. I also performed the multivariate and univariate data statistical analysis. The first year, I worked in Cagliari, Italy, at the Department of Life and Environmental Science where I analysed the lipid profile of faecal samples. In April 2016, I started an internship at the Department of Biochemistry, University of Cambridge in England where I performed the analysis of lipid and polar profile of faecal and plasma samples. The research was difficult, but conducting extensive investigations allowed me to create a robust predictive model for identification of IBD samples, further several metabolic pathways were correlated with the IBD. The microbiota analysis comprehensive of the correlations between the metabolomics data results were performed by the teams of Professor Aldo Manzin and Professor Luigi Atzori and thanks to these goals an original article was published in the Scientific Reports Journal. Although these results need more investigation to clarify the most important metabolic pathway correlated with the IBD pathologies, this project, as it comes, can be considered valuable for the IBD investigations.

I would like to thank my supervisors Professor Pierluigi Caboni and Professor Jules Griffin for their excellent guidance and support during this process.

I, also, wish to thank all the respondents, Sonia Liggi, Maria Laura Santoru, Christine Hinz, Zoe All, James West, Steve Murfitt and Ben McNally without whose cooperation I would not have been able to conduct this analysis.

I hope that this work would be a valid help for people that are studying pathologies with a metabolomics approach and I hope you will enjoy your reading.

Antonio Murgia

Cagliari, January 15, 2017

ABBREVIATIONS

APCI: Atmospheric-pressure chemical ionisation

ADMA: asymmetric dimethyl arginine

ATG16L1: autophagy-related protein 16-1

BMI: body mass index

cAMP: Cyclic adenosine monophosphate

CCS: collisional cross section

CD: Crohn's disease

CDP: cytidine diphosphate

Cer: Ceramides

CRP: C-reactive protein

CT: Computerized tomography

CTP: cytidine triphosphate

DT: drift time

ESI: electrospray ionisation

ESR: erythrocyte sedimentation rate

FA: fatty acid

FMT: faecal microbiota transplantation

GC/MS: gas-chromatography/mass spectrometry

^{1}H NMR: proton nuclear magnetic resonance

IBD: Inflammatory bowel disease

IL23R: Interleukine 23 receptor gene

IL (n): Interleukine (n)

IM-QTOF-LC/ MS: ion mobility coupled with a time of flight quadrupole and a liquid chromatograph

IRGM: immunity-related GTPase family M protein

LC/MS: liquid-chromatography/mass spectrometry

MAPK: mitogen-activated protein kinase

MRI: Magnetic resonance imaging

MDP: muramyl dipeptide

MS: mass spectrometry

MS/MS: tandem mass spectrometry analysis

NAE: N-acylethanolamide

NOD2: Nucleotide-binding oligomerization domain-containing protein 2

OPLS-DA: orthogonal partial least square analysis

PA: phosphatidic acid

PC: phosphatidylcholines

pc: principal component

PCA: principal components analysis

PE: phosphatidylethanolamines

PG: glycerophospholipids

PKC: kinase C

PI: phosphatidylinositols

PLA2: phospholipase A2

PLS-DA: partial least square discriminant analysis

PMN-elastase: polymorph nuclear elastase

PS: phosphatidylserine

QC: quality control

QqQ: triple-quadrupole

Q-TOF: quadrupole time-of-flight

SM: sphingomyelin

TG: triacylglycerols

Th(n): T helper cells (n)

Th-nR: T helper cells receptor

TLRs: toll-like receptors

TNF-α: tumor necrosis factor alpha

UC: Ulcerative colitis

VIP: variable importance in the project

ABSTRACT

Inflammatory bowel disease represents a group of chronic disorders that affect one or more parts of the intestine. Although recently the incidence of inflammatory bowel disease has noticeably increased, its aetiology is still unclear. No specific pathogen has been defined as a causative agent. Serological biomarkers have been recently proposed for diagnosis, but they remain untested in clinical applications. Moreover, current diagnostic and monitoring practices for inflammatory bowel disease are very invasive. Therefore, accurate tools for the early diagnosis, and in particular non-invasive strategies are needed. One area of recent interest is the relationship between the metabolome and microbiota in terms of inflammatory bowel disease pathogenesis and intervention strategies. Direct analysis of metabolites and their interaction with the gut microbial species is a rapidly growing field of research. In this context, metabolomics could represent a useful approach to understand possible pathological mechanisms or metabolite modification in different pathways. Metabolomics is based on the quantitative measurement of dynamic metabolic changes in living systems in response to physiological stimuli or genetic modifications, nutrients and drugs. Thanks to the comprehensive study of low molecular weight metabolites (<1500 Da) in biofluids and in tissues, metabolomics ensures the characterization of the metabolic phenotype of a living organism. In this work, the polar and the lipid metabolite profile of faecal and plasma samples of patients affected by inflammatory bowel disease and healthy patients has been studied by high resolution liquid chromatography coupled with several technological platforms such as a triple quadrupole, a quadrupole time of flight mass spectrometry and an ion mobility prior to multivariate statistical data analysis. By these means, differences between Crohn's disease, ulcerative colitis and control samples were investigated. Results of discriminant analysis were considered with the aim to find the relevant metabolites unique for each class. The results highlight differences in the metabolite profile between the pathological and the control samples. In faecal samples, the most discriminant metabolites for both IBD classes were diacylglycerols, phosphatidylcholines triacylglycerols and tetra pyrrole compounds. Furthermore, plasma samples results showed modification of the lysophosphatidylcholine and phosphatidylcholine pathways strictly related to the arachidonic acid response. These results highlight similarity in the metabolic alteration occurring in both ulcerative colitis and Crohn's disease when compared to healthy

controls. Modification to the endocannabinoid system were also found regarding the ulcerative colitis plasma and faecal samples. All the lipid results were correlated with the targeted analysis of the polar metabolites profile. In conclusion, the application of metabolomics to faecal and plasma samples of IBD patients allows for the identification of metabolites that can be used as possible indicators of metabolic pathways implicated in the onset of these pathologies.

1. INTRODUCTION

1.1. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a group of pathologies characterized by the presence of chronic phlogosis without a clear infective aetiology (1). The two major clinically defined forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (2). When it is not possible to distinguish these two pathologies, due to similarities in clinical manifestations, they are named "indeterminate colitis". Three additional forms of non-specific IBD, termed microscopic ulcerative, microscopic lymphocytic and microscopic collagenous colitis are also documented (3) while other similar pathologies, such as microscopic colitis, and the Behçet syndrome are not classified as strict IBD (4, 5).

1.2. Historical background

The first documented case of UC by Hildenus was in 1612 after an autopsy of a boy who died following abdominal pain (6). Later, Morgagni (1682-1771) described ulceration and perforation in an inflamed distal ileum and enlarged mesenteric lymph nodes in a 20 years old man with a history of diarrhoea and fever culminating in death after 14 days (7). Afterwards, a remarkable increase and geographically spread was observed, and today, these pathologies are recognized worldwide (8). Several reported cases of IBD date back to the 19th and early 20th centuries, appearing initially as isolated cases in Great Britain and northern Europe (9). In 1793 Matthew Baillie strongly suggested that patients were dying from ulcerative colitis during the latter part of the 18th century (9). Other cases were reported by Combe and Saunders, by Abercrombie and by Abraham Colles (10-12). The first "impact" description of "ulcerative colitis" by Samuel Wilks in 1859 concerned a 42 year old woman who died after several months of diarrhoea and fever. In the 20th century, similar conditions associated with lower abdominal (inflammatory) masses, arbitrarily dismissed as "untreatable" were documented in many reports (13, 14). In 1932, Nuboer, Golob and later in 1934 Bissell reported instances of a similar disease (15-17). Nevertheless, it was not until 1936 that one case of 9 patients with combined ileitis and right-sided

colitis was described by Crohn et al. (18). However, this diagnosis was not completely accepted in America until 1960 after the reports of Lockhart Mummery et al. (19).

1.3. Epidemiology

The incidence of IBD varies from country to country. Between 4 and 10 people per 100,000 annually are affected by IBD and the main two forms affect 1.5 million Americans, 2.2 million Europeans, and several hundred thousands more individuals worldwide (3, 20). The highest incidences of IBD have been reported in Northern Europe, with the United Kingdom and North America presenting a greater incidence of UC than CD (21). CD is slightly more common in females and occurs at a younger age than UC (3). However, the incidence of IBD is increasing in emerging areas such as Asia, probably due to changes in environmental factors and in particular diet.

Over the average human lifespan, the peak incidence of IBD is in the second to fourth decade of life and this observation has remained the same over several decades (22). Incidence in established populations is similar in men and women, but is influenced by race and ethnicity. The risk of IBD is higher in the Jewish population and even more so in American and European Jewish populations if compared with those of Israel (23). Furthermore, CD incidence is higher in northern latitudes than in southern. It has also been noticed that IBD incidence is higher in urban populations when compared with rural populations (24). On the other hand, recent studies have supported that IBD is no longer rare in economically emerging populations.

Concerning genetic issues, the risk of IBD can be influenced by the family history e.g. if both of the parents were affected: in this case the probability of developing the disease in the first 30 years of age is estimated to be as high as one-in three. In addition, twin studies have shown that monozygotic twins have a higher risk of contracting the pathology compared with dizygotic twins (25). Furthermore, genetic linkage studies identified an association with a locus on chromosome 16, called subsequently the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) locus (26). Another 163 distinct risk loci associated with other genes were also discovered (27). Genetics studies have also found that the internal microenvironment and immunological response are both influenced by the external environment (28). In fact different studies established that IBD microbial response and innate immunity are

associated with the gut microbiota (29). A marked luminal microbiota dysbiosis was documented in IBD patients, characterized by a decrease in microbial diversity if compared with healthy individuals (29). In addition, early life exposure to pets and farm animals are inversely associated with IBD risk (29). It has also been shown that breastfeeding seems to be protective (29). The external environmental strongly influences the gut microbiota (29). Harries et al. described a reduced frequency in cigarettes use among patients with ulcerative colitis compared with healthy controls (30). As well as smoking, also appendectomies could be protective against the onset of the UC, even if some works report that this surgery could help increase the risk of CD (31). Gut health can also be influenced, by stress. For this reason, IBD is associated with neuroticism, obsessive-compulsive behaviour and psychological stressors. Stress can act using the autonomic nervous system, producing pro-inflammatory cytokines or activating macrophages and altering intestinal permeability (32). Furthermore, different diets could influence the pathogenesis of IBD (33). Different studies found that a fibre rich diet could reduce the risk of Crohn's disease, while a diet rich in saturated lipids can have a role in the pathogenesis of these pathologies (33). In addition, vitamin D is implicated in the pathogenesis and spread of IBD (34). In fact, mice deficient in 1,25-dihydroxy vitamin D3 or knockout for vitamin D receptor seem to tend to contract colitis, while, administration of the vitamin D ameliorate this inflammation (34).

Zinc and iron are also described as implicated in the immune function of IBD; with zinc inhibiting transcription of inflammatory mediators important for autophagy and bacteria clearance (35), and iron inducing colonic inflammation through the interleukin pathway (36).

Through all these suggestions, IBD can be considered as very complex disease induced by several and interconnected factors.

1.4. Aetiology

Different theories have been proposed in the past concerning the causes of CD and UC, however, most of them have failed to find explanations.

These theories were focused on the T cell pathway, a complex set of cytokines, chemokines and other mediators orchestrated by a variety of cell types. The basic idea

was that T cells are activated by an unlucky confluence of genetic and environmental factors that cause an immune imbalance, ending with the characteristic inflammation, prerogative of these diseases (37). In 1913, after this theory, several bacteria (*Diplostreptococci*, *Bacteroides fragilis*, *Pseudomonas maltophilia*, *Helicobacter hepaticus* and *pylori* species and, *Shigella*, *Chlamydia*, *Listeria*, *Escherichia coli*, *Wolinella*) and viruses (both A and B *Coxsakie*, *Reovirus*, *Polio*, *Norwalk*, *Influenze B*, *herpes*, *Paramyxovirus*) were identified as possible etiological agents. Furthermore, atypical forms such as L-forms (cell wall defective bacteria), protoplasts, or spheroplasts, have been suggested (7, 38).

In 1995 Wakefield et al. suggested a connection between CD and Measles virus (39). This theory was strengthened by subsequent epidemiological studies that demonstrated an increase in CD after the exposure to measles virus (39). Even the attenuated vaccine was associated with the increase of the risk. Subsequently, measles was proposed as a cofactor of CD (40).

In the middle of the 20th Century, CD and UC were considered as psychosomatic diseases based on their connection with emotional disturbances (41). This concept died slowly in the early 1980s after the discovery of more significant theories about diet and allergies. In fact, several dietary risk factors have been found regarding infant feeding practices (42). Infant breast-feeding has a protective role against the development of CD and UC, while a diet rich in carbohydrates, in particular simple carbohydrates, or in fast food can increase the risk of IBD (43). These suggestions were found by several studies using elimination diets or dietary challenges.

In 1997, Roediger et al. considered sulfur compounds as etiological agents of UC (44). Recent studies have confirmed this theory, finding a correlation between UC and a diet rich in sulfuric compounds. When the sulfur is not recycled by the intestinal pump, it can pass into the colon where it is metabolized by sulfate-reducing bacteria that use it to create sulfoxides, a family of compound that can damage the intestinal mucosa. (44). Different typologies of diet were found to be correlated with an increase in the risk of IBD (45). For example, the ingestion of toothpaste can cause IBD. Several substances in toothpaste such as tricalcium phosphate and quartz can penetrate the intestinal epithelium creating enteric lesions similar to CD (46).

One of the latest components to be found as a cause of CD is the increase in intestinal permeability (47). This modification across the intestinal epithelium can cause an increase in immune exposure to different antigens. This element was not found in UC patients (47). These observations can be considered the beginning of a long series of theories that are still present nowadays, where CD has been proposed to be a consequence of innate intestinal mucosal barrier deficiency (47). This idea suggests that patients with genetically defined syndromes of neutrophil and monocyte dysfunction syndromes can develop an intestinal phenotype from CD (47).

Recently, changes in the intestinal flora, has been suggested as a permissive factor that can help the emergence of IBD (48). Several animal models confirmed that helminths could have a significant impact on a variety of immune functions like cytokines and T cell pathways (49).

Finally yet importantly, it is the recent proposal that considers fundamental dysbiosis as the root cause of IBD. This dysbiosis is caused by the alteration of gut flora (50). Several studies reported an increased concentration of anaerobes, particularly Gram negative, including *Bacteroides* and *Clostridium perfringens* in pathological samples. Enterobacteriaceae, were found to be increased in CD (51). On the contrary, concentrations of beneficial bacteria, such as *Bifidobacteria*, were found to be decreased in IBD patients (51). Concerning *Bifidobacteria*, in the last decades numerous therapies including probiotics and prebiotics that can have a beneficial effect on immune function and mucosal integrity have emerged (51, 52). Moreover, serological studies revealed specific antibodies and T cell subset in serum and intestinal tissues of IBD patients (53). These studies, gathered with those that revealed the increase of permeability and the reaction to bacteria antigens, confirming the theory of dysbiosis (53, 54). Alterations in faecal flora have been found in healthy relatives of patients with IBD (54). These results suggest that dysbiosis should be considered as a genetic risk factor for the development of IBD.

1.5. Pathogenesis

Given the epidemiological and etiological notions reported so far, it seems clear that genetic predisposition, external environment, intestinal microbial flora and immune response are all convoluted and functionally combined in the pathogenesis of IBD.

This combination is regularly taken into consideration by the scientific community, with the aim to find a clear mechanism of pathogenesis for IBD and to highlight the most peculiar characteristics of each pathology (CD or UC).

During recent years, genetic research was suitable to discover 163 IBD-associated gene loci, of which 110 are associated with both of diseases, 30 only with CD and 22 with UC. These new results could be very helpful in finding differences between the pathogenesis of these two pathologies (55).

The NOD2 was the first susceptibility gene identified for CD. This is a gene encode of an intracellular receptor recognizing a conserved motif of Gram positive and negative peptidoglycan bacteria (56). The stimulation of this motif, called muramyl dipeptide (MDP), induces autophagy with a consequent replication and bacteria antigen presentation control. Furthermore, MDP modulates both innate and adaptive immune response (57).

The autophagy role in IBD immune response was later confirmed by the discovery of a coding mutation in two different autophagy-related genes which codify for the autophagy-related protein 16-1 (ATG16L1) and the immunity-related GTPase family M protein (IRGM) (58, 59). ATG16L1 is essential for all forms of autophagy and its mutation is associated with an increase in CD. Similarly, the reduced expression of IRGM, is connected to the CD polymorphism (60, 61). Moreover, Duerr et al., discovered an association between IBD and the Interleukin 23 receptor gene (IL23R) (62). IL23R encodes, a subunit of the pro-inflammatory cytokine, interleukin (IL)-23, which is associated with the generation of T helper 17 (Th17) cells, already defined as a member of IBD's pathogenesis (62). This increase of gene loci susceptibility in IBD indicates that genetic modifications are critical components of pathogenesis. However, genetics is not the only functional subject of IBD pathogenesis.

A large number of environmental factors are considered as risk factors for IBD. It has been known for a long times that there is an association between UC and smoking as a protective effect on the pathogenesis. On the other hand, smoking increases the risk of CD (63). Vitamin D deficiency was also found correlated with the increasing risk of IBD (64). Furthermore, an association between the prolonged use of nonsteroidal anti-inflammatory drugs and the increased risk of IBD was demonstrated (65). In addition, the use of antibiotics is considered an important environmental factor, due to

their influence on the microbiome. Other factors, like stress, depression or anxiety have long been proposed to increase the IBD pathogenesis (66). Recently, even air pollution has been considered as contributing to an increased risk of IBD. In fact, the highest incidence of IBD is present in the developing countries (66). Moreover, high levels of NO_2 and SO_2 were directly correlated with IBD (67). The above cited factors have a direct connection with the gut microbiome.

Many studies have found a reduced biodiversity in faecal IBD microbiome patients compared with healthy people. The IBD microbiome is also unstable. The gut microbiome found stability in the first 2 weeks of life remaining relatively stable thereafter. It has been noticed a 20-30% of culture in IBD microbiome (68). It is remarkable the importance of microbiome in the field of immunological response, as well as, dysfunction of innate and adaptive immune pathways are considered funding to the intestinal inflammatory response. CD is driven by a Th1 and UC with a non-conventional T helper cell response (Th2). Also Th17 cells are involved in the gut inflammatory response (69, 70).

Regarding the innate immunity, recently it has been discovered that toll-like receptors (TLRs) and NOD proteins are significantly altered in IBD patients. Moreover, in response to trauma, mucosal neutrophil accumulation and production of interleukin have been found reduced in CD but not in UC (71). More studies are required to elucidate the effect of the microbiome and environment in the immune response, with the aim to understand which bacteria, viruses, or fungi can modulate specifically the adaptive or innate response (72).

1.6. Symptomatology

IBD symptoms may range from mild to severe. Common signs of IBD are diarrhoea, fever and weakness (74). Abdominal pain and cramping can be caused by the alteration of normal peristalsis caused by inflammation. Reduced appetite and consequently weight loss are the result of the nutrient malabsorption.

Ulcerative colitis is a relapsing non-transmural inflammatory disease that is usually restricted to the colon. It can affect just the rectum or extend proximally to involve part of the colon. This pathology is classified according to the location of inflammation and severity of symptoms (75):

- Ulcerative proctitis: the inflammation is limited to the area closest to the anus rectum. This form of UC tends to be the mildest.
- Proctosigmoiditis: Inflammation affects the rectum and sigmoid colon.
- Left-sided colitis: Inflammation extends from the rectum up through the sigmoid and descending colon.
- Pancolitis: often affects the entire colon.
- Acute severe ulcerative colitis: This is an uncommon form of colitis that affects the entire colon (78). Patients with fulminant ulcerative colitis usually have more than ten bloody stools daily, anaemia requiring blood transfusion, abdominal painfulness, and colonic increase on plain abdominal radiographs.

Crohn's disease is a relapsing, transmural inflammatory disease characterised by a discontinuous involvement of various portions of the whole gastrointestinal tract, from the mouth to the anus, and the development of complications such as strictures, abscesses, or fistulas. At diagnosis, the disease is located in the terminal ileum in 47%, the colon in 28%, the ileocolon in 21%, and the upper gastrointestinal tract in 3% of cases. Common symptoms can be diarrhoea, abdominal pain, weight loss, fever, malaise as well as UC and anorexia that occur with active disease. Other symptoms can be aphtous ulcerations, abdominal tenderness, right iliac fossa mass, perianal abscesses and anal/rectal strictures. Extra intestinal signs could comprise of erythema nodosum, pyoderma gangrenosum, conjunctivitis, episcleritis, iritis, large joint arthritis, seronegative spondyloarthropathy, fatty liver, primary sclerosing cholangitis, cholangio-carcinoma, renal stones, osteomalacia, malnutrition and amyloidosis. Complications are bowel obstruction, toxic dilatation, abscess formation, fistulae, colovesical, colovaginal, perianal, enterocutaneous, perforation, rectal haemorrhage and colonic carcinoma (75).

Inflammation may be confined to the bowel wall, which can lead to narrowing from inflammation, scarring, or both (fibrostenosis), or may tunnel through the bowel wall (fistula). Narrowing may lead to a blockage (obstruction). Obstructions, stenosis and fistulas are not associated with ulcerative colitis (75).

When IBD has been fully investigated and cannot be recognized as UC or CD, it is called "indeterminate colitis". It tends to resemble UC more than CD, and some cases

may be due to a lack of recognition of unusual variants of UC that involve transmural inflammation or skin lesions. Patients with indeterminate colitis appear to have a higher rate of long-term complications than those with UC. Even if the definition were standardized, in practice, the patients carrying a diagnosis of indeterminate colitis may be a heterogeneous group. Some may have colitis that cannot be sub classified into UC or CD (76, 77).

1.7. Diagnosis

It is clear that most of the symptoms or signs that occurs during the spread of the disease are in common between both pathologies. This can make it difficult to diagnose CD instead of UC and vice versa. IBD can be diagnosed only after excluding other possible causes that could have caused the signs and symptoms. Commonly a combination of tests is helpful to confirm the presence of IBD (75, 76).

- *Blood tests*: They can elucidate the presence of possible signs of anaemia as well as indicate the level of inflammation The most common tests include full blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), urea and electrolytes test, liver function tests and blood cultures. If the anaemia tests are positive, iron, Vitamin B12 and red cell folate are recommended. Lower levels of hemoglobin and albumin and increased levels of ESR, CRP and the white cells count allow for an estimation of the inflammation level.

- *Faecal occult blood test*: To find blood presence in faeces and to exclude infectious diarrhoea: microscopy, culture and sensitivities test *Clostridium difficile* test.

1.7.1. Endoscopic procedures

- *Colonoscopy*: This test allows the entire colon to be viewed using a thin, flexible, lighted tube with an attached camera. During the procedure it is possible to take small samples of tissue (biopsy) for laboratory analysis, which may help confirm a diagnosis. Clusters of inflammatory cells called granulomas, if present, help confirm a diagnosis of CD.

- *Flexible sigmoidoscopy*: A slender, flexible, lighted tube is used to examine the last section of colon (sigmoid). In UC the mucosa looks friable and inflamed, while in CD there would be microscopic granulomas.

- *Upper endoscopy*: In this procedure, a tube is used to examine the *esophagus*, stomach and first part of the small intestine (*duodenum*). While it is rare for these areas to be involved with CD, this test may be recommended only in case of nausea and vomiting, difficulty eating or upper abdominal pain.

- *Capsule endoscopy*: this test is used to help diagnose CD. The patient has to swallow a capsule that has a camera in it. The images are transmitted to a computer worn by the patient, after which the camera exits the body painlessly in his stool.

- *Double-balloon endoscopy*: for this test, a longer scope is used to look further into the small bowel where standard endoscopes cannot reach. This technique is useful when capsule endoscopy shows abnormalities, but the exact diagnosis is still in question.

- *X-ray:* In case of severe symptoms, the use of a standard X-ray of the abdominal area may be considered to rule out serious complications, such as a perforated colon.

- *Computerized tomography (CT) scan*: This test looks at the entire bowel as well as at tissues outside the bowel. CT enterography is a special CT scan that provides images of the small bowel. This test has replaced barium X-rays in many medical centres.

- *Magnetic resonance imaging (MRI):* An MRI scanner uses a magnetic field and radio waves to create detailed images of organs and tissues. MRI is particularly useful for evaluating a fistula around the anal area (pelvic MRI) or the small intestine (MR enterography). Unlike CT, there is no radiation exposure with MRI.

- *Small bowel imaging:* This test looks at the part of the small bowel that can't be seen by colonoscopy. After having drunk a liquid containing barium, the patient will be submitted to an X-ray of the small intestine. While this technique may still be used, it has largely been replaced by CT or MRI enterography (75.76)

Recently, paediatric data suggests that upper endoscopy examinations are useful in differentiating CD from UC, when inflammation is otherwise predominantly confined to the colon. Gastrointestinal involvement follows with equal frequency in children and adults. In many paediatric centres, an upper endoscopy is considered one of the routine evaluation tests of children with suspected IBD (77). Distinguishing the type

of colitis in the earlier life could be a very useful way to apply therapies to prevent the worsening of this disease. Other studies are still trying to reflect same results also among the adult patients.

1.8. Therapies

Medical therapies for IBD have increased dramatically during the last decades especially after the introduction of targeted biological therapies. Current drug treatments induce and maintain the patient in remission, ameliorating the secondary effects of the diseases instead of modifying or reversing the pathogenic mechanism.

Aminosalicylates can be used in combination with steroids to maintain remission. These drugs inhibit IL-1 and tumor necrosis factor alpha (TNF-α) production. Moreover, they inhibit the lipoxygenase pathway, the scavenging of free radicals and oxidants, and the nuclear factor kappa B, an inflammatory mediator transcription factor. Sulfasalazine, was found to be effective in patients with mild or moderately active UC (78, 79), while mesalazine has a role in maintenance in CD (80).

Glucocorticoids, one of the main classes of corticosteroids, are used alone or in combination with aminosalicylates to induce and maintain remission in IBD thanks to their ability to block the inflammatory response.

The response to steroid treatment in individual patients can be diverse, with cases of remission after two weeks of treatment (steroid-responsive), cases of relapses after interruption of the therapy (steroids dependent), and finally cases where no improvement is observed even after prolonged steroid administration (steroids-unresponsive) (81, 82). Drugs with immunosuppressive action were also adapted for the treatment of IBD. These are very important in cases of intractable IBD conditions. Azathioprine and mercaptopurine are the most commonly used. These drugs maintain remission in CD as well as UC (83). Cyclosporine, one of the most important immunosuppressive drug in organ transplantation, is also useful in severely UC patients who are not responding to corticosteroids therapies. Several adverse effect, due to treatment with cyclosporine, have limited its use as a first-line medication (84). Furthermore, drugs that interfere with the inflammatory response, especially with TNF-α, are considered very helpful. The administration of humanized-monoclonal antibodies is one of the latest successful treatments for IBD. Infliximab, a chimeric

immunoglobulin, was the first available product for treating refractory CD. It acts to inhibit TNF- α activity. Unfortunately, this therapy can cause an increase incidence of respiratory infection (85). Adalinumab is another anti-TNF agent used instead of Infliximab. This is a fully humanized antibody. The side effects of this drug are a skin reactions and some respiratory infections (86, 87). More common is also the Certolizumab, a monoclonal antibody that also acts against TNF-α, whose half-life is higher than the others thanks to the presence of a polyethylene compound (88).

Apart from the therapies which act directly on the immune response, other products are used with the aim to reduce the symptoms of IBD. Specifically, antibiotics like Metrodinazole are used with success in the treatment of CD. This use was due to the idea that colonic bacteria may initiate the inflammation of IBD (89, 90)

Concerning the aim to reduce the symptoms, analgesic, anticholinergic and antidiarrheal are also used to support the reduction of symptoms.

Furthermore, sodium cromoglycate reduces degranulation of mast cells inhibiting the passage of calcium ions across the cell membrane while bismuth salts, administrated as enemas, are effective for the effects of UC. In addition, thalidomide has been considered as a modulator of the symptoms (91- 93). Moreover, fish liver oil has also been used in the treatment of IBD, probably because of its high concentration of eicosapentaenoic acid and docosahexaenoic acid and their implication in the pro and anti-inflammatory response (94). Aside from traditional medications, natural remedies were found to be useful in maintaining remission. For example, probiotics, that are a mixture of beneficial lyophilized bacteria, can help to control the number of harmful bacteria, reducing inflammation and giving a protection to the gut. *Aloe vera* gel and *Boswellia* herb as well, have also been demonstrated to have anti-inflammatory power (80).

Finally yet importantly, is the new field which uses faecal microbiota transplantation (FMT) as a potential approach for IBD treatment. It has been noticed that the majority of patients treated with FMT experienced a reduction of symptoms, cessation of IBD medications and disease remission (95, 96).

Roughly 40% of patients with UC and the 80% of those with CD require surgery after diagnosis. The proper role of surgery in patients with CD and UC is controversial (97). In fact, these treatments are defined as multidisciplinary approaches between the

patient, surgeon, and gastroenterologist and the choice of different surgeries depends upon the patient condition.

1.9. Metabolomics

In 1966, Tanaka Kei et al. described for the first time a metabolic disease through the use of a new multi-disciplinary technique (97). After the analysis obtained from the urine of patients affected by isovaleric acidaemia, they noticed that the concentration of an unknown compound was deeply increased compared to healthy people. After the use of different analytical tools including nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and infrared spectrometry (IR), they established the identity of this compound as N-isovalerylglycine. Thanks to this identification, they could suggested that the defective enzyme in these patients was at the level of conversion of isovaleryl coenzyme A to β-methylcrotonyl coenzyme A (97).

Metabolites are the final products of cellular regulatory processes, and their levels are considered as a crucial response of the biological systems to some genetic or environmental change. The set of metabolites synthetized by a biological system constitutes its metabolome (98). Metabolome analyses are based on the identification and quantification of all intracellular and extracellular metabolites with molecular mass lower than 1500 Da, using different analytical techniques (99). In common with the genome and the proteome, the metabolome is associated with the physiological, developmental, and pathological state of a cell, tissue, or organism. Genome and proteome studies are based on target chemical analyses of biopolymers composed of nucleotides or amino acids, respectively. On the other hand, the metabolome consists of several different chemical compounds from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids, and complex natural products. This complexity makes it difficult to simultaneously study the complete metabolome. For this reason, each metabolome study requires an evaluation of the sample preparation and the extraction procedure and a combination of different analytical tools in order to obtain as much information as possible (100). The science that studies the metabolome is called “metabolomics” and it is generally defined as the analysis of intra and extra- cellular metabolites in simple biological systems. When the analysis is related to the quantitative

measurement of the dynamic metabolic response of a living systems to the physio-pathological stimuli or genetic modification it is called metabonomics (101). Different metabolomics analyses were identified by Fiehn et al. with the most important being the untargeted and the target approaches (102). Targeted analysis is useful for studying the primary effects due to genetic alteration. Metabolite identification and quantification are common methods used in this field. On the other hand, untargeted analysis allows to achieve a larger number of metabolites with the objective to find a specific metabolic profile for a particular biological matrix (102). The most common techniques used for metabolomics are MS coupled with either liquid chromatography (LC) or gas chromatography (GC), IR and NMR (103). NMR analyses are very helpful in the characterization of unknown compounds based on their chemical structure and thanks to its non-destructive approach and high reproducibility it is considered one of the most valuable techniques. However, NMR is not as sensitive as the others techniques mentioned above so is not possible to detect those metabolites that are present in lower concentration. MS analyses are very sensitive and permit the determination of those metabolites that are present at a low concentration in biological matrices (103). Furthermore, MS analysis is able to identify and characterize chemical compounds based on their masses or ion mass fragmentation. In order to identify multiple variables measured on multiple samples or at multiple time points, these techniques require the support of multivariate statistical data analysis. Multivariate data, contain much more information than univariate data. Multivariate data analysis techniques can be used to model factors and responses and find the relationships that exist between them. Information resulting from multivariate data is usually very helpful in order to understand the characteristics of metabolic systems and processes (104). Initially used in chemistry to distinguish between useful and unusable chemical data, such as noise, repeated data and non-correlated data, these methods actually find several employments especially in clinical aspects. Real systems are governed by several variables at the same time. These studies allow for the consideration of all the variables and using those to exploit all the information inside of a complex data analysis. They can also be used to highlight their connection to a biological system. This is possible through different methods of modelling, classification, regression and other analysis about the similarity of samples, optimization and experimental design.

1.10. IBD metabolomics state of the art

Through recent years, the number of metabolomic studies concerning IBD has been increased and focused on the research of small metabolite. It has been demonstrated that IBD and healthy patients or, in fewer cases, IBD subtypes can be distinguished using the metabolic profile study (105,106). Several serological markers are currently used in this field. Unfortunately these markers are more targeted in the discrimination between healthy patients and IBD patients, instead of difference between CD and UC. Biomarkers that clearly differentiate the subtypes would be very useful for clinical practice, especially instead of such invasive tests as biopsies. However, the aim of metabolomics is not only the classification between healthy patients and pathological patients, but this field is also suitable to understand how single compounds can act in different metabolic pathways. Mouse models have been studied with the aim to measure difference in serum, urine, faecal and gut tissue extracts of IBD and healthy samples. Almost all of these studies included the IL-10 knockout and $TNF^{\Delta ARE/WT}$ models (108, 109). These studies revealed important changes in the microbiome, serum peptides, and intermediary metabolism. Furthermore, changes in the metabolism of cholesterol triglycerides and phospholipids, through different mouse models, inflammatory lipid mediators have also been considered as IBD biomarkers (110). Another study revealed that compounds involved in several cycles such as the tryptophan, fatty acid ad purine as well as methionine-homocysteine and tricarboxylic acid (TCA), are also valuable with the goal to identify IBD in patients (111). Concerning human tests, non- invasive faecal and serological markers are already used to identify IBD. The most important are lactoferrin, calprotectin, polymorph nuclear elastase (PMN-elastase) and CRP. These are also used to better distinguish the active IBD instead of the inactive forms (112). Several antibodies are also recognized as serum biomarkers, like the anti-*Saccharomyces cerevisiae* or the perinuclear antineutrophil cytoplasmic antibodies (113, 116).

It is well known that the type of biofluid under investigation has a significant impact on the resultant metabolic profile. For example, urine metabolites are more influenced by environmental factors than serum. On the other hand, the urine metabolic profile can help to show possible changes within the bacterial population that colonize the gut (117). Recently ^{1}H NMR studies in humans demonstrated decreased level of hippuric acid in IBD urine samples suggesting this metabolite as a biomarker. This result is in accordance with further studies that found a correlation between hippurate and the

presence of *Clostridia bacteria* in the gut (117). Further studies are required with the aim being to find the specific action of hippurate in the IBD disease, especially because high levels of this metabolite along with other metabolites like 2-oxoglutaric acid and creatinine suggest a connection with general stress metabolism and cannot be used as true IBD biomarkers (118). Up to the present time, no urine studies were able to distinguish between CD and UC patients.

Plasma samples were deeply investigated in several studies by ^{1}H NMR GC/MS and LC/MS. Results suggest a very weak predictability concerning metabolic difference between CD and UC if compared with those one of IBD and healthy patients (119).

Hisamatsu et al. showed changes in the amino acid profile indicating a difference between classes (healthy vs IBD) and even between subclasses (UC vs CD). The amino acid profile was also considered useful in monitoring disease activity (120). GC/MS studies confirmed the same hypothesis adding also TCA cycle metabolites as potent discriminants (121). Despite the propensity to use serum and plasma samples instead of urine, these matrices cannot be used to study changes in the population of the gut (122). This lack has also led to the study of the faecal metabolic profile. ^{1}H NMR studies have already found important changes in faecal metabolite, like the decrease of short chain fatty acids in IBD patient (123,124). Further studies on the metabolic profile correlations between gut microbiota and faecal samples are still in progress.

Biopsies from colon tissue are also used to study metabolomics in IBD. Sharma et al. analysed the macroscopically mucosa from IBD patients by ^{1}H-NMR to distinguish the parts involved in the disease and the ones not involved (125). Other studies were able to distinguish between healthy mucosa samples and IBD samples. Moreover, through these studies it was possible to define difference between the active state of UC and those inactive (126). Concerning the lipid metabolic profile, little has been reported in literature for the IBD. Fan et al. demonstrated different lipids as significantly and negatively associated with CD. These alterations of lipid profiles, particularly plasmalogens may contribute to the pathogenesis of IBD (127). Also, Agouridis et al., highlighted several differences in the IBD lipid profile (128). Bearing in mind all the useful tools used to study IBD we can be assured that the metabolic profile of biofluids represents a powerful instrument in the detection of potential biomarkers and possibly highlight altered metabolic pathways in IBD.

2. AIM OF THE WORK

My PhD study is part of a large project aiming to investigate IBD pathologies through the use of metabolomics and microbiological approaches. This project was in collaboration with the Unità semplice dipartimentale di gastroenterologia dell'azienda sanitaria ospedaliera di Monserrato (CA, Italy) and the Department of biomedical sciences microbiology and clinic microbiology section and clinic pathology section, University of Cagliari (CA, Italy). Furthermore, a part of the analyses have been performed at the Department of Biochemistry, University of Cambridge (United Kingdom). As previously reported, the IBD diagnosis arises from a multidisciplinary method including clinical, endoscopic, radiological, histological and analytical datasets. Unfortunately, this diagnosis is only possible when the pathology is already in a progressive stage and usually it is not possible to clearly define the correct difference between CD and UC. Moreover, the etiological and pathogenic theories for IBD are still not very clear and they need to be improved. In this work, faecal and plasma samples of patients affected by IBD, CD and UC were analysed and compared with healthy patient samples, with the aim to characterize metabolomic profiles and infer possible metabolic pathways implicated in their pathogenesis. The organization and collection of samples was conducted by the gastroenterology unit. The microbiological analyses were completed by the microbiological unit. The main analytical techniques for this project were liquid chromatography coupled with mass-spectrometry and ion mobility, due to their high sensitivity and to their strong reproducibility. Datasets were processed using multivariate statistic packages to fully understand the most significant metabolites implicated in this disease. For the faecal samples, microbiological analyses were also used to study correlation between the gut microbiota and the discriminant metabolites.

3. MATERIALS AND METHODS

3.1. Patients

Patients were recruited at the Unità semplice dipartimentale di gastroenterologia dell'azienda sanitaria ospedaliera di Monserrato (CA, Italy). The study was conducted in accordance with the principles of good clinical practice. The institutional ethics committee approved the study, and written informed consent was obtained from each participant. All eligible UC (n=82) and CD (n=52) patients had their diagnosis confirmed by endoscopic, histological and radiographic data. Disease activity was verified by well-established criteria (129) and were at the time of enrolment graded in accordance with the Mayo-score (130) for UC patients and Harvey-Bradshaw-score (131) for CD patients. Informed written consent was obtained from all patients. The CD group comprised of 25 males, with a range of age and body mass index (BMI) from 21 to 65 and from 16 to 31, respectively, and 27 females with a range of age and BMI from 28 to 78 and from 16 to 31, respectively. UC patients were as follows: 43 males with an age range from 20 to 76 and a BMI range from 19.0 to 32.5 and 44 females from 20 to 76 of ages and a BMI in the range of 15.7 and 35.6. Samples were in different stages of pathological activity: quiescent, mild, moderate and severe. Furthermore, they were subjected to different therapies: adalimumab, azatioprine, infliximab, traditional and naïve.

The healthy volunteers (n=51) were recruited locally (Sardinia, Italy). Exclusion criteria were age above 80 or below 20 years, recent use of antibiotics or probiotics and pregnancy. The group consisted of 31 males with a range of age and BMI from 24 to 66 and from 18.9 to 24.7, respectively, and 20 females from 24 to 65 years old and from 19.3 to 31.2 of BMI. Each participant was given a sample collection kit with instructions. One faecal sample, and one plasma sample from each subject were collected, subsequently delivered to the laboratory within 3 h and stored at -80°C until use. All the patients group's characteristics are reported in table 3.1**.**

3.2. Materials

Methanol, chloroform, dichloromethane, isopropanol, acetonitrile, water and analytical standards were purchased from Sigma Aldrich (Milano, Italy and St. Louis, Missouri, United State). Bidistilled water was obtained from a MilliQ purification system (Millipore, Milan, Italy) before use.

3.3. Internal Standards.

- Amino Acid Standards mix, comprising of physiological acidics, neutrals and basics was used for the faecal and plasma polar phase analysis (A 9906.Sigma-Aldrich, St. Louis, MO, USA).
- 2-Amino-4-methoxy-6-methylpyrimidine 98% (Sigma-Aldrich, St. Louis, MO, USA) was used as internal standard for the faecal samples lipid analysis.
- A mixture of 26 deuterated lipids comprehensive of phosphatidic acid (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE), glycerophospholipids (PG), phosphatidylinositols (PI), phosphatidylserine (PS), sphingomyelin (SM), ceramides (Cer), triacylglycerols (TG) and fatty acids FA was used for the plasma lipid profile analysis. A resume list for the deuterated lipids used is reported in table 3.2**.**

3.4. Sample extraction.

In this project, faecal and plasma samples were analysed with the aim to study the metabolomics profile of IBD. Two different profiles were studied: the polar metabolite profile and the lipid profile.

3.4.1. Faecal samples extraction

Frozen faeces (300 mg) were thawed at room temperature and mixed with 800 µL of methanol and 200 µL of Milli-Q water by vortexing. After 30 minutes, samples were centrifuged at 24,130 g and 4°C for 10 minutes. After the centrifugation 50 µL of supernatant were transferred into an Eppendorf tube (Eppendorf®, 022363204, USA) and mixed with 316 µL of methanol and 633 µL of dichloromethane. Samples were then centrifuged for 10 minutes at 24,130 x g. After centrifugation, the supernatant was transferred into a new Eppendorf tube where 200 µL of water were added to induce phase separation. All samples were centrifuged at 24,130 g for 10 minutes. The

resulting two phases were separated, transferred into glass vials, dried under nitrogen and stored at -80°C until being analysed (132).

3.4.1.1 Sample preparation for the lipid phase analysis of faecal samples.

Dried lipid phases were re-suspended in 300 µL of methanol, filtered with Acrodisc Syringe Filters with 0.45 mm PTFE Membrane (SIGMA, St. Louis, MO, USA) (135) and transferred in a glass vial for the LC/MS Q-TOF analysis.

3.4.1.2 Sample preparation for the polar phase analysis of faecal samples

Dried polar phase samples were suspended in 200 µL of a 10 mM ammonium acetate internal standard solution (paragraph 3.3) and sonicated for 5 minutes. Samples were then transferred to an Eppendorf tube and centrifuged at 24,130 x g for 5 minutes. The supernatant was loaded into a 96 well plate for the LC/MS analysis.

3.4.2. Plasma samples extraction

Samples were thawed and extracted with two different methods: one each for the polar and lipid phases.

3.4.2.1. Sample preparation for the lipid phase analysis of plasma samples

Plasma samples were centrifuged at 24,130 x g for 10 minutes at 4°C and 400 µL of supernatant were transferred in an Eppendorf tube. A modified Folch method (133) was used to extract and separate hydrophilic and lipophilic metabolites. Quality control (QC) samples, which contained 20 µL of each plasma sample analysed, were also prepared using the same method. Briefly, 400 µL of each plasma sample were mixed with 600 µL of methanol, 600 µL of chloroform and 175 µL of Milli-Q water and centrifuged at 5139 x g for 30 minutes at 4°C. The high volume of plasma was chosen with the aim to use a single extraction protocol to analyse the aqueous metabolic profile using both GC/MS and HNMR. The lipid chloroform phase was separated and dried under a gentle stream of nitrogen. Dried samples were send to the Department of Biochemistry at University of Cambridge (Sanger Building, 80 Tennis Court Road) for the lipidomics analysis.

3.4.2.1.1. Total lipid extraction.

Samples were suspended in 1000 µL of methanol, vortexed for 3 minutes and sonicated for 5 minutes. After sonication, 50 µL of the sample was transferred into a vial and diluted with 50 µL of internal standard and 75 µL of Chromasolv ultrapure water.

3.4.2.1.2. Solid Phase Extraction (SPE)

From the pool of samples, 10 from the CD class, 10 for the UC class and 10 for the healthy samples class were submitted to a solid phase extraction. Five males and five girls with an average of age from 20 to 30 years and a BMI from 16 to 31 formed each class. Dried organic phase from the previous Folch analysis were reconstituted in 800 µL of chloroform and sonicated for 5 minutes. Subsequently, 20 µL of each sample was transferred into a vial and diluted with 100 µL of internal standard. The solution was dried under a gentle stream of nitrogen and then suspended in other 800 µL of chloroform. At this stage chromabond aminopropyl-modified silica NH2 (1mL/100mg) columns from Macherey-nagel were conditioned with 3000 µL of hexane. After the conditioning 800 µL of samples was added and different solvents were used to elute separate lipid fraction in the sequence as detailed below:

- 1 mL of chloroform: isopropanol 2:1 (v/v) solution for neutral lipids
- 1 mL of diethyl ether: acetic acid 98:2 (%) solution for fatty acids
- 1 mL of methanol for phospholipids

All the samples were then dried and suspended with 50 µL of isopropanol: acetonitrile: water solution (2:1:1 v/v).

3.4.2.2. Sample preparation for the polar phase analysis of plasma samples

20 µL of thawed plasma were transferred into an Eppendorf tube and 100 µL of 0.1 M of HCl internal standard solution were added. Samples were then sonicated for 5 minutes and centrifuged for 5 minutes at 5139 x G. The obtained surfactant was dried under a gentle stream of nitrogen. Samples were then suspended in 100 µL of a 10 mM ammonium acetate solution, vortexed, sonicated for 5 minutes and centrifuged 5

minutes at 14000 rpm. Samples were then transferred in a 96 multi-well plate for LC/MS analysis.

3.5. Analytical platforms

3.5.1. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is a commonly used technique for chemical analysis, and metabolomics, due to its ability to separate, analyse, and/or purify different samples (135). This technique allows separation of eluted compounds quickly and with high efficiency by exploiting the different chemical affinity of the analysed compounds with solvents (mobile phase), silica particles (stationary phase) under a pressure which can reach 1000 bar. Furthermore, with this technique for every analysis it is possible to use a small volume of sample that imply a smaller use of eluent (136).

There are two variations of HPLC separations depending on the relative polarity of the solvents and the stationary phases. Normal phase separation is based on a column filled with silica particles, and the solvent used is non-polar. Polar compounds in the mixture pass through the column and stay for longer in the polar phase if compared with non-polar compounds that pass more quickly through the column.

Reverse phase separation uses the same 3 µm column size, but the silica is non-polar thanks to the presence of the long hydrocarbon chains to its surface, usually with either 8 or 18 carbon atoms in them. A polar solvent is usually used. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture that will cross the column. Non-polar compounds will spend less time in solution in the solvent and this will slow them down on their way through the column.

For the lipidomic analysis, i.e. the metabolomic lipid analysis, a reversed phase with the use of strong polar solvents as acetonitrile, isopropanol and methanol is recommended (137). Mass spectrometry is the most suitable technique to characterise different compounds after separation.

3.5.2. Mass Spectrometry (MS)

Mass spectrometry is based on the ionisation of molecules that are subsequently moved and influenced by external electric and magnetic fields. Because of the high reactivity of the ions their formation and manipulation must be conducted under vacuum environment. Mass spectrometers are basically formed of three parts. At the beginning, an ion source ionizes the sample following column separation. The ionisation can be conducted in positive or in negative ion mode and that depends on the way how the molecules will be ionised. The most commonly used ion sources are the electrospray ionisation (ESI) and the atmospheric pressure chemical ionisation (APCI). The first is recommended for the analysis of polar molecules, while the APCI for the molecules with low polarity. After ionisation the ions are conducted and separated to the detector according to their mass by the mass analyser. The separated ions are then measured by a detector and then converted in analytical data (137). There are different types of mass analyser.

3.5.3. Triple quadrupole (QqQ)

A triple-quadrupole mass spectrometer, also known as QqQ, is a tandem MS composed of three quadrupoles. The first acts together with the third as a mass filter, while the second fragments the ions through the interaction with a collision gas, usually argon or nitrogen. The second quadrupole can be used in single ion mode or scan mode. This instrument showed successfully power among the structural information and quantification results. The most important advantages are the increased selectivity of compound identification, the improved signal-to-noise ratio, the lower limits of quantitation, the extensive linear range and the improved accuracy. Although QqQ is a particularly useful method for all stages of pharmaceutical development, it is a reliable option for many applications, such as clinical research, food safety, forensics, environmental, and proteomics (138).

3.5.4. Quadrupole Time-of-flight (Q-TOF)

Q-TOF is a mass spectrometry method in which the correct mass of ions is determined by a time measurement. Ions, after ionisation, are accelerated by an electric field that confers a different kinetic energy to the ions according to their charge and mass. The time that each ion subsequently takes to reach the detector through a defined distance

is measured. This time will depend on the velocity of the ion, and therefore is a measure of its mass-to-charge ratio. From this ratio and known experimental parameters, it is possible to identify the ion with a very accurate mass (139). Unfortunately, several compounds have the same mass making their identification difficult. To solve this problem, it is possible to use the tandem mass spectrometry analysis (MS/MS).

3.5.5. MS/MS analysis

MS/MS is a method for structure determination and analysis of molecules. Traditionally, MS/MS uses two mass spectrometers in tandem. Between the two analysers is sited a collision gas cell. Precursor ions selected by the first spectrometer or quadrupole collide with a high pressure gas (helium, argon, nitrogen) in the cell and are fragmented. The resulting ions, called fragments, are analysed by the second spectrometer. In the Q-TOF, the collision cell is located between the quadrupole and TOF analyser. Many large molecules such as peptides or lipids have spectra with only a few fragment ions. Usually each precursor ion has specific fragment ions that can be used for its identification. But in some cases, ions with the same mass have the same fragments and this makes their identification not easier (140). Ion mobility is one of the latest modern techniques that can be used to achieve this goal.

3.5.6. Ion mobility

Ion mobility is an analytical technique used to separate ions in gas phase based on their dimension and shape. Coupled with mass spectrometry, this technique is used to obtain complementary information about analytes. In the instrumental setup used here, a tunnel (drift tube) is placed after the ion source. The ionised molecules in the drift tube move towards the MS thanks to the presence of an electric field, and their velocity is influenced by collision with molecules of a buffer gas (nitrogen or helium) in the tube, as well as their shape and charge. Ions with multiple charges cross the tunnel with more efficiency than those charged singularly because they exert more strength than the others due to their electrical field. On the other hand, ions with a large impact section are held more easily in the tunnel. This happens because the resistance force, resulted from the ions collision with the buffer gas, acts against their acceleration imposed by the electric field. By measuring the time required for an ion to cross the

drift tube and reach the mass spectrometer detector (drift time, DT) it is possible to calculate the collisional cross section (CCS) using the Mason-Schamp equation (141),

$$K = \frac{3}{16}\sqrt{\frac{2\pi}{\mu kT}}\frac{Q}{n\sigma}$$

where, Q is the ion charge, n is the drift gas number density, μ is the reduced mass of the ion and the drift gas molecules, k is Boltzmann constant, T is the drift gas temperature, and σ is the collision cross section between the ion and the drift gas molecules. Hence, the advantages of using ion mobility include obtaining a further layer of separation compared to LC alone, as well as having an extra physicochemical property (CCS) which in combination to the retention time and *m/z* can aid in the identification process (141).

3.6. LC/MS analysis conditions.

3.6.1. LC/MS/MS QqQ polar profile analysis of faecal and plasma samples.

A Thermo scientific UHPLC^{+} series coupled with a TSQ Quantiva mass spectrometer (Thermos fisher scientific, Waltham, Massachusetts, United States) was used with an ESI source, operated in positive and negative ion mode at the same time. The electrospray voltage was set to 3500 V for the positive ionisation and to 2500 V for the negative ionisation. Nitrogen at 48 mTorr and 420 °C was used as a drying gas for solvent evaporation. The organic phases were analysed with an ACE Excel 2 C18 PFP (100A. 150 x 2.1 mm 5μ) column. The mobile phase consisted of: (A) a 0.1% of formic acid water solution and (B) a 0.1% of formic acid acetonitrile solution. The mobile phase was pumped at a flow rate of 500 μL/min programmed as follows: initially stayed at 100% of A for 1.60 min, then subjected to a linear decrease from 100% to 70% of A in 2.4 min and to 10% in 0.5 min then staid constant for 0.5 min and brought back to initial condition after 0.1 min. The Xcalibur software (Thermos fisher scientific, Waltham, Massachusetts, United States) was used for data acquisition. Putative recognition of all detected metabolites was performed using a targeted MS/MS analysis. Calculated masses and mass fragments of the calculated compounds were reported in table 3.3**.**

3.6.2. LC/MS QTOF lipid profile analysis of faecal samples.

An Agilent 1200 series LC/MS QTOF (Agilent technology, Santa Clara, California, United States) was used with an ESI source, operated in positive ion mode. The electrospray capillary potential was set to 60 V, the needle at 5850 V, and the shield at 450 V. Nitrogen at 48 mTorr and 375 °C was used as a drying gas for solvent evaporation. Full-scan spectra were obtained in the range of 100-1500 amu for the faecal extract, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 1500 V. The organic layers were analysed by a Pheomenex Kinetek C18 column EVO (100A. 150x2.1 mm 5µ) (California, USA). The mobile phase consisted of: (A) a 10 mM ammonium formate solution in 60% of milliQ water and 40% of acetonitrile with 0.1% of formic acid (v/v) and (B) a 10 mM ammonium formiate solution containing 90% of isopropanol, 10% of acetonitrile with 0.1% of formic acid (v/v). The mobile phase was pumped at a flow rate of 250 µL/min programmed as follows: initially stayed at 68% of A for 1.30 min, then subjected to a linear decrease from 68% to 3% of A in 30 min and was then brought back to the initial conditions in 10 min. The Mass Hunter software (Agilent technology, Santa Clara, California, United States) was used for data acquisition. Putative recognition of all detected metabolites was performed using the Metlin and the Lipid Maps databases, whereas the most statistically significant metabolites were subjected to further identification with the means of targeted MS/MS analysis. Data were collected in the same m/z range of the MS scan mode and collision energy was set at 30V.

3.6.3. Ion Mobility QTOF LC/MS lipid profile analysis of plasma samples.

3.6.3.1. Agilent Ion mobility QTOF LC/MS analysis

As first step, a new method was set up for the lipid plasma analysis using the Agilent 5650 Ion mobility QTOF.

3.6.3.1.1. Positive ion mode

The electrospray capillary potential was set to 60 V, the needle at 20 kV. Nitrogen at 48 mTorr and 375°C, was used as a drying gas for solvent evaporation. Full-scan spectra were obtained in the ranges of 50 - 1200 amu for the plasma extract, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 2950 V. The organic phases were

analysed by a Waters Acquity CSH C18 column (100 x2.1 mm 1.7 µm). The mobile phase consisted of: (A) 10 mM ammonium formate solution in 60% of milliQ water and 40% of acetonitrile with 0.1% of formic acid (v/v) and (B) 10 mM ammonium formate solution containing 90% of isopropanol, 10% of acetonitrile with 0.1% of formic acid (v/v). The mobile phase was pumped at a flow rate of 400 µL/min programmed as follows: initially stayed at 60% of A, then subjected to a linear decrease from 60% to 50% of A in 2 min, then arrived at 1% in 5 minutes staying at this percentage for 1.9 minutes and then brought back to the initial conditions in 1min. The Agilent MassHunter LCMS acquisition console was used for the data acquisition.

3.6.3.1.2. Negative ion mode

The electrospray capillary potential was set to 60 V, the needle at 20 kV. Nitrogen at 48 mTorr and 375 °C was used as a drying gas for solvent evaporation. Full-scan spectra were obtained in the ranges of 50 - 1200 amu for the plasma extract, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 2950 V. The organic layers were analysed by a Waters Acquity CSH C18 column (100 x2.1 mm 1.7 µm) The mobile phase consisted of: (A) a 10 mM ammonium acetate solution in 60% of milliQ water and 40% of acetonitrile and (B) a 10 mM ammonium acetate solution containing 90% of isopropanol, 10% of acetonitrile with 0.1% of formic acid (v/v). The mobile phase was pumped at a flow rate of 400 µL/min programmed as follows: initially stayed at 60% of A, then subjected to a linear decrease from 60% to 50% of A in 2 min, then arrived at 1% in 5 minutes staying at this percentage for 1.9 minutes and then brought back to the initial conditions in 1min. The Agilent Mass Hunter LCMS acquisition console was used for the data acquisition.

3.6.3.2. Xevo Q2 TOF analysis.

To confirm the strength of the new analytical method, an AcquityTM Waters LC/MS coupled with a Xevo G2 QTOF mass spectrometer was used with an ESI source, operated in both positive and negative ion mode.

3.6.3.2.1. Positive ion mode.

The electrospray capillary potential was set to 60 V, the needle at 20 kV. Nitrogen at 48 mTorr and 375 °C was used as a drying gas for solvent evaporation. Full-scan spectra were obtained in the ranges of 50 - 1200 amu for the plasma extract, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 2950 V. The organic layers were analysed by a Waters Acquity CSH C18 column (100 x2.1 mm 1.7 µm) The mobile phase consisted of: (A) a 10 mM ammonium formate solution in 60% of milliQ water and 40% of acetonitrile with 0.1% of formic acid (v/v) and (B) a 10 mM ammonium formiate solution containing 90% of isopropanol, 10% of acetonitrile with 0.1% of formic acid (v/v). The mobile phase was pumped at a flow rate of 400 µL/min programmed as follows: initially stayed at 60% of A, then subjected to a linear decrease from 60% to 50% of A in 2 min then arrived at 1% in 5 minutes staying at this percentage for 1.9 minutes and then brought back to the initial conditions in 1min. The Masslynx v4.1 software was used for data acquisition.

3.6.3.2.2. Negative ion mode.

The electrospray capillary potential was set to 60 V, the needle at 20 kV, and the shield at 450 V. Nitrogen at 48 mTorr and 375 °C was used as a drying gas for solvent evaporation. Full-scan spectrum was obtained in the ranges of 50-1200 amu for the plasma extract, scan time of 0.20 amu, scan width of 0.70 amu, and detector at 3100 V. The organic layers were analysed by a Waters Acquity CSH C18 column (100 x2.1 mm 1.7 µm). The mobile phase consisted of: (A) a 0.1% triethylamine solution in LC/MS grade chromasolv water and (B) a 0.1% triethylamine solution containing 40% acetonitrile and 60% isopropanol (v/v). The mobile phase was pumped at a flow rate of 400 µL/min programmed as follows: initially stayed at 80% of A, then subjected to a linear decrease from 80% to 0% of A in 5.50 min staying at this condition for three minutes and was then brought back to the initial conditions in 2 min. The Masslynx v4.1 software was used for data acquisition.

3.6.3.3. IM-QTOF-LC/MS analysis of SPE samples

For this analysis, the same parameters of the Ion Mobility QTOF LC/MS lipid profile analysis of plasma samples were used. The mobile phase was pumped at a flow rate of 400 µL/min programmed as follows: initially stayed at 60% of A, then subjected to a

linear decrease to 50% of A in 2.10 min and kept decreasing to 30% of A in 10 minutes and to 1% of A in 6 minutes staying at this condition for 0.1 minutes and was then brought back to the initial conditions in 2 min. The Agilent Mass Hunter LC/MS acquisition console software was used for data acquisition.

3.6.4. MS-MS analysis

Data were collected in the same m/z range of the MS scan mode and injected using different collision energy: 20, 30 and 40 V. The analysis was conducted in a targeted MS/MS mode, choosing the parent masses and the respective retention time. All the information about the MS/MS data are reported in table 3.4 and 3.5**.** Examples of a MS/MS experiment are reported in figure 3.1**.**

3.7. Chromatogram pre-processing.

LC/MS chromatograms were processed to obtain a matrix of features present across all samples. The R (R Core Team 2013.R Foundation for Statistical Computing, Vienna, Austria) library XCMS (https://bioconductor.org/biocLite.R) was utilized for peak detection and retention time correction (144,145). Parameters utilized for peak deconvolution for LC/MS matrices were optimised using the R library IPO (146). Grouping of features into pseudospectra was performed using the standard parameters of the R library CAMERA (147). Matrices obtained from IM-QTOF-LC/ MS analyses were processed with the MassHunter suite to perform mass and CCS calibration as well as feature extraction.

The resulting matrices were processed using a KNIME pipeline comprising both KNIME native nodes and integrated R scripts to: eliminate noise/background signals, keep only the most abundant feature per pseudospectrum, impute missing values using a Random Forest approach and perform median fold change normalization (148).

3.8.Chemometric analysis

From the chromatogram pre-processing a data matrix was created and subsequently was elaborated using SIMCA Software (14.0, Umetrics, Umeå, Sweden). Among the several mathematical and statistical analysis of data exploration, in this thesis principal

component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) with its orthogonal extension (OPLS-DA) were achieved.

3.8.1. Chemometrics

Chemometrics is the science that allows the relation of measurements from a data system or process with the state of itself through mathematical and statistical methods (149). A multivariate system, showed in figure 3.2, is represented by a matrix X of N rows (samples) and K columns (variables). Generally, the observed samples can be analytical samples, clinical samples or chemical reactions, while variables can have different origins such as, spectral intensities (from NIR, NMR, IR, UV analysis), chromatographic response or physical measurements. In this thesis several extracts of plasma and faecal samples were used as observations, while spectral peak intensities were used as variables.

3.8.2. Scaling

In a multivariate analysis variables have to be comparable each other. For this reason data set have to be pre-treated with the aim to be converted in a more eligible form for the analysis. In the case of variables that differentiate in order of size, we need to give the same importance to each one. This signifies that the length of each coordinate in the variable space has to be regulated following a predetermined criteria. In order to accomplish this task, a mathematical transformation of the data, termed scaling, needs to be used. In this work, pareto scaling was chosen as preferred method. Pareto scaling uses the square root of the standard deviation as the scaling factor. This action reduces the large differences in intensity between different features, which would otherwise bias the results by conferring a higher importance to most abundant variables (150).

3.8.3. Principal component analysis (PCA)

To show the complexity of a phenome constituted by a large number of variables using a small number of summary indicators (latent variables) a PCA is. PCA is an explorative method that identifies the distribution of a data set, highlights similarities and differences no suspected among the data and compresses the data reducing their dimensions into a smaller number of new variables called principal components (pc). Principal components describe the data variability, reveal groups of normal and

abnormal objects (outliers) and find the relations between the objects and the variables and even between the variables. After the data pre-processing and scaling, samples that formed the matrix take place into a space creating a set of points. This distribution causes a redisposition of the coordinated system that matches the average point with the origin of a new reference system (Figure 3.3). This new direction is called principal component and the new coordinate system it is called a scores plot. Usually, to show all the system variations it is not sufficient using only one pc and for this reason a second pc is also calculated. This component is shown as an orthogonal line to the first pc that passes across the average point (Figure 3.4). The loadings analysis is required to interpret the scores plot and to discover the correlation between samples and variables. The loadings describe the data structure on the basis of the variables correlations. They represent the orientation of plan set into the multivariate space. The first pc direction compared to original variables is formed due to the angles of cosine α1, α2 and α3. These values indicated as the original variables x1, x2 and x3 influenced the first pc. These are called loadings. Similarly, a second set of loadings creates the direction of a second pc compared to the original variables (Figure 3.5). The number of pc is related to the variance of the model and by that it is possible to understand how much information is explained by the model. PCA results are reported into different plots. The scores plot reported the samples projection into the model space, calculated by the use of principal components, while the loadings plot reports the projection of variables, using the same rules. The quality of the model can be evaluated by the R^2 and Q^2 parameters. The R^2 is the fraction of the variation of the variables explained by the model. Q^2 is an estimate of the predictive ability of the model. It is calculated by cross-validation. The sample data set is divided into n parts (n = 7 in SIMCA by default), then the model is built on n-1 parts (training set) and tested on the remaining part (test set). By performing these steps for as many iteration as allowed by the initial division of the dataset into sub-sets, it is possible to calculate the sum of square error for the whole dataset. This is then called the Predicted Residual Sum of Squares. The closer the R^2 is close to 1 the more the model is close to the referred experimental models. If the system is homogenous R^2 and Q^2 will be similar, whereas if the system is heterogeneous Q^2 will be lower than R^2. Furthermore, it is possible to highlight the outliers, namely samples that differ excessively if compared with the others and are not very well described by the model. These observations have a high leverage to the model. The leverage is proportional to the distance between an

observation and the dataset centre. The variance explained, usually measured as the total variance percentage, is a variance proportional measurement of data taken in consideration by the pc. The variance that is not explained is called residual. To greater appreciate the presence of outliers it is possible to use two analyses: Hotelling's T^2 and DmodX analysis (151).

3.8.4. Partial least square-discriminant analysis and its orthogonal variation (O/PLS-DA)

PLS-DA is a discriminant analysis that classifies one object (sample) on the basis of its belonging class. This is a supervised analysis that uses as a prerogative the necessity of a sample classification that is collected in a second matrix called Y matrix. The generated two matrices, the X matrix (constituted by the observation and the variables) and the Y matrix (constituted by the samples classes) are put in relation and the quality of the model is valuated with different parameters (Figure 3.6). The generated R^2 and Q^2 values described the reliability and the predictive ability of the fitting, respectively. Q^2 is made on the basis of a cross-validation analysis. R^2Y describes the classificatory power of the model. Permutation test, a non-parametric test, is also achieved to highlights the classificatory power of the model. This test use random shuffles of the data to get the correct distribution of a test statistic under a null hypothesis. Three parameters are used for this analysis: the null hypothesis which determines what we permute, the test statistic which affects the power and the number of permutations, which affects precision of the estimated p-value. This test collect the correct false positive amount from the distributional characteristics of the data.

In the case of a non-directly correlation between Y matrix and variables an Orthogonal Projections to Latent Structures (OPLS) can be useful to interpret the problem by incorporating an orthogonal signal correction (OSC) filter into a PLS model. This model separates the Y -predictive variation from the Y -uncorrelated variation in X. It is important to highlight that OPLS-DA does not provide predictive advantage over PLS-DA. With the aim to study the possible discriminant variables for the project, different analyses can be used. A variable importance in the project test (VIP) gives a measure of a variables importance in the PLS-DA model. It summarizes the influence of a variable in the model. The VIP score of a variable is calculated as a percentage variation sum of the squared correlations between the PLS-DA components and the

original variable. All the variables that have a value of VIP lower than 1 are not influencing the classification. In the case of OPLS-DA it is more correct using the S-plot to study the discriminants. S-plot, combines covariance and correlation of loading profiles (152).

3.9.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 (153). To verify the significance of metabolites obtained using multivariate statistical analysis and to find differences in the microbiome, a Mann Whitney *U* test was also performed (154). For the SPE analysis all the univariate analysis was made using a one way non parametric test with the Bonferroni's correction (155).

4. RESULTS

4.1. Lipid metabolite profile analysis of faecal samples

Using LC/MS QTOF, 255 metabolites from 177 samples of CD patients, UC patients and healthy subjects were detected. Peaks were identified and attributed to endogenous metabolites that included steroids, Cer, PS, PC, PE, diacylglycerols (DAG), TG and NAPE. One example of faecal lipid extract chromatograms are shown in figure 4.1. To investigate the correlations between compositional and analytical data, and to observe sample distribution in multivariate space, unsupervised PCA was performed within SIMCA 14.0 software. The Hotelling's T^2 and the DmodX analysis showed five outliers that, after a deep investigation with the aim to find valid correlations for their different distribution, were excluded from the project. The analysis reported five significant principal components for the project with an R^2 and a Q^2 of 0.54 and 0.46, respectively. The first two components could describe the 29% and 39% of the total variance, respectively. From a visual analysis of the score plot, no clusters were highlighted based on pathology, pathological localisation or disease activity. With the aim to find possible discriminant metabolites between the pathologies and the healthy patients a PLS-DA was performed with its orthogonal extension (OPLS-DA). OPLS-DA analysis displayed a clear separation between healthy subjects and both pathological classes. For the separation between CD and healthy patients the R^2Y and Q^2 resulting from the OPLS-DA models were, 0.78 and 0.45, respectively. The permutation test was verified and the Q^2 resulted of -0.48 demonstrating the good predictability of the model. For the separation between UC and healthy patients the R^2Y and Q^2 resulted from the OPLS-DA were 0.62 and 0.48, respectively. The permutation test verified the Q^2 resulted of -0.14. It was not possible to build a statistically significant model to distinguish between CD and UC patients. OPLS-DA scores plots and permutation tests are reported in figures 4.2 and table 4.1**.** From the coefficient analysis and the loadings plot analysis, and after verifing the significance of the metabolites by the univariate Mann Whitney *U* test, seventeen metabolites were interpreted to be different between CD patients and healthy controls. Using MS/MS fragmentation data and consulting the Metlin and Lipidomics libraries, six compounds were annotated. DAG 18:0/22:2 was significantly increased in CD patients, while

urobilin, PC 3:1/16:0, urobilinogen, PA 16:1/19:0 and PS 18:0/22:2 were found to be decreased (Figure 4.3 and table 4.2).

Comparing UC patients with healthy controls, sixteen metabolites differentiated the two classes. Nine of these were in common with the CD patients-healthy controls comparison. DAG 16:0/18:2, DAG 18:0/22:2 and NAPE 16:1/18:0/18:1 were identified to be significantly increased in UC patients faeces, while PC 3:1/16:0, urobilinogen, PC 14:1/22.2, PS 18:0/22:2 and Cer 18:1/22:0 were decreased in UC patients (Figure 4.4 and table 4.2).

4.1.1. Correlation between the metabolome and the microbiome

Microbiological faecal analysis was carried out by the Department of Biomedical Sciences and the Department of Medical Sciences and Public Health and Gastroenterology Unit, University of Cagliari. A correlation analysis between faecal microbiota and lipidomics data was conducted. The analysis was restricted to the microbial genera whose relative abundance resulted in being statistically different among the different groups of subjects and the most discriminant metabolites among the patient groups. Spearman correlation analysis of the microbiome and metabolome data revealed strong associations between some members of the microbiota and metabolites for CD patients. Five bacterial genera revealed an association with 10 discriminant metabolites in CD patients. The most correlated genera was Oscillospira, particularly with PC 3:1/16:0 and urobilin (all positive correlations). The Escherichia genus was negatively correlated with PC 3:1/16:0. For patients affected by UC, the Spearman correlation coefficient was lower than for CD patients. Three bacterial genera were correlated with discriminant metabolites. Particularly, a strong positive correlation was observed between bacteria from the Flavobacterium genus and PC 3:1/16:0 (Figure 4.5). Spearman's correlation analysis was also performed between the bacterial species and the discriminating metabolites. Fifteen bacterial species showed a good correlation with 14 discriminant metabolites in Crohn disease patients. Seven species belonging to the *Firmicutes* phylum (*Faecalibacterium prausnitzii, Oscillospira eae, Oscillospira guillermondii, Anaerobranca zavarzinii, Veillonella montpellierensis, Ruminococcus albus and Alkaliphilus crotonatoxidans*), four species to the Proteobacteria phylum (Desulphonauticus Autotrophicus, Serratia entomophila, Escherichia albertii and Candidatus Endobugula sertula) three species to the

Bacteroidestes phylum (Dysgonomonas wimpennyi, Rikenella microfusus and Parabacteroides johnsonii) and one, Bifidobacterium adolescentis to the Actinobacteria phylum. In particular, a strong positive correlation was detected between Oscillospiraeae PC 3:1/16:0 and urobilin; between *Oscillospira guillermondii* and PC 3:1/16:0; and finally between *Desulphonauticus autotrophicus* and PC 3:1/16:0. On the other hand, a strong negative correlation between *Faecalibacterium prausnitzii* and phenylethylamine was documented (Figure 4.6**) (**156**).**

4.2. Polar metabolite profile analysis of faecal samples

LC/MSMS QqQ was used to measure the polar metabolite profile of 166 faecal samples. In total 61 polar compounds (Figure 4.7, table 3.2) were selected and their area ratios calculated by dividing the detected area of the most abundant fragment with that for the relevant internal standard. These area ratios were submitted to a Mann Whitney *U* test. From the Mann Whitney *U* test analysis 8 and 7 compounds were found statistically significant changed in UC and CD, respectively, when compared with the control group. Cystine, arginine, carnosine, β-aminoisobutyric acid, α-aminoisobutyric acid, asymmetric dimethylarginine and methionine were found increased in CD samples, while only malic acid was found decreased in CD (Figure 4.8) (table 4.3). For the UC class cysteine, arginine, carnosine, β-aminoisobutyric acid, asymdimethylarginine and ornithine were found to be increased, while AMP was decreased (Figure 4.9) (table 4.3). No statistical difference was found between CD and UC.

4.3. Lipid metabolite profile analysis of plasma samples

4.3.1. Positive ionisation analysis

The lipid profiles of IBD and healthy plasma samples were studied by LC/MS-QTOF and IM-QTOF-LC/MS. After processing the data from LC/MS-QTOF and IM-QTOF-LC/MS, 780 and 776 mass spectral features, respectively, were selected and undertaken for the statistical analysis. Chemical composition analysis indicated that the lipid fraction was composed of lipids from the following classes: LysoPC, PC, PS, PE, DAG, CE, SM, Cer and TG. Example chromatograms from the ion-mobility analysis and from the Q-TOF analysis of plasma samples are reported in Figures 4.10

and 4.11. Initially, to study sample distribution, to detect outliers and to highlight differences or common features, PCA was performed on the LC/MS data. The results of the PCA were for the IM-QTOF-LC/MS analysis R^2X=0.69 and Q^2=0.44 and for the LC/MS-QTOF analysis: R^2X=0.82 and Q^2=0.70. Considering gender, age, BMI, therapies, state of disease and localization of inflammation no clusters were indicated in both analyses. DmodX and Hotelling's T^2 analysis showed 7 and 3 outliers, respectively. To identify metabolites that can discriminate for the 3 classes of samples (CD, UC, and C), three different OPLS-DA models of the LC/MS data were performed: CD vs C, UC vs C and CD vs UC. All the validation results and the differences between the two instrument analyses are showed in table 4.4.

OPLS-DA analysis displayed a clear separation between healthy subjects and both pathological classes. From the LC/MS-QTOF data, the OPLS-DA between CD and healthy patients showed a R^2Y and Q^2 of 0.67 and 0.51, respectively. The permutation test was verified and the R^2 resulted of -0.20 highlighting a good prediction power. The separation between UC and healthy patients reported R^2Y and Q^2 of 0.84 and 0.59 respectively. The permutation test was verified and the Q^2 resulted of -0.31 highlighting a good classification. Unfortunately, the two pathological classes could not be distinguished by their plasma lipid profile. Using IM-QTOF-LC/MS CD and healthy patients were differentiated by their lipid profiles (R^2Y and Q^2 of 0.50 and 0.37 respectively). The permutation test verified the model (Q^2 = -0.18). UC and healthy patients were also distinguished by lipidomics data from IM-QTOF-LC/MS (OPLS-DA: R^2Y and Q^2 of 0.54 and 0.34, respectively). The permutation test was verified and the R^2 resulted of -0.18. Results of OPLSDA are shown as scores plot and permutation tests in figures 4.12 and 4.13**.** From the coefficient analysis and the loadings plot analysis, and after verifying the significance of the metabolites by the Mann Whitney *U* test, thirteen metabolites were interpreted as different between CD patients and healthy controls and for UC patients and healthy controls. Using MS/MS fragmentation data and consulting the Metlin and Lipidomics libraries, we could identify the most discriminant compounds. LysoPC 18:0, LysoPC 18:1, LysoPC 18:2, PC 18:0/18:1, PC 18:1/18:1, PC 18:2/18:2 and PC 18:3/20:4 were decreased on CD samples, while PC16:0/22:6, PC18:0/22:6, PC 18:3/20:3, SM 16:1/20:1, TG 18:1/18:2/20:4 and TG 16:0/18:1/18:2 were increased in CD samples. On the other hand, LysoPC 18:1, LysoPC 18:2, PC 18:0/18:1, PC 18:1/18:1, PC 18:2/18:2, , PC

18:3/20:4, TG 14:0/16:0/18:2 and TG 16:0/18:0/18:1 where decreased for UC samples, while PC 16:0/20:4, PC 16:0/22:6, SM 18:2/24:0 and TG 18:1/18:2/20:4 were increased in UC samples. All the discriminant metabolites with *p* values are reported in figures 4.14 and 4.15 and in table 4.5.

4.3.2. Negative ionisation analysis

From the data analysis of IM-QTOF-LC/MS and LC/MS QTOF data acquired in negative ionisation mode 1688 and 605 mass spectral features were selected and undertaken for the statistical analysis, respectively. The chemical composition analysis by exact mass searches, indicated that the lipid fraction was composed of free fatty acids, phosphatidylserines, bile acid diacylglycerols and eicosanoids. Chromatogram examples from the IM-QTOF-LC/MS analysis and LC/MS-QTOF analysis of plasma samples are reported in figures 4.16 and 4.17**.** PCA of the IM-QTOF-LC/MS data gave model statistics of R^2X=0.5 and Q^2=0.4 and for the LC/MS-QTOF analysis: R^2X=0.82 and Q^2=0.60. Considering gender, age, BMI, therapies, state of disease and localization of inflammation no clusters were indicated in either of the analysises. DmodX and Hotelling's T^2 analysis showed 3 outliers only for the LC/MS QTOF analysis which were subsequently excluded. OPLS-DA analysis displayed a clear separation between healthy subjects and both pathological classes. All the validation results and the differences between the two instrumental analyses are shown in table 4.4**.** OPLS-DA from the LC/MS-QTOF data comparing between CD and healthy patients showed a R^2Y and Q^2 of 0.7 and 0.5, respectively. The permutation test was verified and the Q^2 resulted of -0.19 highlighting a very powerful prediction power. The separation between UC and healthy patients reported R^2Y and Q^2 of 0.7 and 0.5, respectively. The permutation test was verified and the Q^2 resulted of -0.11 highlighting a very powerful classification. The comparison between the two pathological classes did not show a good separation. From the IM-QTOF-LC/MS the OPLS-DA between CD and healthy patients and UC vs healthy patients the analysis suggested good classificatory powers (table 4.4). Unfortunately, permutation tests suggested these models were over fitted. For this reason, to deeply investigate the predictability of the model a manually cross-validation analysis was set up using two-thirds of the total number of samples to train the model and use one-third as a set test. 76% of samples were classified correctly. Results of the OPLSDA are showed as

scores plot and permutation test in figures 4.18 and 4.19**.** From the coefficient analysis and the loadings plot analysis, and after verifying the significance of the metabolites by the Mann Whitney test, ten metabolites were interpreted to be different between CD patients and healthy controls and for UC patients and healthy controls. Using co-chromatography identification, after MS/MS fragmentation and consulting the Metlin and Lipidomics libraries, several discriminant metabolites were identified. PC 18:1/18:2, PC 16:0/22:6 and PC 18:0/18:2 were decreased on CD samples, while free fatty acid such as oleic acid, linoleic acid, palmitic acid and palmitoleic acid were increased in CD and UC samples. Only PC 18:1/18:2 was found decreased in UC samples. All the discriminant metabolites are reported in figures 4.20 and 4.21 and in table 4.6.

4.3.3. Lipid class analysis of plasma samples

To more deeply investigate the plasma lipid profile of the IBD patients and to describe possible variations among the different lipid categories in IBD and healthy samples, 33 samples were subjected to differential solid phase extractions and the resulting fractions were analysed by IM-QTOF-LC/MS. The extraction resulted in three different lipid profiles: a phospholipid profile, a neutral lipid profile, and a free fatty acids profile (Figure 4.22).

4.3.3.1. Phospholipid fraction analysis

Analysis of the phospholipid fraction revealed over 262 mass spectral features. These were analysed using the Metlin and and lipid maps databases and included 44 PC, 20 SM, 16 Cer, 15 DAG and 11 LysoPC. The intensities of all compounds in each lipid class were summed. Next, total lipids in each class were compared across the different groups (CD, UC C) using one way ANOVA test. Results were adjusted for multiple testing using a Bonferroni's correction.

Several significant differences were observed. The glycerophospholipids were found to be increased in UC samples when compared with CD samples. On the other hand, unsaturated LysoPC were decreased in CD. Saturated DG were increased in CD. (Figure 4.23).

To confirm these changes and to find more discriminant metabolites for these classes, a multivariate data statistical analysis was carried out. PCA analysis didn't showed any outliers or clusters, while R^2X and Q^2 for the first two principal components were 0.5 and 0.3 respectively. Three discriminant analyses were performed: UC *vs* C samples, CD *vs* C samples and UC *vs* CD samples. The two discriminant analysis regarding the classification between disease classes versus the control samples reported the same results of the all ion analysis and the same discriminant metabolites (data not showed). On the other hand, the discriminant analysis between CD vs UC for the first time reported good validation parameters as follows: R^2Y=0.7 Q^2=0.3 and Q^2 of permutation test=-0.07. After the variable analysis and after the Mann-Whitney test, twelve metabolites were identified as increased for UC samples and only one was increased in CD samples. For the UC classes the identified compounds were PC16:1/16:0, PC 16:0/18:1, PC 16:0/18:2, PC 16:0/20:3, PC 16:0/22:5, PC 18:0/18:1, PC 18:0/18:2, PC 18:0/20:3 and PC 18:1/18:1. Discriminant compounds are reported in tables 3.4 and 4.7.

4.3.3.2. Neutral lipid fraction analysis

The neutral lipid fraction was composed of 249 mass spectral features. These were identified by lipid maps and XCMS annotation and included 98 TG, 27 DAG, 16 Cer, 9 CE and 8 SM lipids. Compounds were treated as explained above for the phospholipids analysis. In this case, the only category that was found significantly changed was the CE category. These compounds level were found increased in UC compared to CD (Figure 4.24).

4.3.3.3. Free Fatty acids fraction analysis

The free fatty acid fraction was composed of 138 spectral features which subsequently identified 43 free fatty acids or related structures. The areas of all the fatty acids were summed for each sample. After that, samples were submitted to a one way ANOVA test, taking in consideration all of the 3 classes of samples: CD, UC and C samples. All the comparisons were subjected to the Bonferroni correction. Results showed a significant increase in fatty acid in CD compare to controls. Fatty acids were then divided into two different categories based on their level of saturation: saturated fatty acids and unsaturated. These two categories were treated similarly to the previous

analysis. The analysis of unsaturated lipids showed a significant separation between CD and C samples, with an increase being detected in the CD group. A separation between monounsaturated and polyunsaturated fatty acids was achieved and both analyses demonstrated a significant increase of the fatty acids in the IBD samples. On the other hand, saturated fatty acids didn't show significant changes (Figure 4.25).

4.4. Polar metabolite profile of plasma samples

Using LC/MSMS QqQ, 55 polar compounds from 166 plasma samples were measured (Figure 4.26 and table3.2) and their area ratios, was calculated by dividing the detected area of the most abundant fragment by that of the related internal standard. Group means for the area ratios were compared using the Mann Whitney *U* test. Overall, 16 and 21 compounds were found to be statistically significantly changed in CD and UC, respectively, when compared with the control group. β-amino isobutyric acid, α-hydroxy butyric acid, histidine, creatine, isocitric acid and citric acid levels were increased in CD samples, while alanine, betaine, citrulline, threonine, hydroxyproline, proline, valine, methionine, tyrosine, methionine sulfoxide where decreased in CD. For the UC class cysteine, β-amino isobutyryc acid, AMP, α-hydroxybutyric, c AMP, creatine, isocitric acid, GMP, citric acid, uridine, GABA, β-hydroxy isovaleric acid and ketoleucine were increases while, arginine, alanine, betaine, citrulline, threonine, proline, methionine, tyrosine and tryptophan were decreased. (Figures 4.27 and 4.28) (table 4.8).

5. DISCUSSION

In this project, to improve the understandings of IBD pathogenesis, a metabolomics approach was performed. Several compounds were found discriminant comparing both UC and CD pathologies with healthy individuals in faecal and plasma samples. Numerous compounds were found discriminant and linked with different metabolic pathways.

5.1. Faecal samples

Increased levels of DAG were found in both pathological classes. DAG are a lipid category derived from glycerol. Their chemical structure is composed by two fatty acids both bounded with glycerol through an ester bond. They also, are associated in the biosynthesis and degradation of triglycerides, glycerol phospholipids and glycerol glycolipids (157). Moreover, their accumulation in the human cells may effect changes in the physical membrane properties (157). In addition, they act as second messengers in cell signalling like in the activation of the mitogen-activated protein kinase (MAPK) (158). The MAPK is a heterogeneous group of enzymes responsible for phosphorylating serine and threonine amino acids in many proteins (158). Phosphorylation of a specific amino acid sequence, which is specific for each MAPK, is required for their full activation. MAPKs activation causes a cascade of extracellular stimuli that initiate inflammation, including production of pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-2 and IL-6) (159). Furthermore, these proteins are involved in mediating several hormonal, mitogen, and tumour promoting signals in cells (160). Among these proteins, the kinase C (PKC) plays very crucial roles in many aspects of several pathogenesis like IBD, taking part in physiological and pathological processes such as development, inflammation i.e. intestinal inflammation and tumorigenesis (161). DAG are involved in the activation of the protein kinase (PK) family. Specifically, the classical (cPKCα, β and γ) and novel (nPKCδ, ϵ, η and θ) PKC isotypes display a physiological and specific requirement of DAG to express their activity. The mechanism of this pathway has been described in many cell types, and numerous reviews have confirmed this hypothesis (161). The inflammatory response implication in IBD pathogenesis is also confirmed by the asymmetric dimethyl arginine (ADMA) levels that, in this thesis, were found increased in the pathological samples. High levels of ADMA have been previously associated with biomarkers of

inflammation, like TNF-α and IL-8, in a variety of chronic conditions like IBD (162, 163). Furthermore, in previous studies, it was demonstrated that DAG are produced even by intestinal microflora. (164). In fact, Morotomi et al. showed that the incubation of bacterial, obtained by normal human faeces, with ^{14C}labeled phosphatidylcholine cause the production of diacylglycerols, monoacylglycerols, and free fatty acids in faecal samples (164). Correlations with the changes in the intestinal microflora are highlighted even by the increased levels of cysteine and methionine in CD and UC. Methionine together with cysteine are two sulfur-containing proteinogenic amino acids. Methionine is an essential amino acid while cysteine is semi-essential due to its production in the human being from the same methionine (165). Sulphur amino acids in human being maintain and support immune function modulating the actions of oxidant stress and related reactive oxygen species caused by bacteria infections or environment factors (166). Furthermore, in vitro experiment highlighted that a consumption of methionine causes an increase of plasma homocysteine concentration stimulating inflammatory events (167). Morgan et al., have also found increased levels of methionine and cysteine in CD indicating an activation of the further mechanism of maintaining redox homeostasis caused by a microbiome dysfunction (168).

Elevated levels of DAG can also be correlated with the decreased levels of PC found in UC and CD patients when compared with C samples. PC is a class of phospholipids derived from DAG that incorporates choline as a head group (169). They are a major component of biological membranes (170) also playing a role in membrane-mediated cell signalling and in the activation of other enzymes (171). Schneider et al. found that mucus from UC patients had a decreased phosphatidylcholine content, while clinical trials discovered that therapeutic addition of phosphatidylcholine to the colonic mucus alleviates the inflammatory activity (171). It has been known that several pathologies such as IBD and tumours are correlated to the phospholipid pathway (172). Epidemiological and experimental studies had already suggested how high fat diets could increase the risk of cancer of the colon (173). Remarkable is the use of the phospholipase A2 (PLA2) to achieve these goals (174, 175). Phospholipase is a group of enzymes that hydrolyses phospholipids into fatty acids and other lipophilic substances. There are four major classes called A, B, C and D that use different pathways to catalyse their reaction (174). PLA2 releases fatty acids from the phospholipids second carbon group of glycerol releasing arachidonic acid and

lysophosphatidic acid. Later, arachidonic acid is modified into active anti-inflammatory and inflammatory mediators called eicosanoids (174). Cytosolic PLA2 is activated by low concentrations of Ca^{2+}. After the activation, the lipase is transferred from the cytosol to the cell membrane to initialise the arachidonic acid cascade (175). A modification of the PLA2 activity can cause a change in the phosphatidylcholine synthesis or in the degradation pathway. The synthesis of phosphatidylcholine starts from the uridine. This pyrimidine is converted to cytidine triphosphate (CTP) and reacts with PC to form cytidine diphosphate (CDP)-choline (176). CDP-choline and DAG then form PC by a CDP-choline: diacylglycerol phosphocholine transferase (177). Changes of several metabolites implicated in the PC synthesis have been observed in our samples: in fact, DAG levels were found increased in both CD and UC when compared with C while PC were found decreased. This can suggest a variation in the CDP-choline: diacylglycerol, phosphocholine- transferase step. Furthermore, no variations have been found in the IBD uridine and choline levels suggesting that the previous enzymatic steps of the phosphocholine synthesis did not reported any change. Certainly, further enzymatic studies are needed to understand variations in the CDP-choline: diacylglycerol phosphocholine transferase activity.

Urobilinogen was found decreased in both IBD classes, while urobilin only in CD samples. Urobilinogen and urobilin are open tetrapyrrole compounds formed from bilirubin by gut microorganisms present in the distal part of the small and large intestines. These compounds are excreted with the urines and faeces. A small fraction of urobilinogen is reabsorbed from the gut and re-excreted predominantly by the liver. In the presence of excessive bile pigment formation or liver disease, urobilinogen is also excreted by the kidney (178). Intestinal bacterial action on deconjugation of conjugated bilirubin and reduction of unconjugated bilirubin has been shown to be decreased due to a modification of gut microflora in cirrhosis, which may cause a further decrease in faecal urobilinoids (179,180). This decrease confirmed what Midtvedt et al. have already noted (181). In fact, CD patients showed a significantly lower conversion rate of urobilin as compared to the controls in faecal samples (181). Our results might indicate altered entero-metabolic functions in CD patients (181). Furthermore, urobilinogen and urobilin were strongly correlated with *Oscillospiraea* bacteria species (Figure 4.5 and 4.6), one of the most discriminant bacteria found in

the microbiome analysis in the IBD cohort (156). This correlation confirms the connection between microbiome variation and the bilirubin metabolic pathway.

It has been noted that the endocannabinoid system can affect the intestinal motility acting into different metabolic pathways. For example, this is involved in the regulation of numerous gastrointestinal functions including gut homeostasis, modulating gastrointestinal motility, visceral sensation, and inflammation, as well as being recently implicated in IBD pathogenesis. (182,183). Increased levels of NAPE 18:1/16:1/18:0 in UC patients can suggest a modification of this pathway. N-acylphosphatidylethanolamines (NAPEs) are synthesized in the small intestine in response to feeding and reduce food intake and obesity (184). These are precursors to the N-acylethanolamide (NAE), family of potent anorexigenic lipids. After their synthesis, NAPEs are converted to the active NAEs. NAPE are converted also into phosphatidic acid and anandamide by N-acylphosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) enzyme. Anandamide and 2-arachidonoylglycerol are endogenous bioactive lipids that bind and activate the cannabinoid receptors, and together with the enzymes responsible for their biosynthesis and degradation (fatty acid amide hydrolase and monoacylglycerol lipase) constitute the endocannabinoid system (185). Levels of anandamide and its synthesizing enzyme (N-acyl phosphatidylethanolamine phospholipase D) were decreased in biopsies of patients with ulcerative colitis. (186). The increased levels of the NAPE 18:1/16:1/18:0 can suggest a modification of the NAPE-PLD activity.

5.2. Plasma samples

The second part of this project was based on an investigation of predictive biomarkers for the diagnosis of CD and UC in plasma samples and possible peculiarities that can distinguish between CD and UC. All the analyses reported a clear classification between the two typologies of disease when compared with healthy samples. In the positive ion mode decreased levels of LysoPC 18:0, 18:1 and 18:2 were observed in IBD samples. LysoPC is a group of lipids derived by the partial hydrolysis of PC through the action of the PLA2 that removes one of the two fatty acid groups. As already reported in the previous paragraph (paragraph 5.1.), the PLA2 represents a category of enzymes that releases fatty acids from the second carbon group of glycerol with the aim to produce eicosanoids starting from the arachidonic acid (187). PLA2

has been reported several times as an important enzyme that helps different inflammatory processes like the IBD pathogenesis (188). Recently, Peterson et al. found an increased localization of the activator antigen of PLA2 in the monocytes and granulocytes of IBD intestinal sections (189). In this thesis, a modification of the PLA2 metabolic pathway was found in IBD faecal samples (paragraph 5.1). These results suggested a variation of PLA2 activity in the mucosal tissues. The decrease of LysoPC in IBD samples can confirm this variation. Moreover, in the LC/MS negative ionisation analysis, levels of several free fatty acid were found increased in IBD. These can suggest an increase of the PLA2 enzymatic activity. Also, Ehehalt et al., in 2004 showed a significantly decrease of PC and LysoPC in inactivating the rectal mucus of ulcerative colitis patients when compared to CD and healthy samples (190). The most abundant species found in the Ehehalt work were PC 16:0/18:1, PC 16:0/18:2, PC 18:0/18:1, PC 18:0/18:2, LysoPC 16:0 and LysoPC 18:0 (190). These results are in accordance with our results, showing the variation of LysoPC and PC groups. In fact, in the C *vs* CD and the C vs UC discriminant analyses, LysoPC and PC were the most discriminant compounds. LysoPC18:0, LysoPC18:1, LysoPC18:2, PC18:2/18:2, PC18:1/18:0 and PC18:1/18:1 were decreased in pathological samples, while PC16:0/22:6, PC18:0/22:6 and PC16:0/20:4 were increased in CD and UC. The increased levels of those PCs that contain docosahexaenoic acid and the arachidonic acid as fatty acids, suggest a requirement from the metabolic system of fatty acid implicated in the inflammatory response. This hypothesis strengthens the fact that several FA like 18:2, 18:3, 20:3 20:4 22:4, 22:5 and 22:6, already known as implicated in the arachidonic acid cascade (191), were found increased in our pathological samples. Furthermore, in the targeted analysis of the polar metabolite profile of plasma, levels of cyclic adenosine monophosphate cAMP were found increased in UC. Moore et al. described how the production of cAMP in leucocytes is due to 3-adrenergic catecholamines, histamine and the E serie prostaglandins by a receptor coupled activation of adenylate cyclase (192). Several in vitro studies reported that the agents that increase cAMP level were implicated in the reduction of several inflammatory metabolic pathway as release of histamine or leukotrienes or the proliferation of lymphocytes (193,194).

Decreased levels of PC can suggest a damage of the intestinal mucosa organization. It is already known as a change of the intestinal endothelium increase the bacteria permeability (195).

From the aqueous fraction, we found a remarkable decrease of amino acid levels in IBD patients like, alanine, betaine, citrulline, threonine, hydroxyl-proline, proline, valine, methionine, tyrosine, tryptophan and methionine sulfoxide. This result confirm what Hisamatsu et al. had already reported. In fact, the author observed significant alterations in the plasma amino acid composition suggesting the use of their model as a diagnostic approach for IBD (196). In addition, dietary protein and amino acid supplementation in the IBD colonic mucosa, afflicted by changes in the microbiota, it has been already suggested as a therapy for the IBD (197). The effects of production of amino acids by microbes on whole-body fluxes and human health are still under examination. Up to now, it is noticeable that the amino acids production by bacteria could lead to pathological conditions as type 2 diabetes where systemic concentrations of amino acids have been found elevated (198). The amino acid variation in the IBD samples can suggest a remarkable mucosa damage due to microbiota changes. For example, decreased plasma levels of citrulline correspond to severe and extensive mucosal barrier injury of the small intestine (199).

5.2.3. Overview of IBD plasma lipidomic profile.

In the previous plasma analysis discrimination between the pathological classes were not observed. The aim of this analysis was to investigate possible differences between CD and UC. This result can be caused by a strong variability of samples intra-classes. For this reason, with the aim to reduce the intra-class variability only samples in a selected range of age, gender, BMI and therapy were chosen. To investigate the lipid samples profile of IBD cohort a SPE method was used. Several lipid categories were identified and some of them resulted altered in the different sample classes. Among the phospholipid fraction, the glycerol phospholipids levels were found increased in UC when compared to CD. Saturated diacylglycerols were decreased in UC when compared to CD. From the neutral fraction, the cholesteryl esters were found strongly upregulated in UC when compared to CD. These variations, found between CD and UC, suggest that in a wide human being cohort metabolomics study, information can be hidden due to the large variability between patients. Among these findings,

variations in the cholesteryl esters levels can suggest an implication of the lipoprotein system. Changes in the lipoprotein levels are strictly correlated to the patients BMI (200). Reducing the BMI range helped to clarify these observations. Byyany et al., in a lipoprotein profile comparison between IBD patients and the National Health and Nutrition Examination patients found lower total cholesterol and high density lipoprotein cholesterol levels in IBD while higher low density lipoprotein cholesterol levels (199). Hypocholesterolaemia is a common symptom in patients with various types of acute disease, including surgery, trauma, burn injury and sepsis (201) and this variation can be caused by the chemokine action (202). Chemokines are chemotactic cytokines that stimulate the leukocytes migration to areas of inflammation and lead cell activation events (203). It has been noticed that the local inflammation and tissue damage in UC and CD is caused by a local expression of specific chemokines in IBD tissues (203). Further experiments are recommended to understand why the levels of cholesteryl esters were strongly higher in UC when compared with CD as the same for glycerol phospholipids and DAG that were found also variated. Regarding the fatty acid fraction, no variation between CD and UC were highlighted. However, a strong increase of monounsaturated and polyunsaturated fatty acid was found in both CD an UC when compared to healthy samples. These results agree with the central role of the arachidonic acid pathway in the activation of inflammatory responses.

6. CONCLUSIONS

The aim of this PhD thesis was to characterise lipid and polar profiles of IBD with the use of different mass spectrometric platforms. The study was based on the investigation of predictive biomarkers for the diagnosis of CD and UC and possible peculiarities able to distinguish between CD and UC. IBD patients and C stool and plasma samples were analysed, and several compounds were recognized as discriminant for IBD samples.

From the statistical analysis of stool samples, the comparison between IBD *vs* C showed good classificatory and predictive powers while those of CD *vs* UC didn't reported good classificatory powers. Several metabolites were found discriminants for the pathological classes and were correlated with different metabolic pathways. Decreased levels of urobilin and uribilinogen in IBD samples suggested a variation of the bilirubin metabolic pathway and further correlation was found when the tetra pyrrole compounds where correlated with the most discriminant bacteria from the microbiological analysis, highlighting an implication of the gut microbiome in the IBD pathogenesis. Increased levels of NAPE in IBD samples were also found, indicating a modification in the endocannabinoid system. Increased levels of several DAG and decrease levels of PC in IBD samples suggest a correlation with the PKC and phospholipase A2 pathways, highlighting an inflammatory response and resulting as products from the variation of the intestinal mucosa permeability. These results were confirmed with the variation of several amino acids in the IBD samples. In fact, methionine, cysteine and asymmetric dimethyl arginine levels were found increased in IBD samples.

Statistical analysis of plasma samples showed good classificatory and predictive powers when IBD were compared with C samples. The discriminant compounds such as PC, LysoPC and Fatty acids showed a modification in the phospholipase A2 pathway reminding to inflammatory response implication. In addition, a variation of cAMP and several amino acids levels like citrulline suggested and confirmed what previously reported for the faecal samples analysis: a correlation with the intestinal mucosa damage. Comparison between CD and UC did not show good classificatory power. No discriminant metabolites between CD and UC resulted in both faecal and

plasma analysis. These results were linked to the strong variability intra-class of samples. To avoid this problem and to deeply investigate possible differences between CD and UC a SPE method of 30 samples, chosen based on similar age, gender and BMI was studied.

The SPE fraction analysis of IBD samples reported difference comparing UC *vs* CD and the disease *vs* healthy samples. Cholesteryl esters levels resulted increased in UC when compared with CD suggesting an implication of the lipoproteins and chemokines pathways. Unsaturated fatty acid levels resulted increased in IBD samples when compared with C confirming the implication of the arachidonic acid pathway in the activation of inflammatory responses.

In conclusion, the metabolomics study based on the analysis of both polar and lipid metabolite profiles though the use of LC/MS/MS and IM platforms can be considered a valuable tool for the IBD pathogenesis investigation. Further studies are strongly recommended to understand the two most important related metabolic pathways found in this PhD thesis: the inflammatory response and the mucosa damage.

7. REFERENCES

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8. TABLES

Patients class	Gender	Number	Age	BMI(body mass inddex)
CROHN DISEASE	Male	23	21 - 65	16 - 31
	Female	27	28 -78	17.2 - 31.3
ULCERATIVE COLITIS	Male	40	20 - 76	19.1 - 32.5
	Female	42	20 - 76	15.7 - 35.6
CONTROLS	Male	31	24 - 66	18.9 - 24.7
	Female	20	24 - 65	19.3 - 31.2

Groups		C	CD	UC	clinical information		UC	CD
Diet	Mediterranean	49	50	82	**Surgery**	Yes	0	11
	High-protein	2	-	-		No	82	39
Smoke	Yes	18	12	8		Adalimumab	10	15
	No	33	38	74	**Therapy**	Azatioprine	12	9
	Ex-smokers (since 2 years)	5	24	20		Infliximab	7	4
						Naive	2	6
Alcohol	Yes	28	15	10		Traditional	51	16
	No	23	35	72		Quiescent	43	16
Coffee	Yes	44	26	51	**Activity**	Mild	15	5
	No	7	24	31		Moderate	17	7
Total		51	50	82		Severe	7	18

Table 3.1. Samples clinical record.

Name	trivial name	class	store
N-palmitoyl-d31-D-erythro-sphingosine (C16-d31 Ceramide)	C16-d31 Ceramide	Cer	avanti
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphate (sodium salt) (16:0-d31-18:1 PA)	16:0-d31-18:1 PA	PA	avanti
1-palmitoyl(D31)-2-oleyl-sn-glycero-3-phosphocholine (16:0-d31-18:1 PC)	16:0-d31-18:1 PC	PC	avanti
1-palmitoyl(D31)-2-oleyl-sn-glycero-3-phosphoethanolamine (16:0-d31-18:1 PE)	16:0-d31-18:1 PE	PE	avanti
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (16:0-d31-18:1 PG)	16:0-d31-18:1 PG	PG	avanti
1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphoinositol (ammonium salt) (16:0-d31-18:1 PI)	16:0-d31-18:1 PI	PI	avanti
1,2-dimyristoyl-d54-sn-glycero-3[phospho-L-serine] (sodium salt)(14:0 PS-d54)	14:0 PS-d54	PS	avanti
N-palmitoyl(D31)-d-erythro-sphingosylphosphorylcholine (16:0-d31 SM)	16:0-d31 SM	SM	avanti
1,2-dipalmitoyl-d62-sn-glycero-3-[phospho-L-serine] (sodium salt)	16:0 PS-d62	PS	avanti
cholesteryl-2,2,3,4,4,6-d6-octadecanoate (18:0-d6 CE)	18:0-d6 CE	Cer	qmx
Pentadecanoic-d29 Acid (15:0-d29 FA)	15:0-d29 FA	FA	qmx
Heptadecanoic-d33-acid (17:0-d33 FA)	17:0-d33 FA	FA	qmx
Eicosanoic-d39 Acid (20:0-d39 FA)	20:0-d39 FA	FA	qmx
Tetradecylphosphocholine-d42 (14:0-d29 LPC-d13)	14:0-d29 LPC-d13	LysoPC	qmx
Glyceryl Tri(pentadecanoate-d29) (45:0-d29 TG)	45:0-d29 TG	TAG	qmx
glyceryl-tri(hexadecanoate-d31) (48:0-d31 TG)	48:0-d31 TG	TAG	qmx
Glyceryl Tri(octadecanoate-d35) (54:0-d35 TG)	54:0-d35 TG	TAG	qmx
arachidonic acid d8	arachidonic acid d8	PUFA	Cayman Chemicals
linoleic acid d4	linoleic acid d4	PUFA	Cayman Chemicals
11(12)-EET-d11	11(12)-EET-d11	oxFA	Cayman Chemicals
Prostaglandin E2-d4	Prostaglandin E2-d4	oxFA	Cayman Chemicals
12-HETE-d8	12-HETE-d8	oxFA	Cayman Chemicals
TxB2-d4	TxB2-d4	oxFA	Cayman Chemicals
LTB2-d4	LTB2-d4	oxFA	Cayman Chemicals

Table 3.2. List of lipids used for the plasma internal standard mixture. oxFA = oxidated fatty acid.

compound	Rt(min)	ionisation	parent mass (m/z)	fragment (m/z)	CE (v)
5- hydroxy-tryptophane	4.1	positive	221.2	204.1	11
alanine	0.7	positive	90.1	44.2	11
α-amino isobutyric acid	0.8	positive	104.05	85.9	5
α-hydroxy-butyric acid	2.1	negative	103.025	73.1	10
AMP	1.2	positive	348.1	136.1	20
arginine	0.7	positive	175.15	70.2	23
asparagine	0.7	positive	134.175	74.1	15
aspartate	0.7	positive	133.1	116.0	8
asymmetric dimethyl-arginine	1.2	positive	203.3	46	12
β-hydroxy-isovaleric acid	3.4	negative	117.075	59.1	12
β-amino-isobutyric acid	0.9	negative	103.025	57.1	12
β-hydroxy-butyric acid	1.9	negative	103.025	57.1	12
betaine	0.8	positive	118.1	58.2	26
cAMP	3.4	positive	330.1	136.2	25
carnosine	0.7	positive	227.25	110.2	21
choline	0.9	positive	104.1	60.1	15
citrate	1.4	negative	191	111	15
citrulline	0.8	positive	176.15	159.0	8
creatine	1	positive	131.975	43.1	29

creatinine	0.9	positive	114.1	86.1	11
cystine	0.7	positive	122.025	59.1	23.6
cytidine	1.1	positive	112	95.1	18
γ-amino-butyric acid	0.8	positive	104.039	58	10.2
glutamic acid	0.7	positive	148	84.1	16
glutamine	0.7	positive	147.1	84.1	16
glycine	0.6	positive	76.1	48.2	6
guanidine	3.4	positive	152.1	135	19
GMP	1.2	positive	364.1	152.1	16
guanosine	3.4	positive	168	151.1	19
histidine	0.6	positive	156.1	110.1	13
hydroxy-proline	0.7	positive	132.025	86.2	8
inosine	3.2	positive	269.15	137.1	10
isocitrate	1.1	positive	191	111	15
isoleucine	1.8	positive	132.025	86.2	8
keto-isoleucine	4.2	negative	129.05	84.8	6
Keto-leucine	4.5	negative	129.1	129.1	6
leucine	1.9	positive	132.025	86.2	8
lysine	0.6	positive	147.1	84.1	16
malate	1.1	negative	133	115.0	12
methionine	1.2	positive	150.05	61.1	23
methionine sulfoxide	0.8	positive	166	149.1	12
methyl-histidine	0.7	positive	170.2	95.1	30
NADPN	1.2	negative	742.8	408.1	35
nicotinic acid	1.2	positive	124.07	80.1	20.8

nicotinamide	1.1	positive	123.152	80.1	19.5
N-monomethyl arginine	0.8	positive	189.3	70.2	23
ornithine	0.6	positive	133.2	70.1	16
phenylalanine	3.6	positive	166.1	120.1	13
phosphocreatine	0.8	negative	210	79.0	16
proline	0.9	positive	115.975	70.1	15
putrescine	0.6	positive	89.252	30.3	20.2
pyruvic acid	0.8	negative	87	43	10
serine	0.7	positive	106.075	60.2	10
succinate	1.8	negative	117	73	10
taurine	0.7	positive	126.1	44.1	14
threonine	0.7	positive	120.1	74.1	9
trimethylamine	1.1	positive	60.152	44.1	20.1
Trimethylamine-N oxide	0.9	positive	76.122	58.1	18.1
tryptophan	4.2	positive	205.2	188.1	11
tyrosine	2.3	positive	182.1	136.1	12
UDP N-acetyl glucosamine	0.9	negative	606.1	159	28
UMP	1.2	positive	325.2	97.1	12
uridine	1.9	positive	245.1	112.9	12
valine	1.1	positive	118.1	72.2	10

Table 3.3. LC/MS QqQ detected metabolites from the MS/MS analysis of faecal and plasma samples.

compound	adduct	*m/z* measured	*m/z* theoretical	Δppm	Rt (min)	Fatty acid composition	Product ion (*m/z*)	Discriminant class
urobilin	Na^+	617.335	617.331	5.5	1.3		291.2	C
PC 19:1	neutral	549.3461	549.3431	4.4	1.4	16:0, 3:1	130.1	C
Unknown1		279.7294			1.7			CD
urobilinogen	H^+	597.367	597.3647	2.4	2.2		471. 3	C
Unknown2		508.4934			6.3			CD
Unknown3		647.9453			11			UC
Unknown4		515.3659			11.6			C
DAG 34:2	$NH4^+$	634.5398	634.5405	1.9	12.3	18:2, 18:2	353.3	UC
PA 35:1	H^+	689.504	689.5043	0.7	14.8	19:0, 16:1	355. 2	C
PC 36:3	neutral	783.5768	783.5778	1.9	14.9	22.2, 14:1	393.1, 184.1	CD
Unknown5		371.3179			15			C
Unknown6		474.3812			15.5			C
Unknown7		465.376			15.8			CD,
DAG 40:2	$H\text{-}2H_2O$	641.5974	641.5878	14	16.4	18:0, 22:2	267.1, 341.1	CD, UC
PS 40:2	H^+	844.6053	844.6062	1.7	16.5	18:0, 22:2	413.2,341.1, 185.1	C
Unknown8		1238.874			20.8			C
Cer 40:1	H^+	622.6167	622.6133	4.5	22.7	18:1, 22:0	282.1, 339.2	C
Unknown9		1147.911			23.2			CD
Unknown10		975.7294			23.2			CD, UC
Unknown11		1149.92			23.2			CD, UC
Unknown12		1076.719			23.5			CD, UC
Unknown13		465.3796			25.9			C
NAPE 52:2	H^+	982.7768	982.7762	0	24	18:1, 16:1, 18:0	281, 265, 341	UC

Table 3.4. Discriminant metabolites of IBD faecal samples identification by LC/MS/MS.

Lipid	Adduct	m/z experimental	m/z theoretical	Δppm	Fatty acid composition	Rt (min)	CCS ($Å^2$)	Product ion (m/z)
LysoPC 18:2	H^+	520.3351	520.3398	10	18:2	1.0	226.1	337.3, 258.1, 184.1, 104.1
LysoPC 16:0	H^+	496.3393	496.3398	2.1	16:0	1.2	228.5	313.3, 258.1, 184.1, 104.1
LysoPC 18:1	H^+	522.3543	522.3554	3.1	18:1	1.3	231.3	339.3, 258.1, 184.1, 104.1
LysoPC 18:0	H^+	524.3706	524.3711	1.9	18:0	1.8	236.0	341.3, 258.1, 184.1, 104.1
Unknown		356,352				1.8*	200.9	
PC 36:4	H^+	782.5638	782.5694	7.8	18:2, 18:2	4.4	285.0	599.5, 520.3, 337.2, 263.2, 184.1
PC 38:7	H^+	804.5511	804.5538	4	18:3, 20:4	4.4	287.9	621.5, 544.3 527.3, 502.3, 277.4, 184.1
PC 34:3	H^+	756.5536	756.5538	0.9	16:1, 18:2	4.5	282.1	573.4, 476.4, 494.3, 184.1
PC 34:2	H^+	758.5684	758.5694	2	16:0, 18:2	4.6	281.5	575.5, 502.3, 478.3, 337.2, 313.3, 184.1
SM 36:2	H^+	729.5857	729.5905	7	16:1/20:1	4.6	285.9	546.3, 237.1, 184.1
PC 38:6	H^+	806.6038	806.5913	14	18:3/20:3	4.9	292.2	624.5, 528.3, 500.3, 184.1
PC 36:4	H^+	782.572	782.5694	2.6	16:0, 20:4	4.7	287.6	599.5, 526.3, 496.3, 313.3, 184.1

PC 38:6	H^+	806.5677	806.5694	2.8	16:0, 22:6	4.9	291.6	623.5, 550.3, 313.3, 184.1
PC 40:6	H^+	834.6008	834.6007	0.5	18:0, 22:6	4.9	296.6	651.5, 341.3, 184.1
SM 42:2	H^+	813.6823	813.6844	3.2	18:2, 24:0	5.8	303.3	629.5, 337.3, 184.1
PC 32:1	H^+	732.5529	732.5538	1.9	16:1, 16:0	6.93*	279.4	549.5, 479.3, 477.3, 184.07
PC 38:5	H^+	808.5829	808.5851	3.3	22:5, 16:0	7.2*	290.2	625.49, 552.49, 341.30, 313.3, 184.07
Unknown		369.3484				7.4	202.19	
PC 36:3	H^+	784.5841	784.5851	1.9	16:0, 20:3	7.96*	287.5	601.5, 528.3, 478.3, 184.07
PC 36:2	H^+	786.6003	786.6007	1.2	18:2, 18:0	9.8*	289.3	603.53, 502.3, 506.38, 520.36, 524.39, 341.30, 184.07
PC 36:1	H^+	788.616	788.6164	1.2	18:1, 18:0	12.2*	291.8	605.53, 504.35, 524.36, 184.07
Unknown		698,255				8.74*	295,7	
PC 34:1	H^+	760.585	760.5851	0.8	18:1, 16:0	9.2*	285.7	577.5, 504.3, 478.3, 339.2, 313.3, 184.1
PC 36:2	H^+	786.5990	786.6007	2.8	18:1, 18:1	9.4*	289.5	604.53, 504.38, 522.36, 184.07
PC 38:3	H^+	812.6151	812.6164	2.2	20:3, 18:0	10.6*	294.0	629.56, 527.37, 506.37, 341.6, 267.1, 184.07

TG 56:7	$NH4^+$	922.7844	922.7858	2.1	20:4, 18:2, 18:1	6.9	323.6	601.5, 625.5, 361.2, 339.2, 337.2, 287.2, 265. 263.2
TG 52:3	$NH4^+$	874.7833	874.7858	3.4	18:1, 16:0, 18:2	7.1	318.1	575.1, 577.1, 601.1, 339.3, 337.2, 313.2, 265.3, 263.2, 239.2
TG 52:1	$NH4^+$	878.8144	878.8171	3.7	18:1, 18:0, 16:0	7.5	322.0	603.5, 577.5, 339.3, 313.3, 265.3, 247.2
FA 16:0	$-H^-$	255.231	255.2330	9	-	1.9	201.3	-
FA 18:2	$-H^-$	279.232	279.2330	5	-	1.6	203.8	-
FA 24:2	$-H^-$	363.251	363.2541	10	-	1.9	226.6	-
FA 16:1	H^-	253,217	253,217	0	-	1.5	213.7	-
PC 36:2	$+OAc^-$	844.608	844.6073	0	18:0, 18:2	5.2	304.3	770.5, 283.3, 279.2
FA 18:0	$-H^-$	283.262	283.2643	10	-	2,6	207.6	-
FA 22:1	$-H^-$	337.236	337.2384	8	-	1,9	222.3	-
FA 24:3	$-H^-$	361.235	361.2384	10	-	1.5	191.2	-
PC 38:6	$+OAc^-$	864.576			16:0, 22:6	4,5	296.4	790.5, 327.2, 255.2
FA 18:1	$-H^-$	281.243	281.2486	10	-	1.9	271.5	-
PC 36:3	$+OAc^-$	842.592	842.5917	0	18:1, 18:2	4,9	294.1	768.5, 281.3, 279.2

Table 3.5. Discriminant metabolites of IBD plasma samples identification by IM-C-MS/MS. * = Retention time related to the SPE LC/MS method.

ANALYSIS	**UC *vs* C**				**CD *vs* C**			
	R^2X	R^2Y	Q^2	Ptest Q^2	R^2X	R^2Y	Q^2	Ptest Q^2
PCA	0,6		0,5		0,5		0,4	
OPLS-DA	0,5	0,6	0,5	-0,5	0,4	0,8	0,5	-0,5

Table 4.1 Validation parameters of multivariate statistical data analysis of IBD faecal samples. Ptest= permutation test.

UC *vs* C		CD *vs* C	
compound	**MW *p***	**compound**	**MW *p***
PC 16:0/3:1	<0.0001	urobilin	<0.0001
urobilinogen	<0.0001	PC 16:0/3:1	< 0.0001
DAG 18:2/18:2	0.002	urobilinogen	< 0.0001
PC 22:2/14:1	0.002	PA 19:0/16:1	< 0.0001
DAG 18:0/22:2	0.003	DAG 18:0/22:2	0.001
Cer 18:1/22:0	0.004	PS 18:0/22:2	0.002
NAPE 18:1/16:1/18:0	0.004		
PS 18:0/22:2	0.004		

Table 4.2. Discriminant metabolites from the Mann-Whitney *U* test of faecal samples. UC= ulcerative colitis, CD= Crohn's disease, C= healthy samples, MW*p*= Mann-Whitney *p* value.

UC *vs* C		CD *vs* C	
compound	**MW *p***	**compound**	**MW *p***
carnosine	<0.0001	β-amino isobutirric acid	<0.0001
AMP	0.002	carnosine	< 0.0001
cystine	0.002	cystine	< 0.0001
ornithine	0.003	methionine	< 0.0001
Arginine	0.007	adma	0.0005
ADMA	0.02	α-amino isobutirric acid	0.001
β-amino isobutirric acid	0.03	malic acid	0.01
		arginine	0.03

Table 4.3 Discriminant metabolites from the Mann-Whitney *U* test of faecal polar metabolite profile samples. UC= ulcerative colitis, CD= Crohn's disease, C= healthy samples, MW*p*= Mann-Whitney *p* value.

Platform	Analysis	UC *vs* C				CD *vs* C			
		R^2X	R^2Y	Q^2	PtestQ^2	R^2X	R^2Y	Q^2	PtestQ^2
Q-TOF (+)	PCA	0.8		0.6		0.8		0.6	
	OPLS-DA	0.8	0.8	0.6	-0.31	0.7	0.7	0.5	-0.20
IM (+)	PCA	0.6		0.4		0.8		0.3	
	OPLS-DA	0.2	0.5	0.3	-0.18	0.2	0.5	0.4	-0.18
Q-TOF (-)	PCA	0.8		0.6		0.7		0.6	
	OPLS-DA	0.7	0.7	0.5	-0.19	0.7	0.7	0.5	-0.11
IM (-)	PCA	0.5		0.4		0.5		0.4	
	OPLS-DA	0.5	0.7	0.6	0.10	0.4	0.7	0.4	0.10

Table 4.4. Validation parameters of multivariate data analysis from the Xevo and the Ion Mobility IBD plasma samples. Ptest= permutation test.

UC vs C		CD vs UC	
compound	**MW*p***	**compound**	**MW*p***
LysoPC 18:1	< 0.0001	LysoPC 18:1	< 0.0001
PC 18:2/18:2	0,0001	PC18:3/20:4	< 0.0001
TG 18:1/18:2/20:4	0,0001	PC 18:2/18:2	0,0008
PC 18:3/20:4	0,0001	TG 18:1/18:2/20:4	0,0013
LysoPC 18:2	0,0003	LysoPC 18:2	0,0016
PC 18:0/18:1	0,0009	PC 16:0/22:6	0,0031
PC 16:0/22:6	0,0017	PC 18:0/18:1	0,0052
PC 18:1/18:1	0,0045	SM 16:1/20:1	0,0075
PC 16:0/20:4	0,0108	PC 18:1/18:1	0,0132
TG 16:0/18:0/18:1	0,0382	TG 16:0/18:1/18:2	0,0191
SM 18:2/24:0	0,0419	PC18:3/20:3	0,0246
TG 14:0/16:0/18:2	0,0469	PC 18:0/22:6	0,0347
		LysoPC18:0	0,0398

Table 4.5. Discriminant metabolites from the Mann-Whitney *U* test of plasma samples, positive ionisation. UC= ulcerative colitis, CD= Crohn's disease, C= healthy samples, MW*p*= Mann-Whitney *p* value.

UC *vs* C		CD *vs* C	
compound	**MW*p***	**compound**	**MW*p***
FA18:1	< 0.0001	FA18:1	< 0.0001
FA18:2	< 0.0001	FA16:0	< 0.0001
FA16:0	< 0.0001	FA18:2	< 0.0001
PC 18:1/18:2	< 0.0001	FA24:2	< 0.0001
FA24:2	< 0.0001	FA16:1	< 0.0001
FA16:1	< 0.0001	PC 18:1/18:2	< 0.0001
FA18:0	< 0.0001	FA18:0	< 0.0001
		FA22:1	< 0.0001
		FA24:3	< 0.0001
		PC 18:0/18:2	< 0.0001

Table 4.6. Discriminant metabolites from the Mann-Whitney *U* test of plasma samples negative ionisation. UC= ulcerative colitis, CD= Crohn's disease, C= healthy samples, MW*p*= Mann-Whitney *p* value.

Var ID (lipid maps annotation)	MW*p*	Class
PC 18:2/18:0	0,0002	UC
PC 18:1/18:0	0,0007	UC
PC 22:5/16:0	0,0011	UC
PC 20:3/18:0	0,0015	UC
PC 16:0/18:2	0,0029	UC
PC 16:0/18:1	0,0039	UC
PC 18:1/18:1	0,0039	UC
PC 18:0/22:6	0,0068	UC
PC 16:0/20:3	0,0089	UC
Unknown	0,01	CD
PC 16:1/16:0	0,01	UC
Unknown	0,04	UC

Table 4.7. Discriminant metabolites from the Mann-Whitney *U* test of SPE plasma phospholipid fraction. UC= ulcerative colitis, CD= Crohn's disease, MW*p*= Mann-Whitney *p* value.

UC *vs* C		CD *vs* C	
compound	**MW *p***	**compound**	**MW *p***
β-amino isobutirric acid	<0.0001	α -hydroxy butirric acid	<0.0001
α -hydroxy butirric acid	<0.0001	histidine	<0.0001
GABA	<0.0001	proline	<0.0001
Proline	<0.0001	tyrosine	0.0006
citric acid	0.0001	citric acid	0.0008
tryptophane	0.0008	β-amino isobutirric acid	0.001
Creatine	0.003	betaine	0.002
methionine	0.003	creatine	0.003
Alanine	0.005	hydroxy proline	0.003
threonine	0.005	methionine	0.009
AMP	0.006	citrulline	0.01
GMP	0.007	isocitric acid	0.01
Camp	0.008	alanine	0.02
isocitric acid	0.008	threonine	0.03
Uridine	0.008	methionine sulfoxide	0.04
β -hydroxy valeric acid	0.01	valine	0.04
Tyrosine	0.01		
citrulline	0.02		
ketoleucine	0.02		
Arginine	0.04		
Betaine	0.04		

Table 4.8. Discriminant metabolites from the Mann-Whitney *U* test of plasma polar metabolite profile samples. UC= ulcerative colitis, CD= Crohn's disease, C= healthy samples, MW*p*= Mann-Whitney *p* value.

9. FIGURES

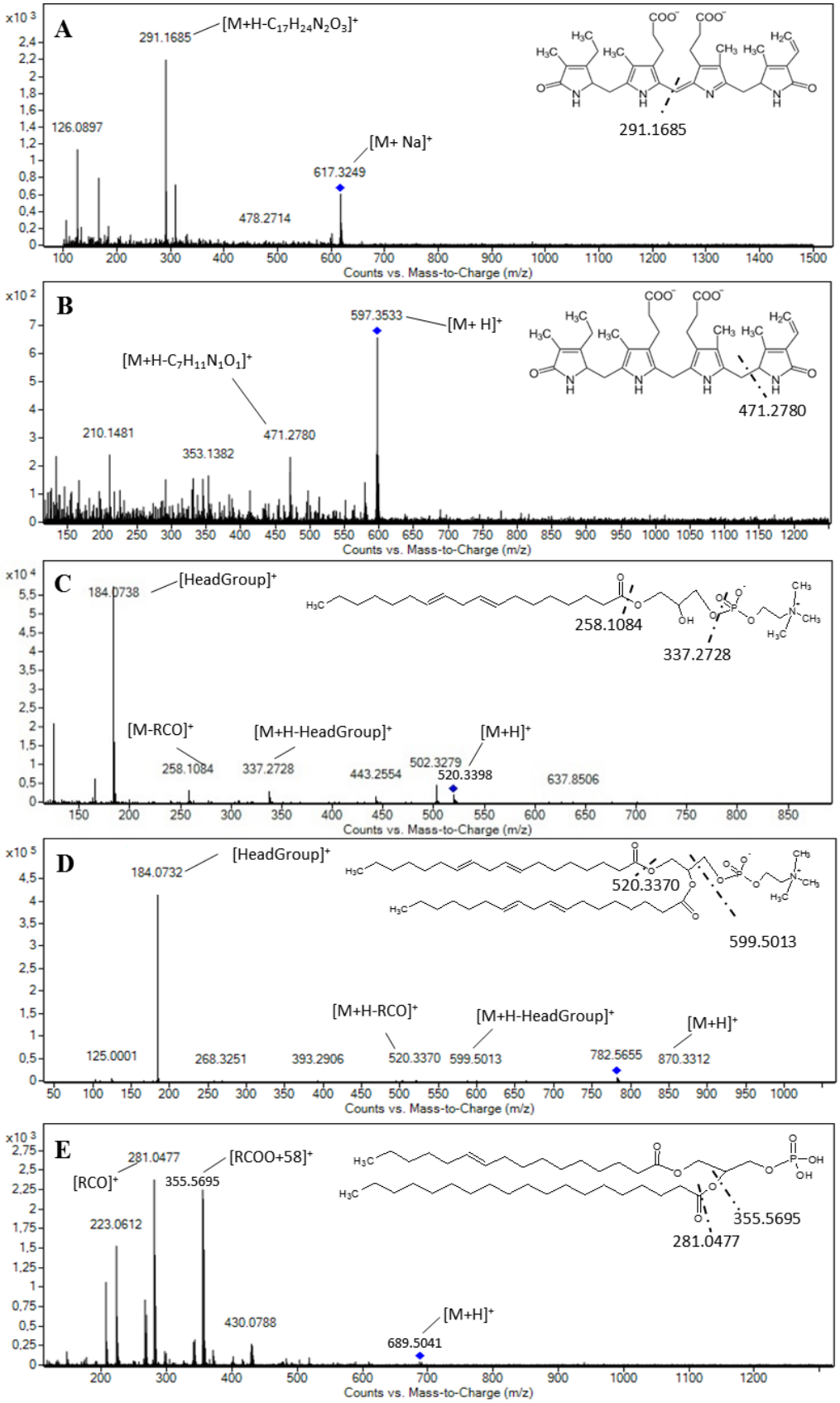

Figure 3.1. MS/MS fragmentation of different lipid categories: A= urobilin, B= urobilinogen, C=LysoPC 18:2, D= PC 36:4, E= PA 35:1, F= PS4 0:2, G= DAG 34:2, H= NAPE 52:2, I= SM 42:2, L= TG 52:1

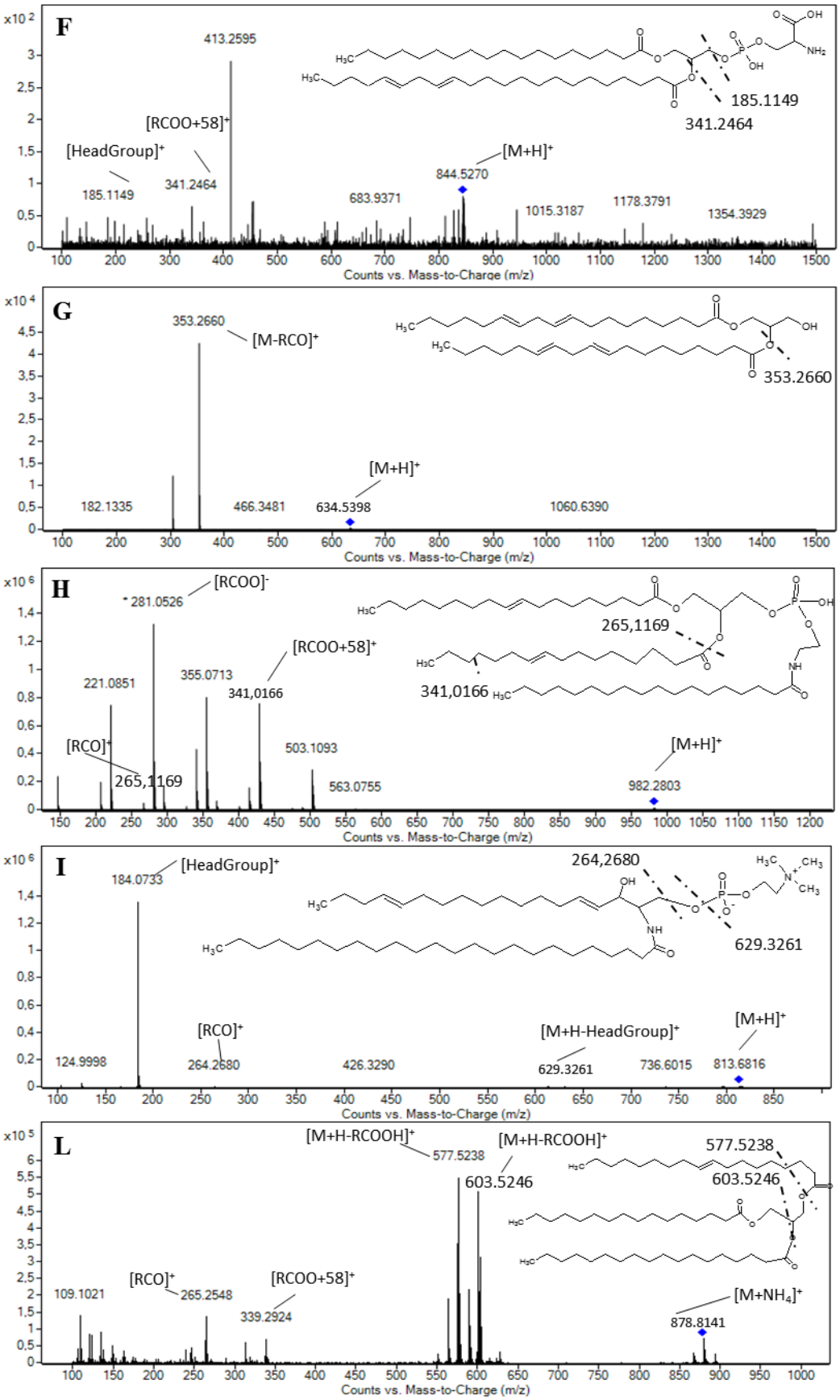

Figure 3.1. MS/MS fragmentation of different lipid categories: A= urobilin, B= urobilinogen, C=LysoPC 18:2, D= PC 36:4, E= PA 35:1, F= PS4 0:2, G= DAG 34:2, H= NAPE 52:2, I= SM 42:2, L= TG 52:1

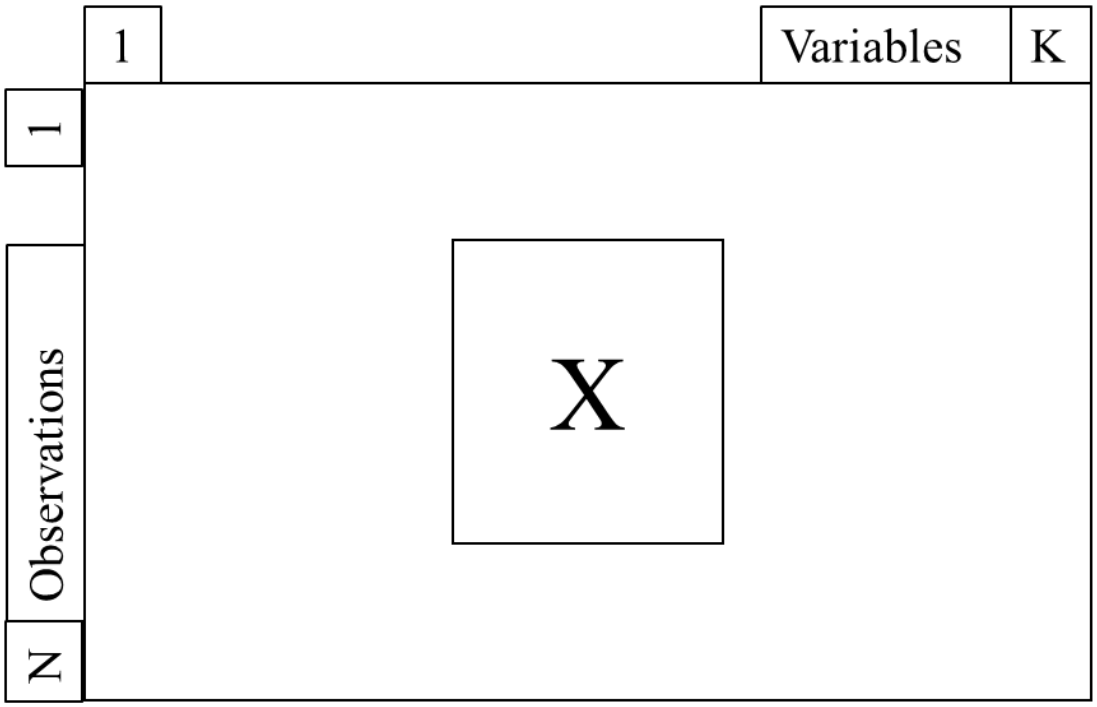

Figure 3.2. Matrix annotation of a typical dataset.

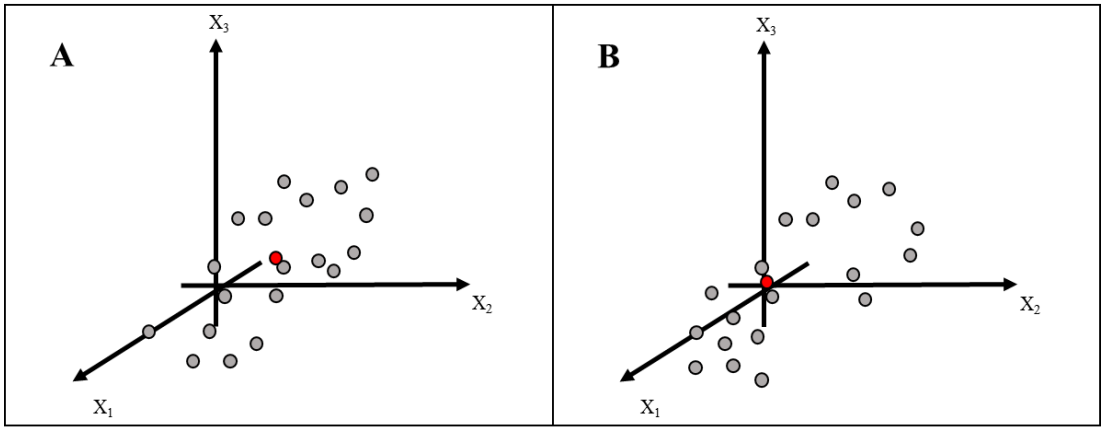

Figure 3.3. The mean-centering correspond to the movement of the origin system for coincide with the median point represented with a red circle. A: distribution before mean centering, B: distribution after mean-centering.

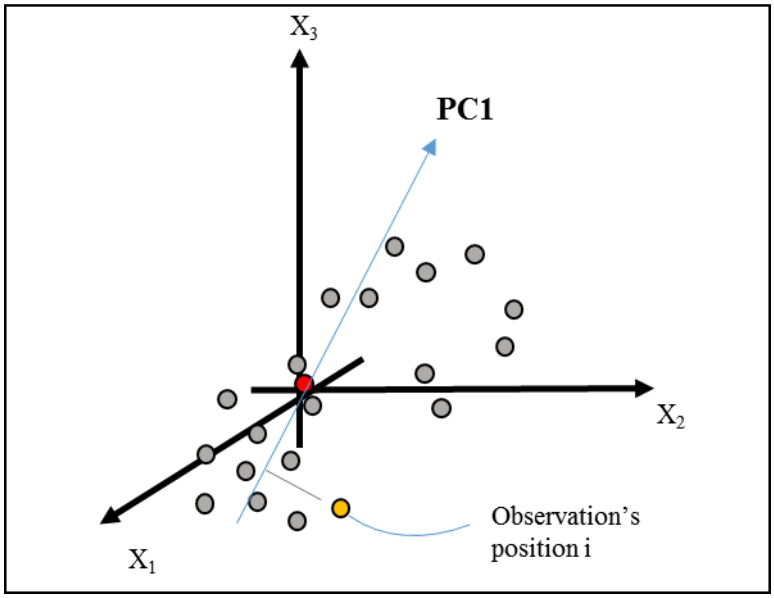

Figure 3.4. First principal component: is the line that describes the shape of set point. It represents the direction of maximum data variance. PC1= first principal component.

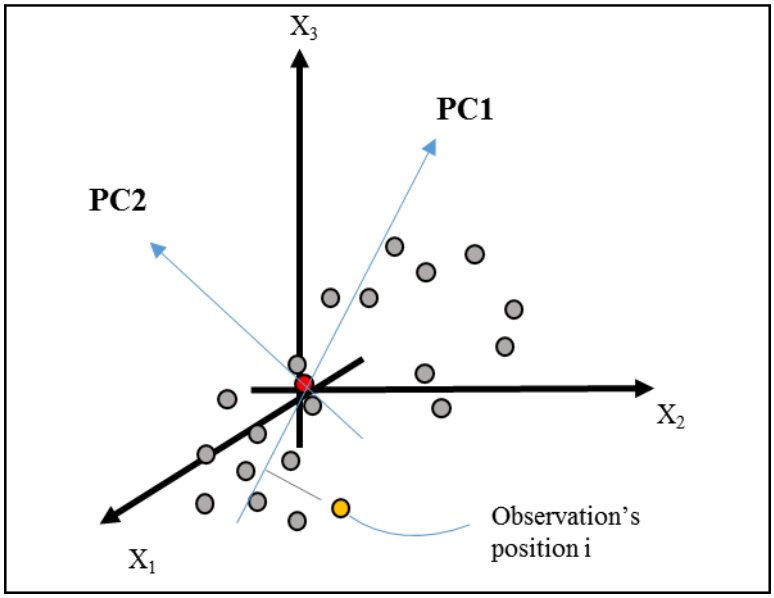

Figure 3.5. Scores Plot. The two principal components build a plan. This plan represents a window inside the multidimensional space that can be showed graphically. PC1,2: first and second principal components respectively.

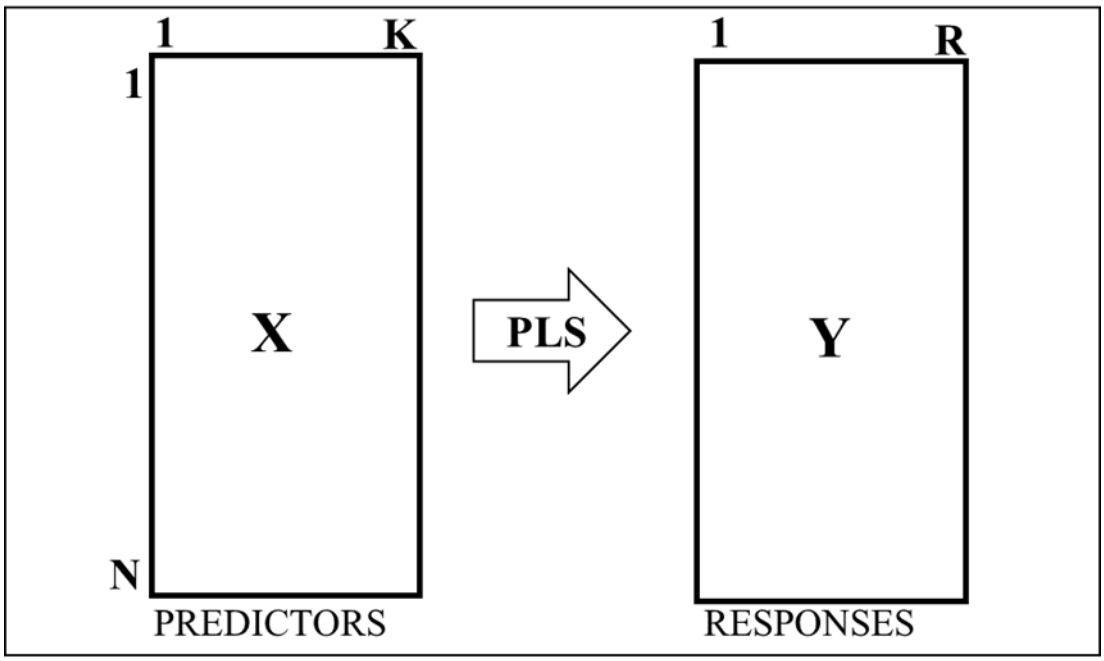

Figure 3.6. PLS-DA model. The generated two matrices, the X matrix (constituted by the observation and the variables) called predictors and the Y matrix (constituted by the samples classes) called response, are put in relation.

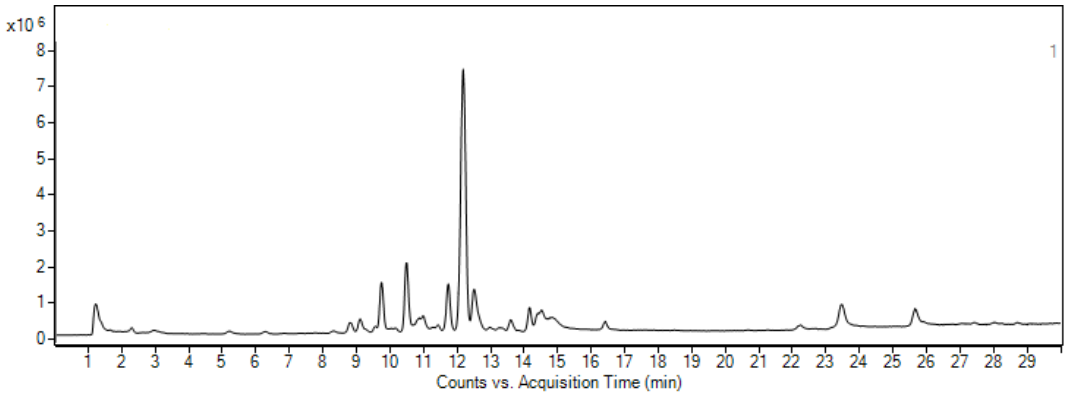

Figure 4.1. Example of LC/MS QTOF chromatograms from the faecal lipid analysis.

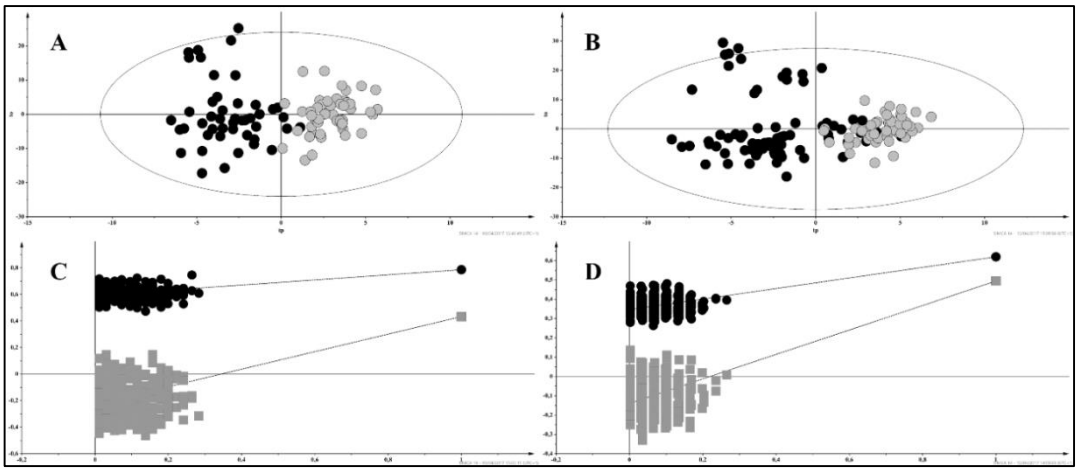

Figure 4.2. Scores plot from the LCMS-QTOF positive ionisation analysis. A) Scores plot from the OPLS-DA of CD samples (black filled circles) vs control samples (gray filled circles): $R^2Y=0.78$ $Q^2=0.45$. B) Scores plot from the OPLS-DA of UC samples (black filled circles) vs healthy patient samples (gray filled circles). $R^2Y=0.62$ $Q^2=0.48$. C) Permutation test from the PLS-DA of CD samples vs healthy patient samples ($R^2X=0.59$ $Q^2=-0.22$). D) Permutation test from the PLS-DA of UC samples vs control samples ($R^2X=0.59$ $Q^2=-0.22$). Grey squares represent the Q^2 and black circles the R^2Y.

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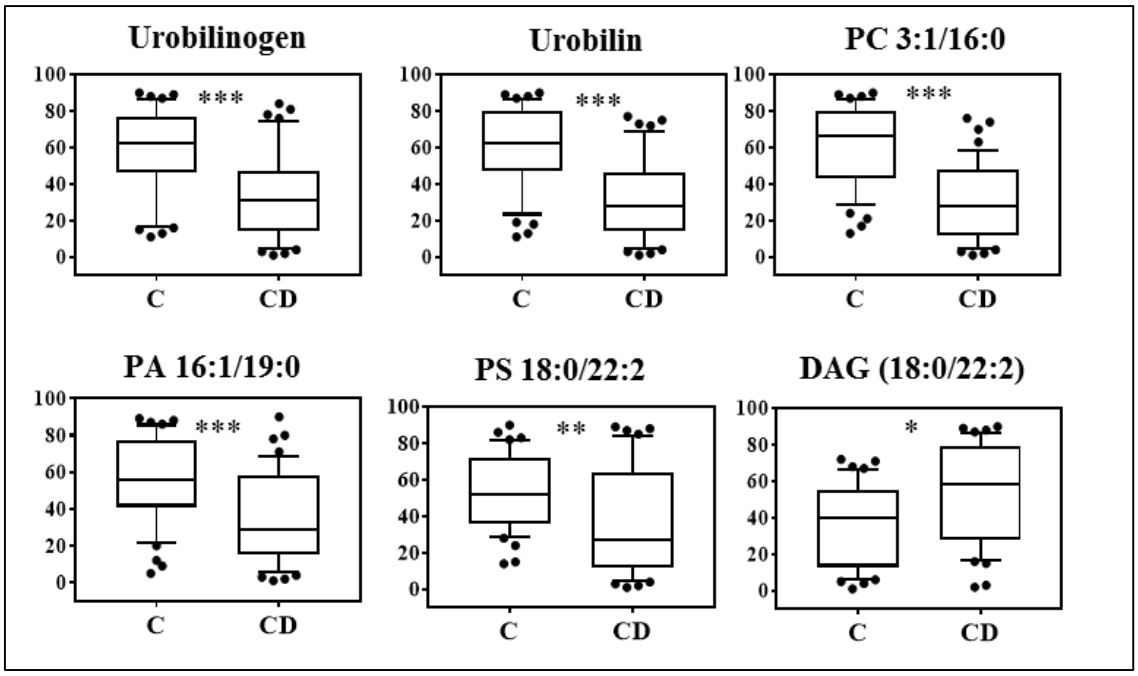

Figure 4.3. Box plot related to the most discriminant metabolites of faecal samples underwent from the Mann-Whitney *U* test. The relative analysis was the OPLSDA of CD *vs* control samples. Variable intensities are shown in the y axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

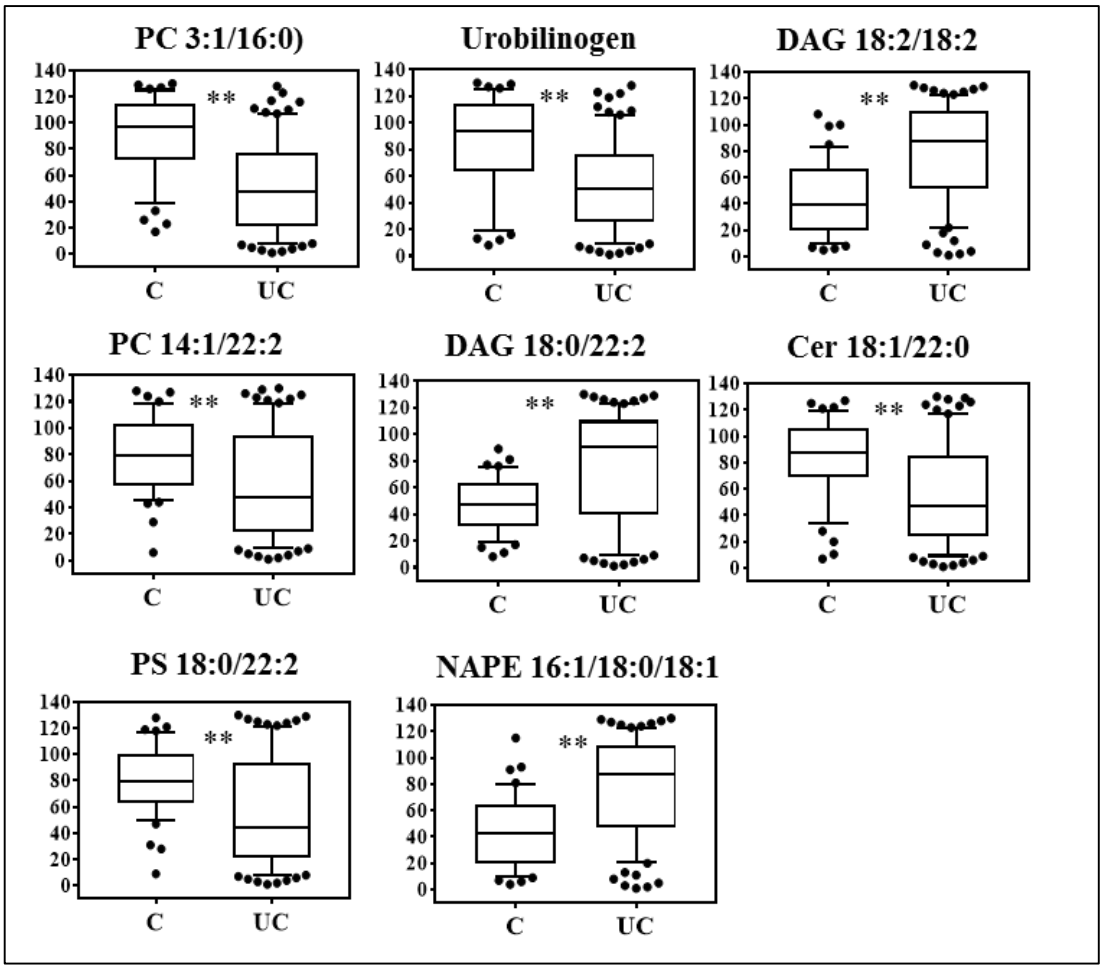

Figure 4.4. Box plot related to the most discriminant metabolites of faecal samples underwent from the Mann-Whitney *U* test. The relative analysis was the OPLSDA UC *vs* control samples. Variable intensities are shown in the y axis. *,**, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

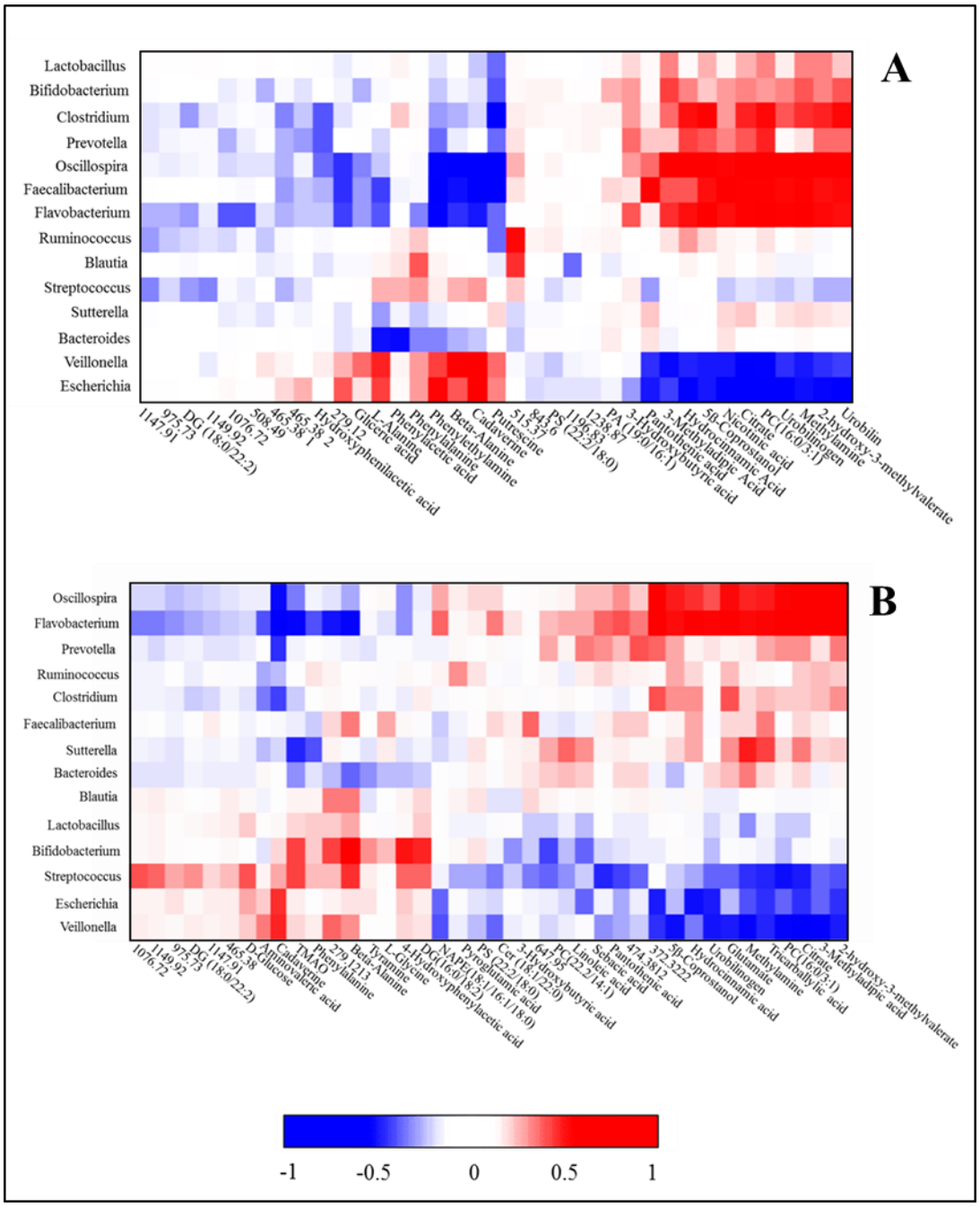

Figure 4.5. Inter-omic Spearman's rank correlation between metabolites and bacterial genera. Spearman correlation between statistically different metabolites and bacterial genera was calculated for both CD (A) and UC (B). All calculated correlations are showed.

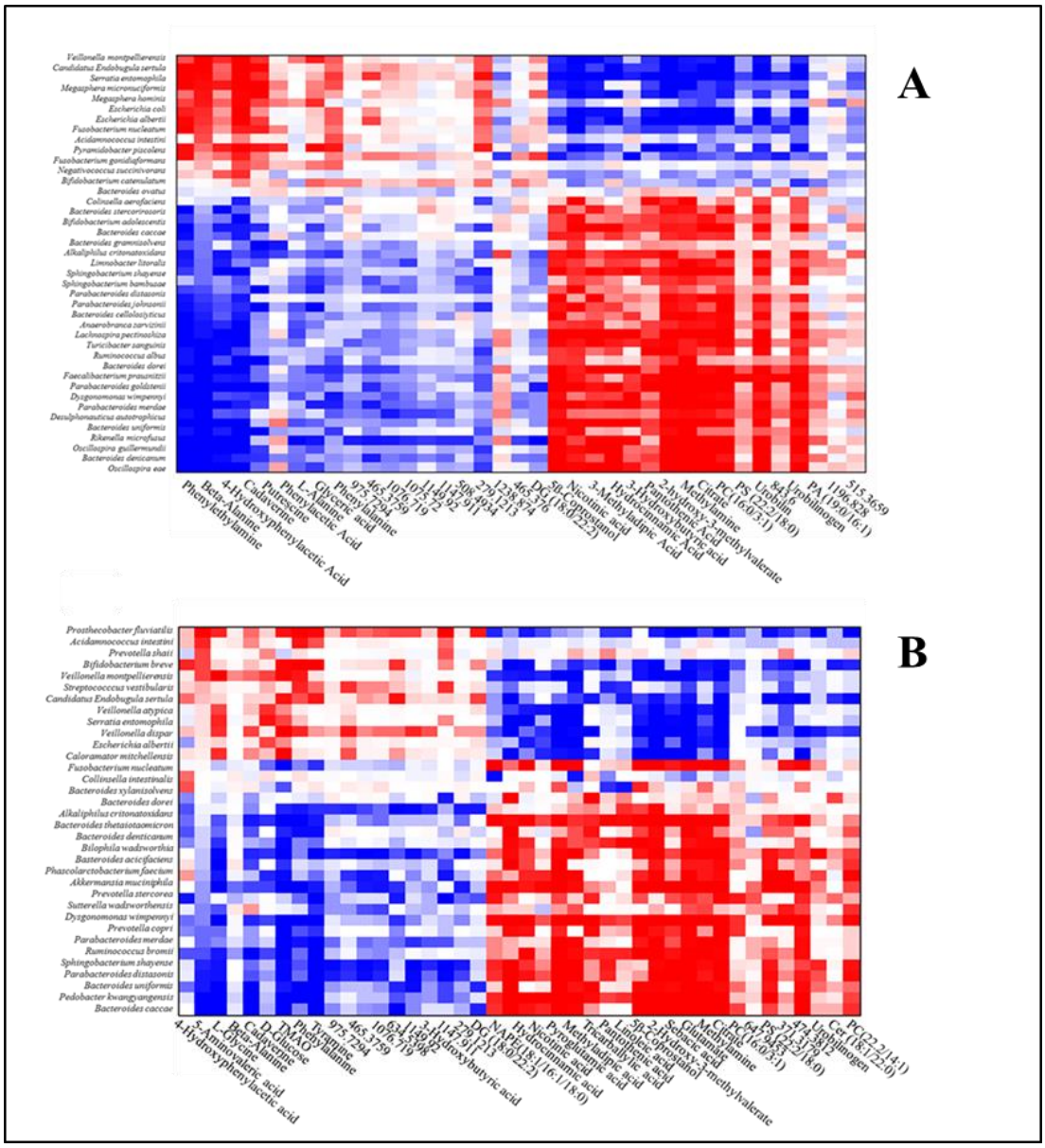

Figure 4.6. Inter-omic Spearman's rank correlation between metabolites and bacterial species. Spearman correlation between statistically different metabolites and bacterial species was calculated both for CD (**A**) and UC (**B**). All calculated correlations are shown.

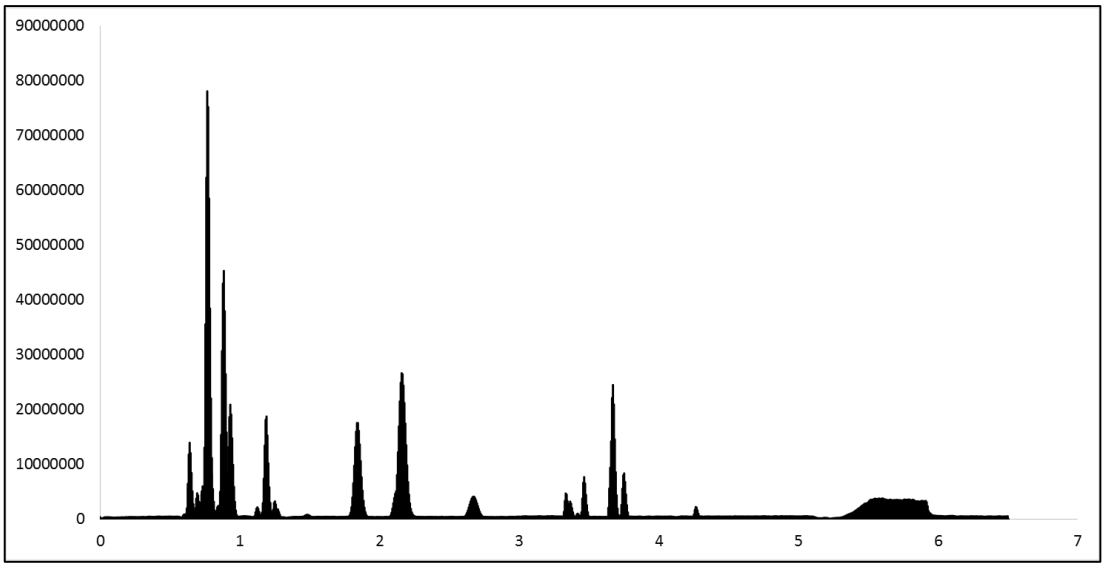

Figure 4.7. Example of a LC/MS QqQ chromatograms from the faecal polar metabolite profile analysis.

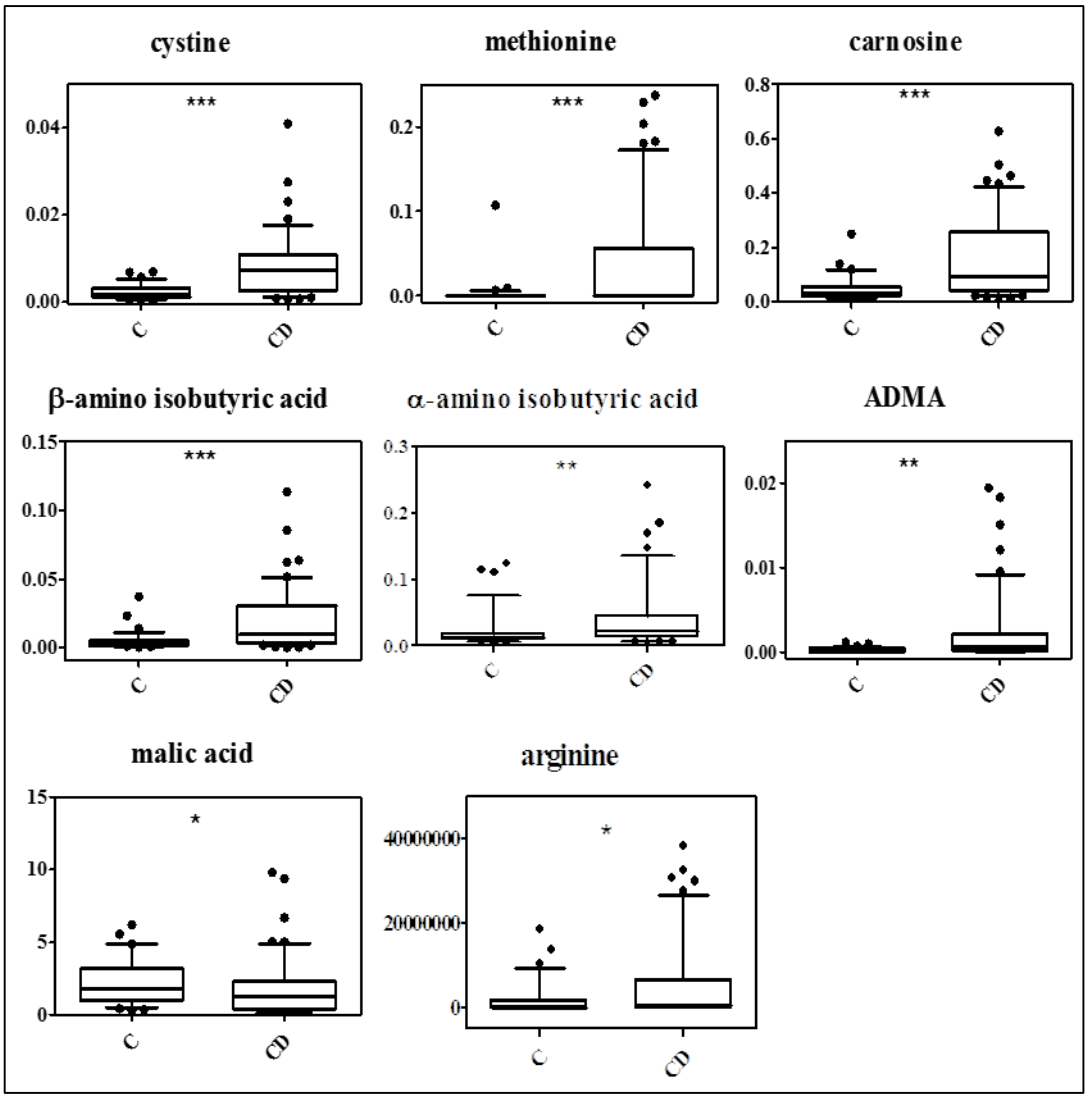

Figure 4.8. Box plot related to the most discriminant metabolites of faecal samples underwent from the Mann-Whitney *U* test between CD vs C samples. Variable intensities are shown in he y-axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

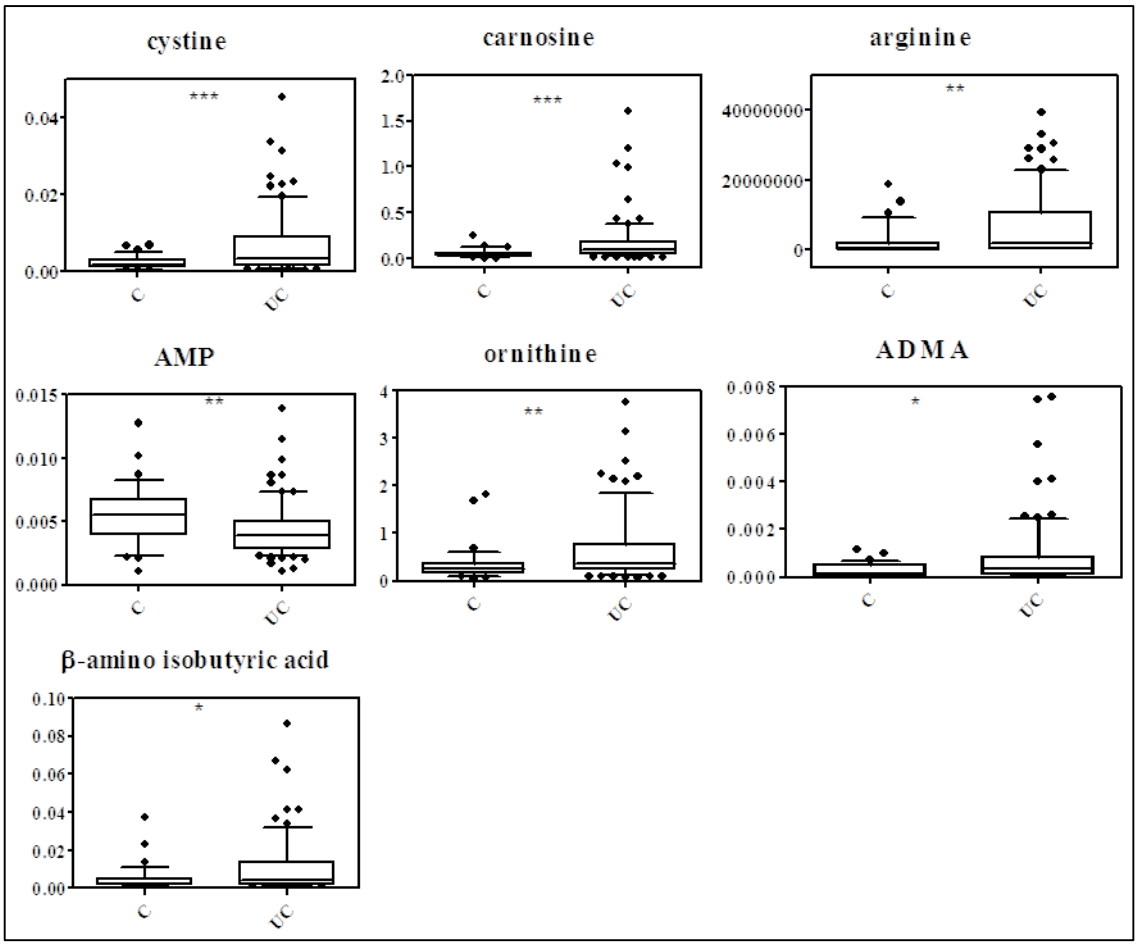

Figure 4.9. Box plot related to the most discriminant metabolites of faecal samples underwent from the Mann-Whitney *U* test between UC vs C samples. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

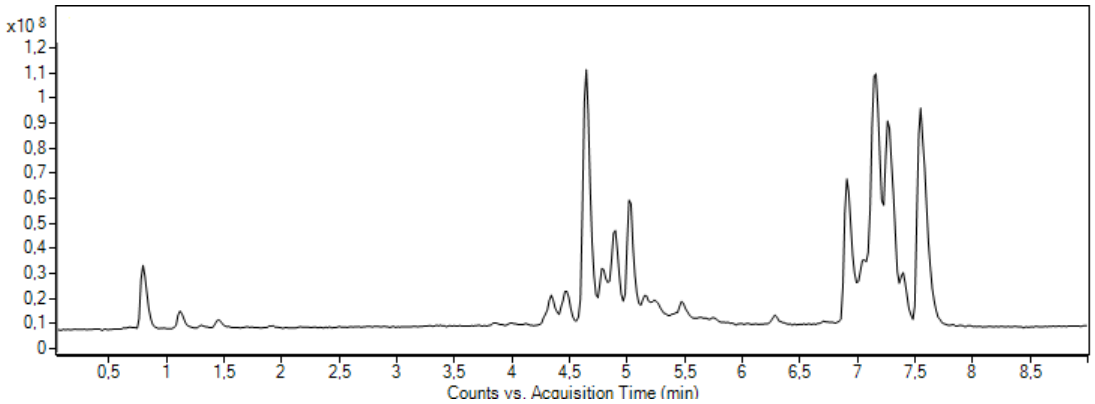

Figure 4.10. Example of IM-Q TOF-LC/MS chromatogram from the plasma lipid profile analysis in positive ionisation.

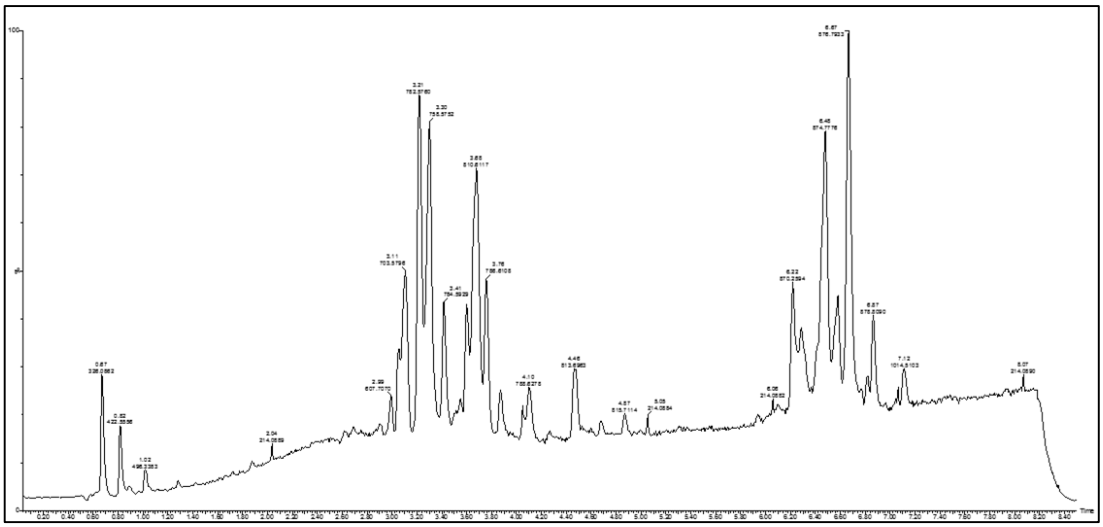

Figure 4.11. Example of XEVO Q TOF-LC/MS chromatogram from the plasma lipid profile analysis in positive ionization.

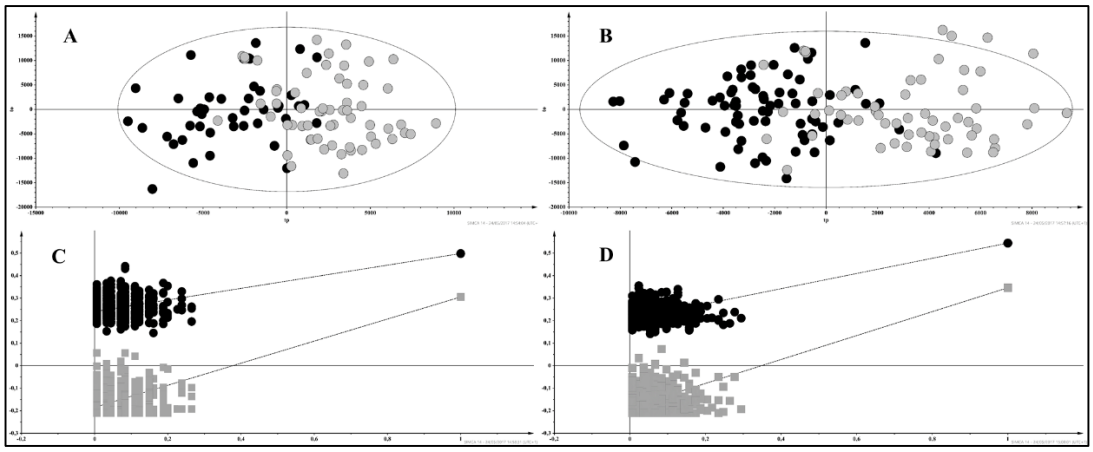

Figure 4.12. Scores plot from the Agilent Ion mobility-Q-TOF positive ionisation analysis. A) Scores plot from the OPLS-DA of CD samples (black filled circles) vs control samples (gray filled circles): $R^2Y=0.50$ $Q^2=0.37$. B) Scores plot from the OPLS-DA of UC samples (black filled circles) vs healthy patient samples (gray filled circles): $R^2Y=0.54$ $Q^2=0.34$. C) Permutation test from the PLS-DA of CD samples vs healthy patient samples ($Q^2=-0.18$). D) Permutation test from the PLS-DA of UC samples vs control samples ($Q^2=-0.18$). Grey squares represent the Q^2 and black circles the R^2Y.

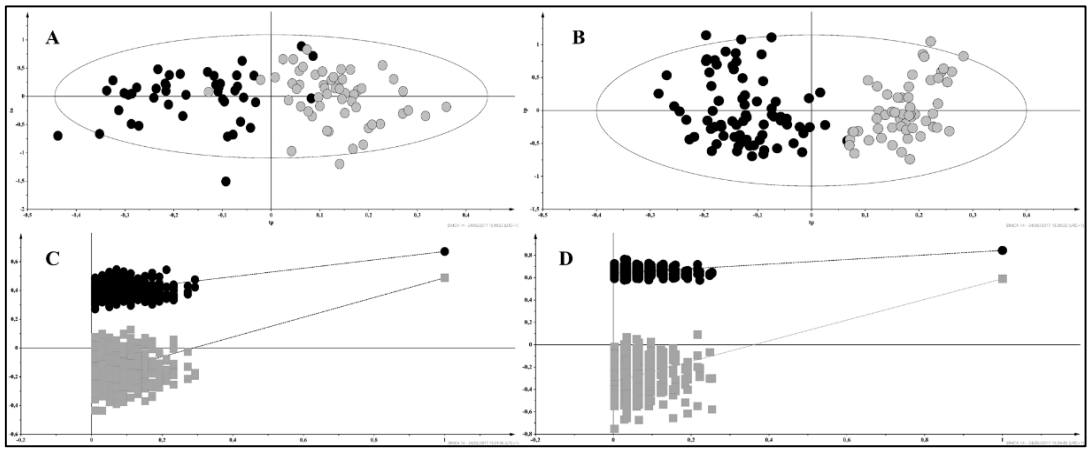

Figure 4.13. Scores plot from the Xevo Q-TOF positive ionisation analysis: A) Scores plot from the OPLS-DA of CD samples (black filled circles) vs control samples (gray filled circles): $R^2Y=0.67$ $Q^2=0.51$. B) Scores plot from the OPLS-DA of UC samples (black filled circles) vs healthy patient samples (gray filled circles): $R^2Y=0.75$ $Q^2=0.84$. C) Permutation test from the PLS-DA of CD samples vs healthy patient samples ($Q^2=-0.20$). D) Permutation test from the PLS-DA of UC samples vs control samples ($Q^2=-0.31$). Grey squares represent the Q^2 and black circles the R^2Y.

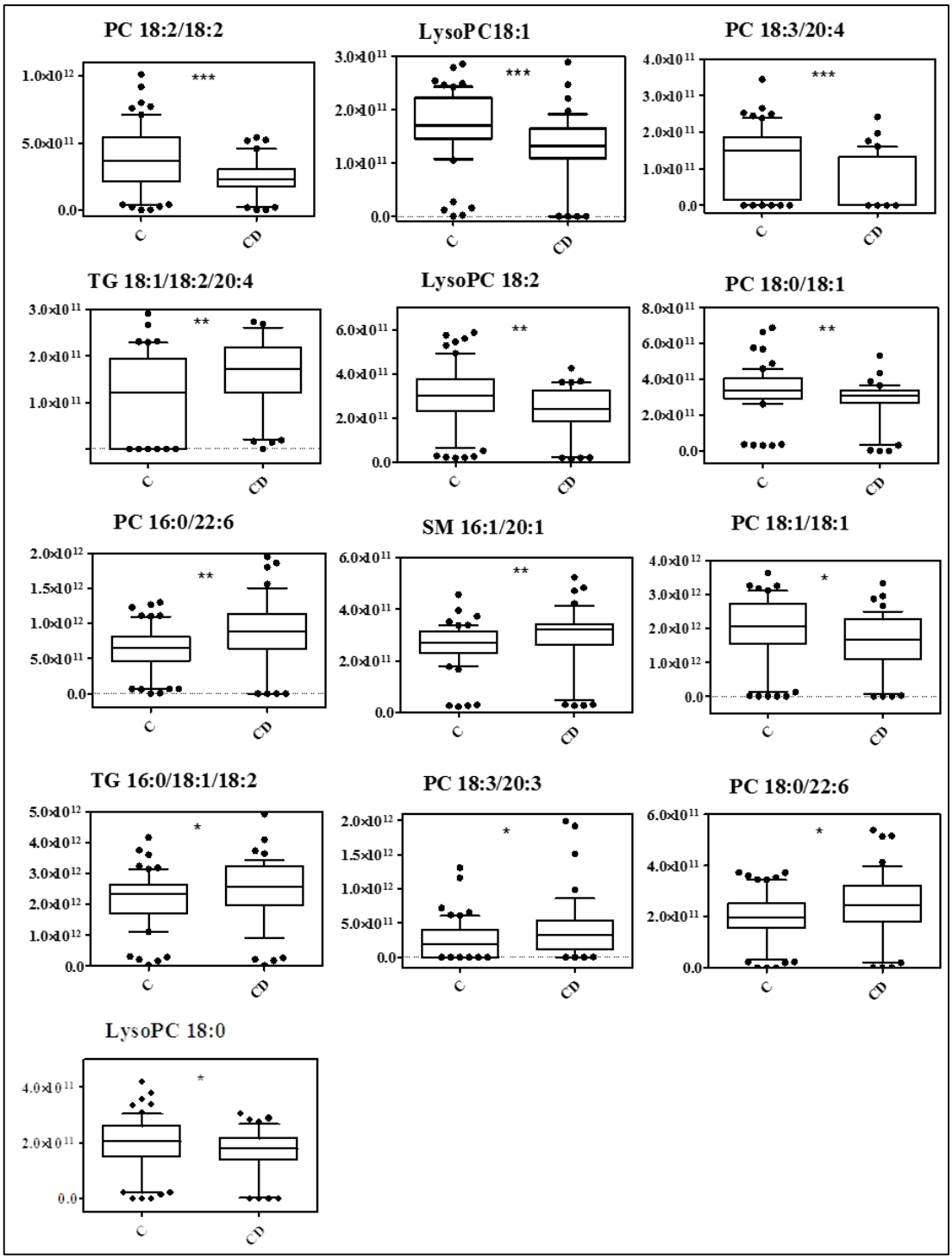

Figure 4.14. Box plot related to the most discriminant metabolites of plasma samples underwent from the Mann-Whitney test of the all ion positive analysis. The relative analysis was the OPLSDA of CD *vs* control samples. Variable intensities are shown in the y axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

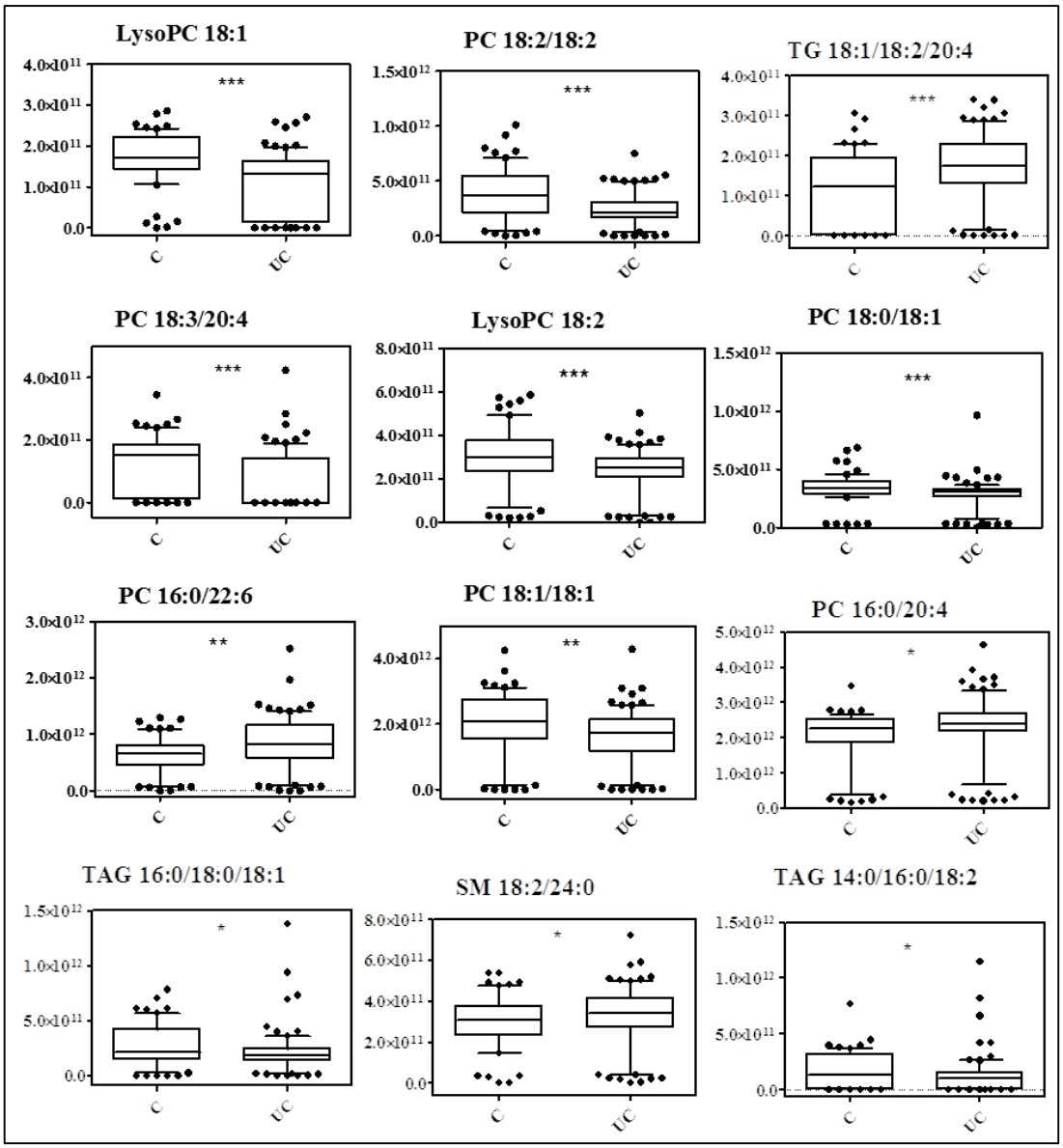

Figure 4.15. Box plot related to the most discriminant metabolites of plasma samples underwent from the Mann-Whitney test of the all ion positive analysis. The relative analysis was the OPLSDA of UC *vs* control samples. Variable intensities are shown in the y axis. *,**, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

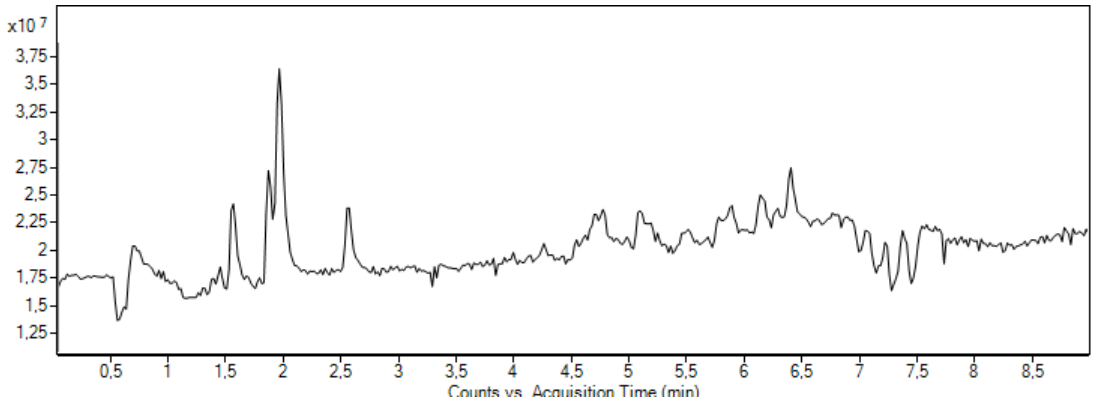

Figure 4.16, Example of IM-Q TOF-LC/MS chromatogram from the plasma lipid profile analysis in negative ionisation.

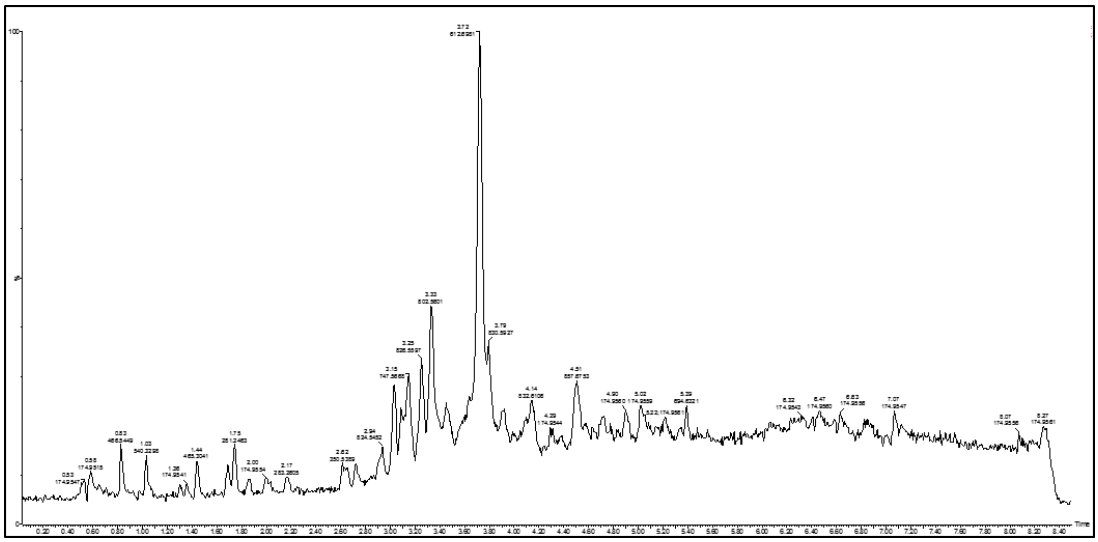

Figure 4.17. Example of LC/MS XEVO chromatogram from the plasma lipid profile analysis in negative ionisation.

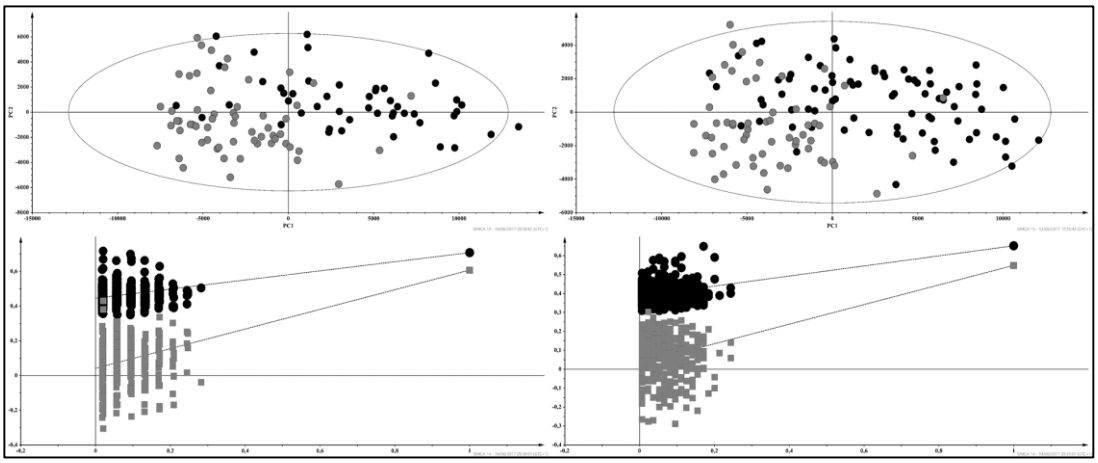

Figure 4.18. Scores plot from the Agilent Ion mobility-Q-TOF negative ionisation analysis. A) Scores plot from the PLS-DA of CD samples (black filled circles) vs control samples (gray filled circles) $R^2Y=0.70$ $Q^2=0.6$. B) Scores plot from the PLS-DA of UC samples (black filled circles) vs healthy patient samples (gray filled circles): $R^2Y=0.5$ $Q^2=0.36$. C) Permutation test from the PLS-DA of CD samples vs healthy patient samples ($Q^2=0.05$). D) Permutation test from the PLS-DA of UC samples vs control samples ($Q^2=-0.02$). Grey squares represent the Q^2 and black circles the R^2Y.

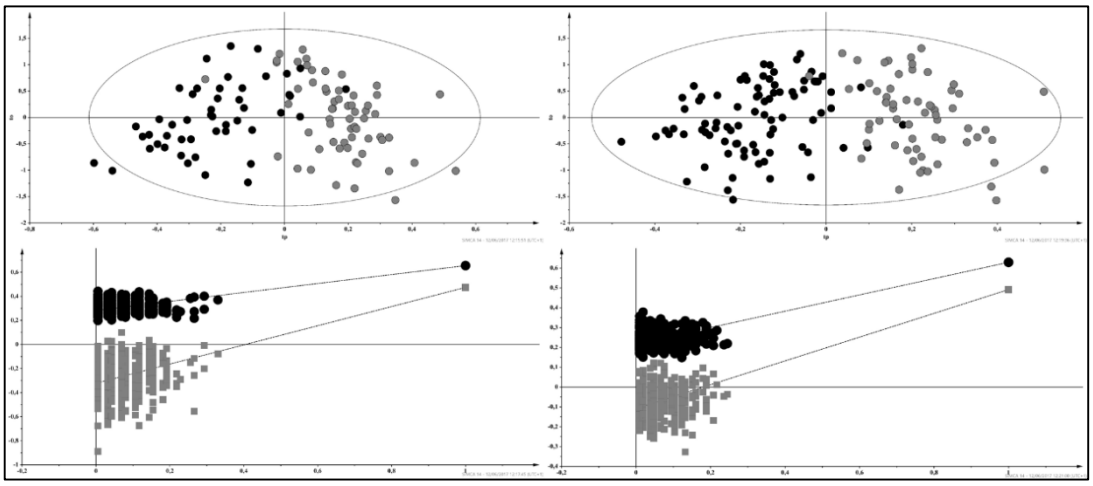

Figure 4.19. Scores plot from the Xevo Q-TOF negative ionisation analysis. A) Scores plot from the OPLS-DA of CD samples (black filled circles) vs control samples (gray filled circles): $R^2Y=0.74$ $Q^2=0.62$. B) Scores plot from the OPLS-DA of UC samples (black filled circles) vs healthy patient samples (gray filled circles): $R^2Y=0.69$ $Q^2=0.58$. C) Permutation test from the PLS-DA of CD samples vs healthy patient samples ($Q^2=-0.10$). D) Permutation test from the PLS-DA of UC samples vs control samples ($Q^2=-0.11$). Grey squares represent the Q^2 and black circles the R^2Y.

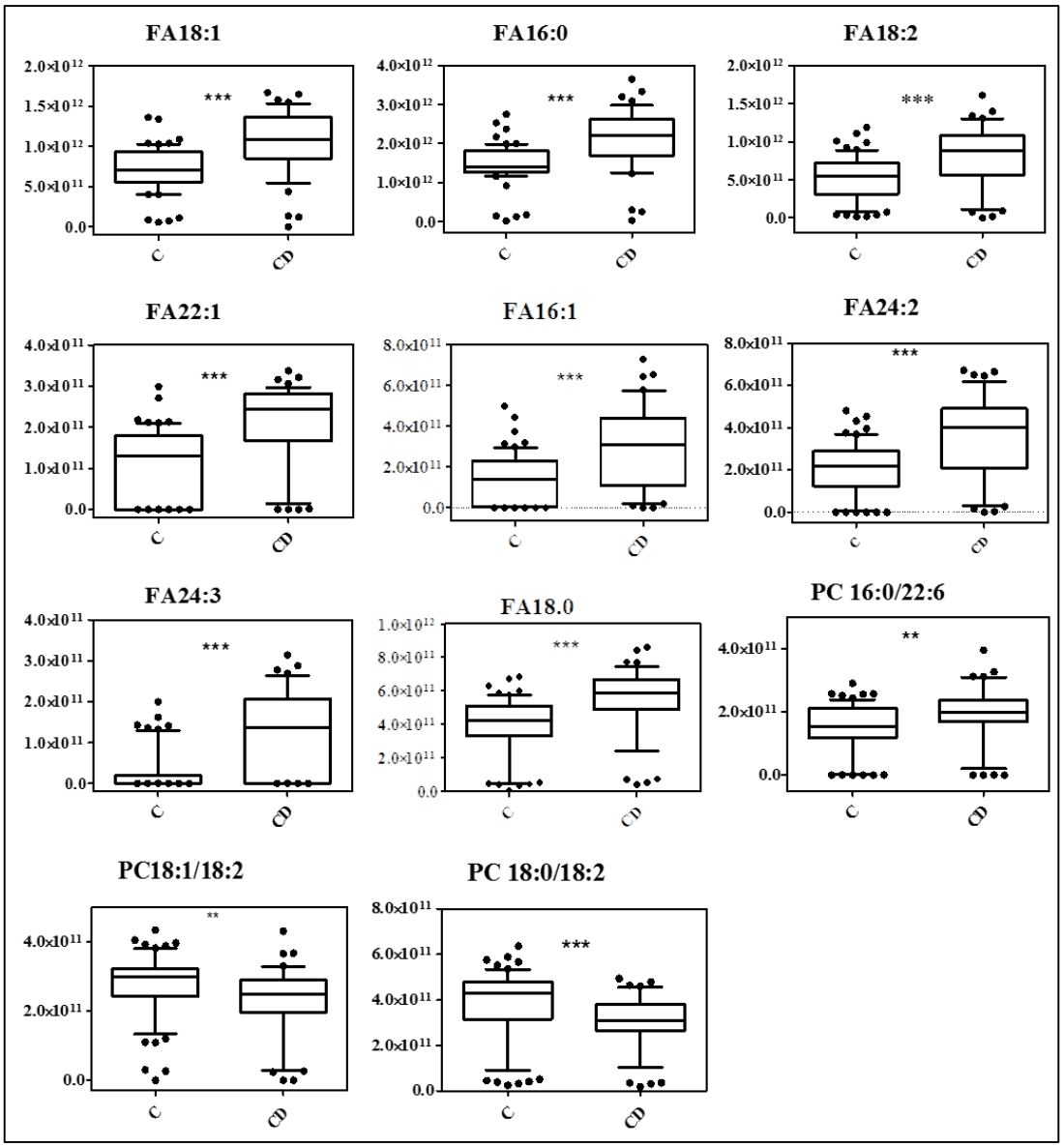

Figure 4.20: Box plot related to the most discriminant metabolites of plasma samples underwent from the Mann-Whitney test of the all ion negative analysis. The relative analysis was the OPLSDA of CD *vs* control samples. Variable intensities are shown in the y axis. *,**, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

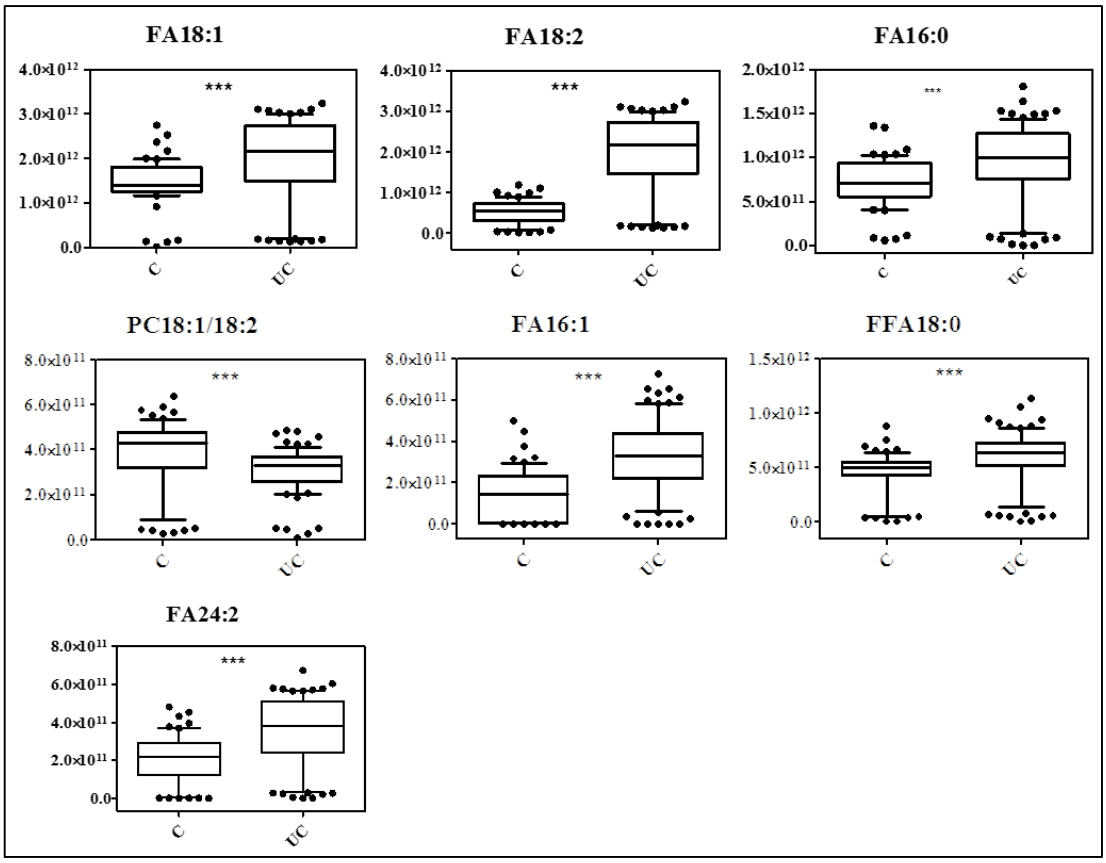

Figure 4.21: Box plot related to the most discriminant metabolites of plasma samples underwent from the Mann-Whitney test of the all ion negative analysis. The relative analysis was the OPLSDA of UC vs control samples. Variable intensities are shown in the y axis. *,**, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

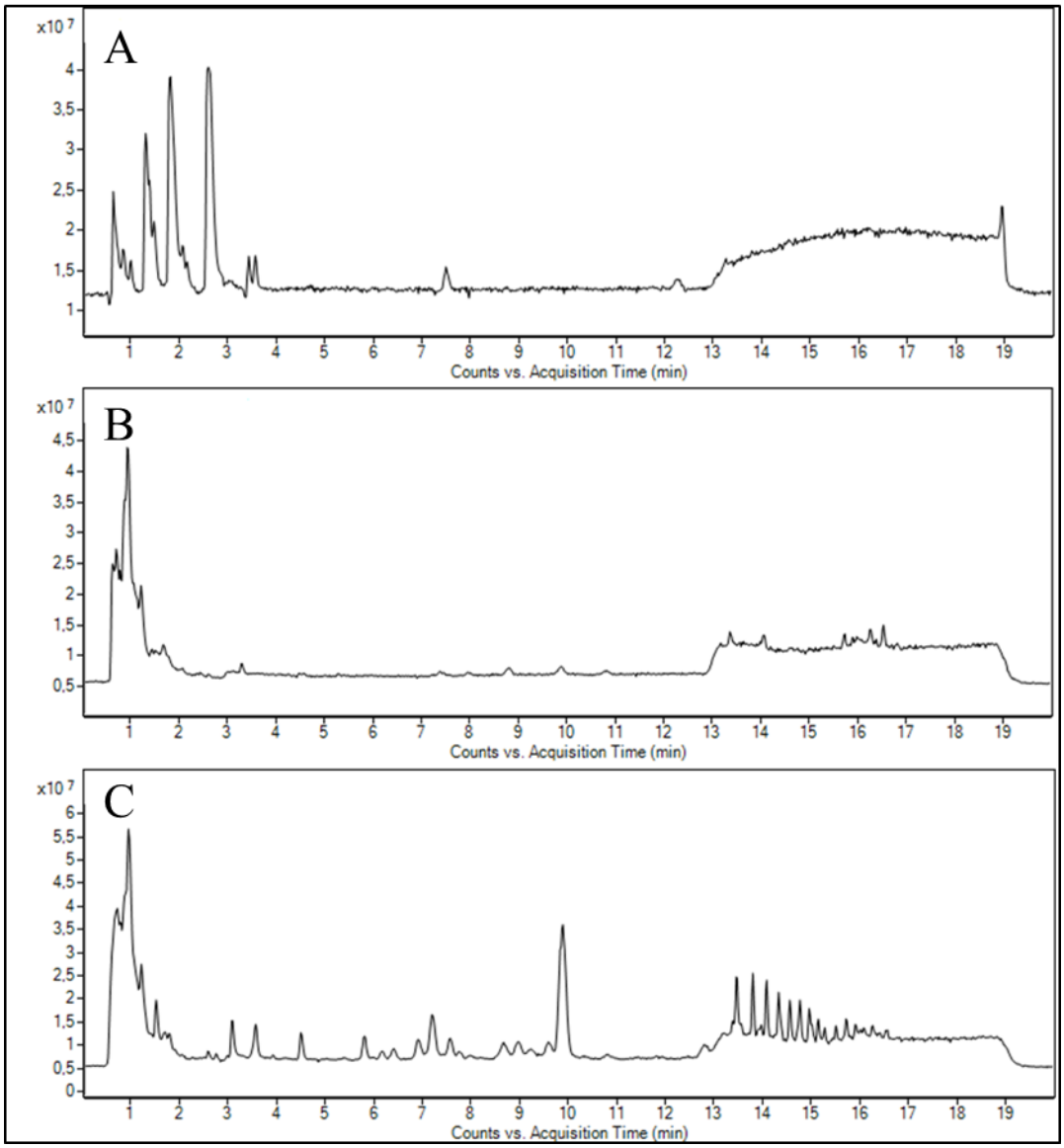

Figure 4.22. Example of an IM-Q TOF-LC/MS chromatogram from the SPE extraction analysis. A) fatty acid fraction B) neutral fraction, C) glycerophospholipid fraction.

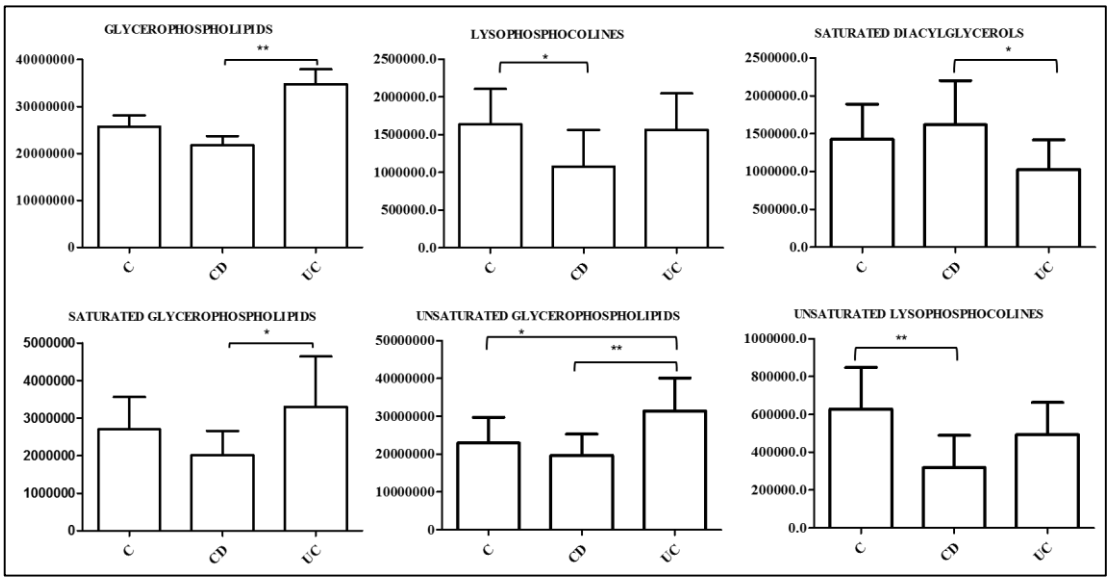

Figure 4.23. Graph related to the levels of different categories of glycerophospholipids of plasma glycerophospholipids plasma fraction samples, underwent from the one-way ANOVA test between CD vs UC vs C samples with the Bonferroni correction. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.01, <0.05, <0.001 respectively.

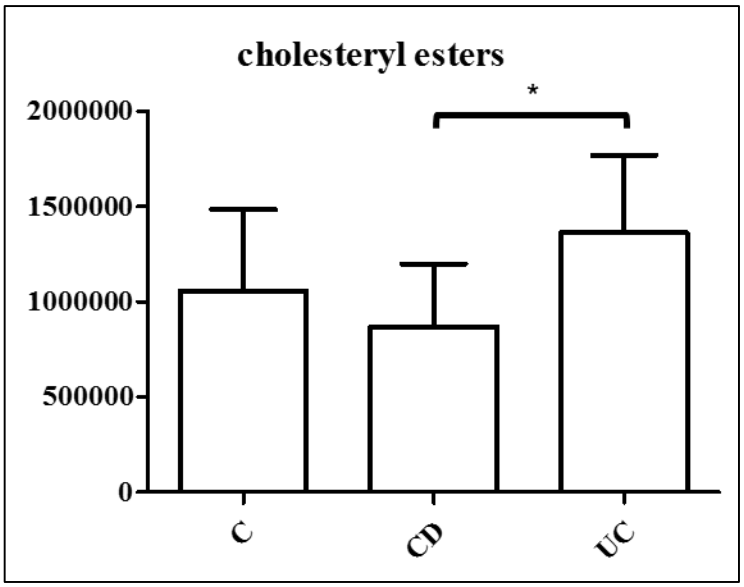

Figure 4.14. Graph related to the levels of cholesteryl esters of plasma neutral fraction samples underwent from the one-way ANOVA test between CD vs UC vs C samples with the Bonferroni correction. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.01, <0.05, <0.001 respectively.

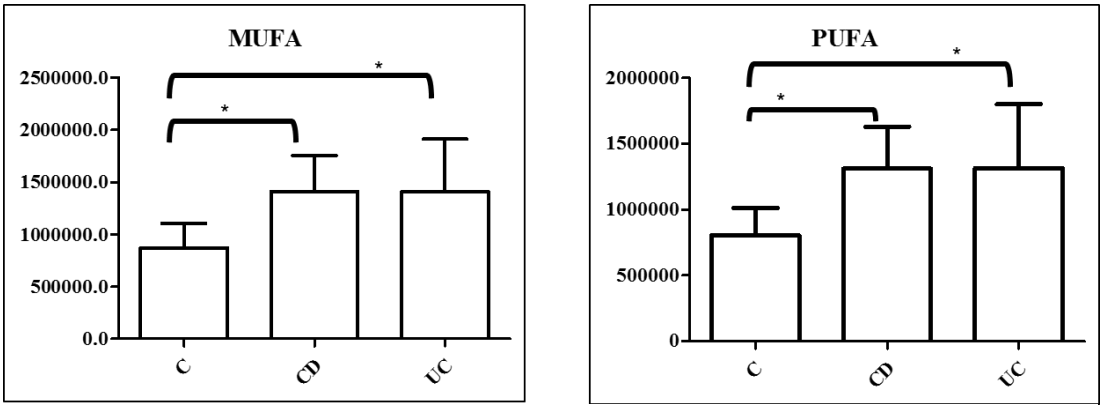

Figure 4.25. Graph related to the levels of MUFA and PUFA of plasma fatty acid fraction samples underwent from the one-way ANOVA test between CD vs UC vs C samples with the Bonferroni correction. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.01, <0.05, <0.001 respectively.

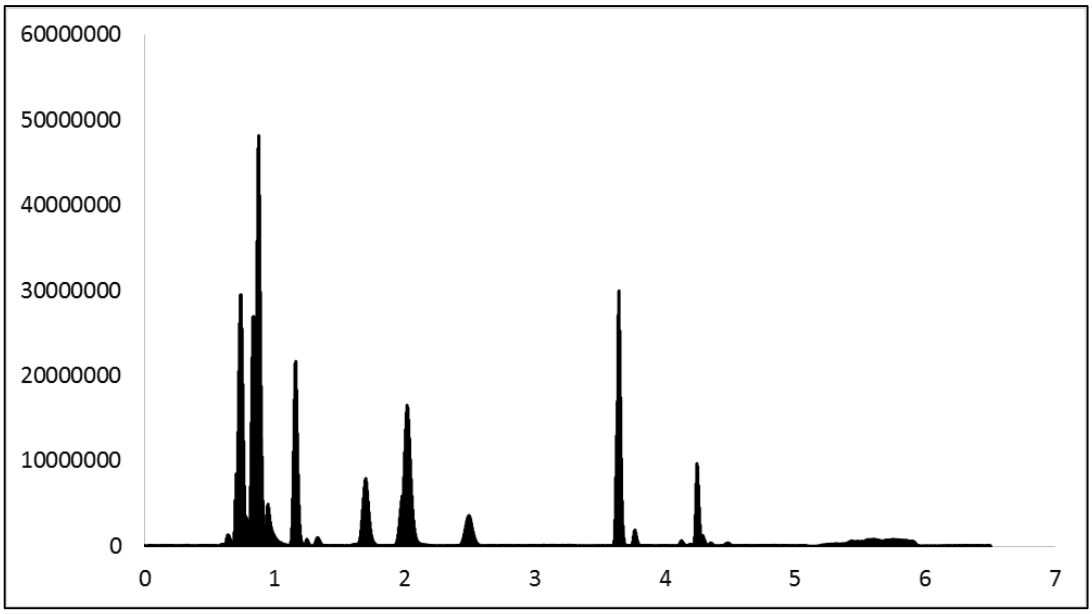

Figure 4.26. Example of a LC/MS QqQ chromatograms from the plasma polar metabolite profile analysis.

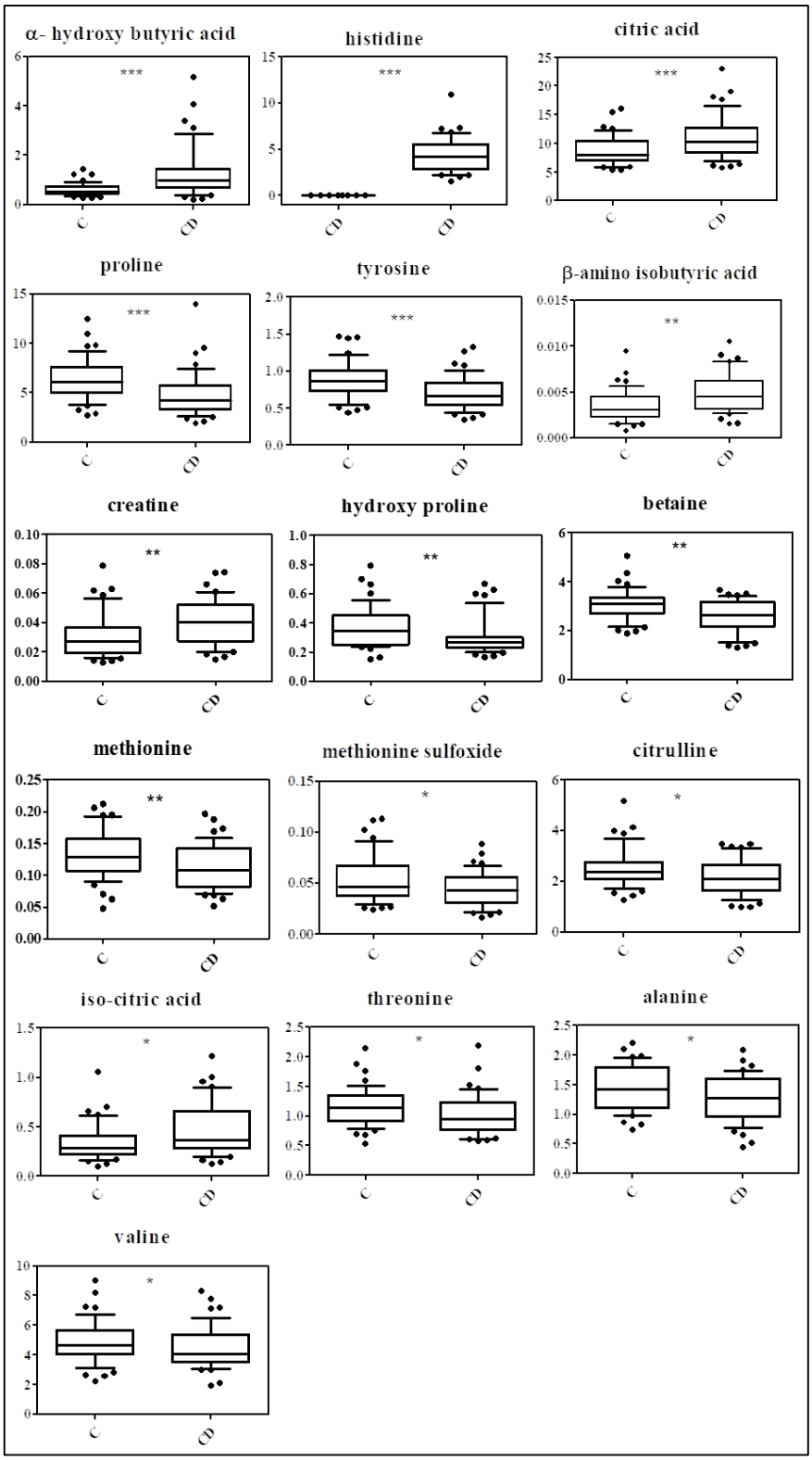

Figure 4.27. Box plot related to the most discriminant metabolites of plasma samples underwent from the Mann-Whitney *U* test between CD vs C samples. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.

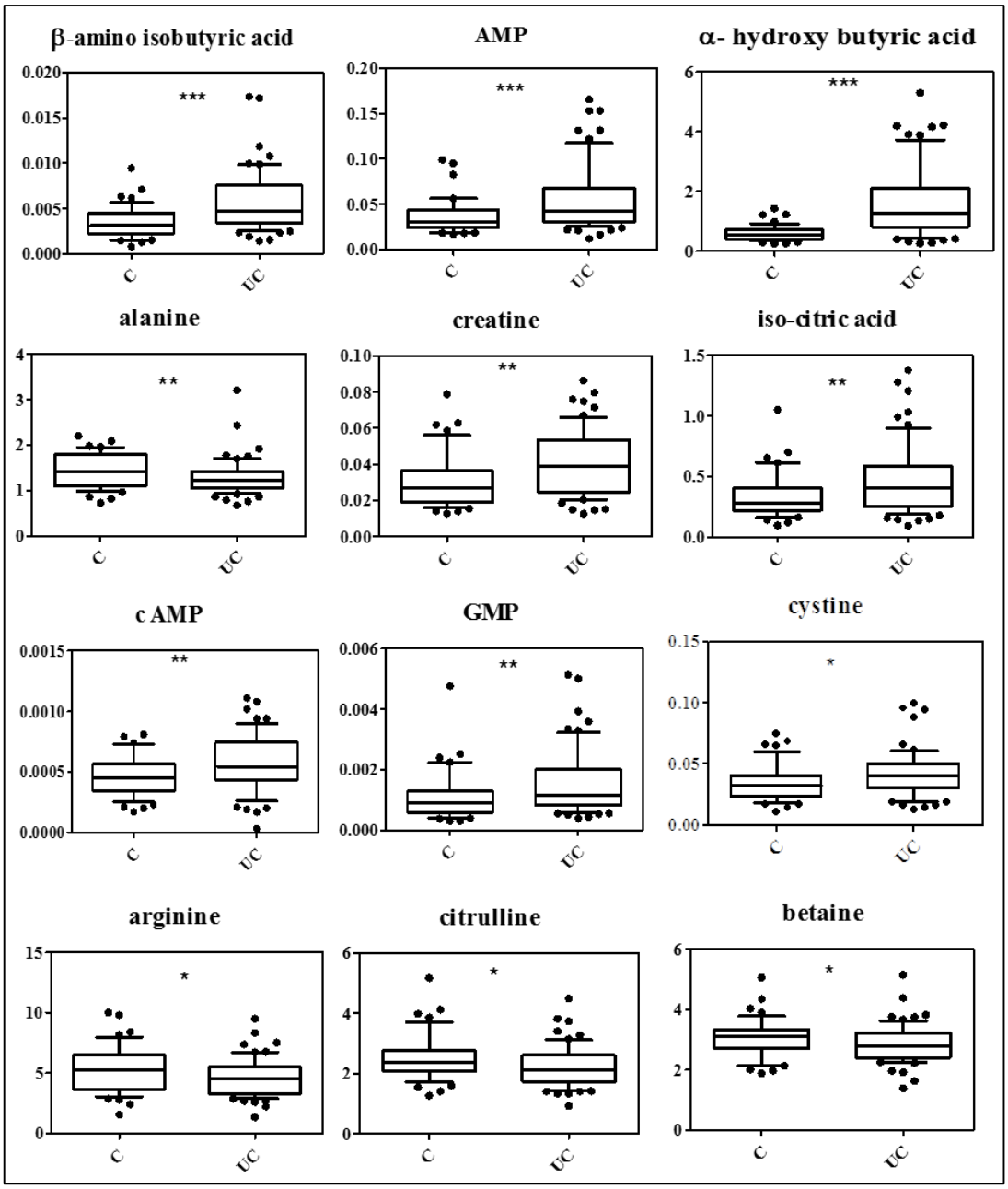

Figure 4.28. Box plot related to the most discriminant metabolites of plasma samples underwent from the Mann-Whitney *U* test between UC vs C samples. Variable intensities are shown in the y-axis. *, **, and *** indicates levels of significance with a p value<0.05, <0.01, <0.001 respectively.