ITALIAN COHORT OF PATIENTS AFFECTED BY INFLAMMATORY BOWEL DISEASE SHOWED VARIATIONS IN GLYCEROPHOSPHOLIPID, FREE FATTY ACIDS AND AMINO ACID LEVELS

Antonio Murgia[1,2], Christine Hinz[2], Sonia Liggi[2], Jùlìa Denes[2], Zoe Hall[2], James West[2], Maria Laura Santoru[3], Cristina Piras[3], Cristina Manis[1], Paolo Usai[4], Luigi Atzori[3], Julian L. Griffin[2], Pierluigi Caboni [1]

[1] Department of Life and Environmental Sciences, University of Cagliari, Italy

[2] Department of Biochemistry and Cambridge Systems Biology Centre, University of Cambridge, UK

[3] Department of Biomedical Sciences, University of Cagliari, Italy

[4] Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, Italy

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ABSTRACT

Background Inflammatory bowel disease is a group of pathologies characterised by chronic inflammation of the intestine and an unclear aetiology. Its main manifestations are Crohn's disease and ulcerative colitis. Currently, biopsies are the most used diagnostic tests for these diseases and metabolomics could represent a less invasive approach to identify biomarkers of disease presence and progression.

Objectives The lipid and the polar metabolite profile of plasma samples of patients affected by inflammatory bowel disease have been compared with healthy individuals with the aim to find their metabolomic differences. Also, a selected sub-set of samples was analysed following solid phase extraction to further characterise differences between pathological samples.

Methods A total of 200 plasma samples were analysed using drift tube ion mobility coupled with time of flight mass spectrometry and liquid chromatography for the lipid metabolite profile analysis, while liquid chromatography coupled with triple quadrupole mass spectrometry was used for the polar metabolite profile analysis.

Results Variations in the lipid profile between inflammatory bowel disease and healthy individuals were highlighted. Phosphatidylcholines, lyso-phosphatidylcholines and fatty acids were significantly changed among pathological samples suggesting changes in phospholipase A_2 and arachidonic acid metabolic pathways. Variations in the levels of cholesteryl esters and glycerophospholipids were also found. Furthermore, a decrease in amino acids levels suggests mucosal damage in inflammatory bowel disease.

Conclusions Given good statistical results and predictive power of the model produced in our study, metabolomics can be considered as a valid tool to investigate inflammatory bowel disease.

1 Introduction

Inflammatory bowel disease (IBD) is a group of pathologies characterised by a chronic phlogosis, and a not specified etiology (Baumgart and Carding 2007). The main clinically defined forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC) (Kaser et al. 2010, Kumar and Clark 2016). The incidence of IBD fluctuates from country to country but the main two typologies affect 1.5 million Americans, 2.2 million Europeans, and several hundred thousands more worldwide (Kumar and Clark 2016, Cosnes et al. 2011). Diagnosis of IBDs is particularly challenging, as other diseases causing similar signs and symptoms need to be excluded first through a combination of tests, but the ultimate diagnostic tool remains endoscopic examination coupled with biopsies. Furthermore, discrimination between the two manifestations, UC and CD, is particularly complicated given the similarity of the symptoms, resulting in 10-15% of cases lacking a defined diagnosis (undefined colitis) (Kumar and Clark 2016). Different causative agents have been proposed in the past for CD and UC diagnosis. One of these theories is based on the T-cell pathway, that proposes the idea that T cells activation is due to a confluence of genetic and environmental factors that causes an immune imbalance, ending with the characteristic inflammation, prerogative of these diseases (Maul et al. 2005). Moreover, change in the intestinal flora, has been suggested as a crucial factor that can trigger the emergence of IBD (Weinstock et al. 2002, Summers et al. 2003). However, it is clear that most of the symptoms that occur during the progression of disease are common for both pathologies.

Metabolomics and lipidomics could both represent useful tools in elucidating the mechanisms and suggesting diagnostic and therapeutic approaches for IBDs. Metabolomics is the study of metabolites in a biofluid, cell, tissue or organism to understand the mechanism behind the response to a given stimulus/condition. By extension lipidomics aims to understand how lipid metabolism is perturbed by a biological stimulus. A number of studies have recently used metabolomics and lipidomics to examine IBD, demonstrating differences in the metabolic profile of IBD patients when compared with healthy individuals (Lu et al. 2012) or, in a smaller number of cases, discriminating between

IBD subtypes (Storr et al. 2013, Lin et al. 2011). Lipid species are also altered in these diseases, with Fan et al. (2015) demonstrating that triaclyglycerol and cholesterol levels were significantly higher and lower in CD when compared with UC, respectively. These alterations of lipid profiles, including suggested changes in plasmalogens may contribute to the pathogenesis of IBD (Balasubramanian et al. 2009). Moreover, Agouridis et al. (2011) highlighted lower total cholesterol and high density lipoprotein cholesterol levels in IBD samples when compared with healthy individuals, while low density lipoprotein cholesterol levels were higher when compared with healthy patients . Santoru et al. in 2017 have reported higher levels of several lipids like diacylglycerol (18:0/22:2), diacylglycerol (16:0/18:2) and n-acylphosphatidylethanolamines (18:1/16:1/18:0) in IBD patient when compared with healthy individuals, while urobilin, phosphatidylcholine (16:0/3:1), urobilinogen, phosphatidic acid (19:0/16:1), phosphatidylserine (22:2/18:0), phosphatidylcholine (22.2/14:1) and ceramide (18:1/22:0) were decreased. Recently, Scoville et al. (2018), studying the metabolomic profile of the serum of IBD patients, showed variations in a number of lipid, amino acid, and tricarboxylic acid cycle related in IBD patients: for example deoxycholate, sphingomyelin, glycochenodeoxycholate glucuronide, taurolithocholate 3-sulfate, dehydroepiandrosterone sulfate, as well as long chain, polyunsaturated, branched chain, and monohydroxy fatty acids were significantly decreased in CD when compared with healthy individuals. Also several compound related to the tricarboxylic acid cycle, like citrate, aconitate, α-ketoglutarate, succinate, fumarate and malate were found decrease in CD and UC when compared with healthy individuals.

A major challenge in the field of lipidomics is the identification of individual lipid species, in part reflecting the similar chemical structure across the class of compounds as well as the high proportion of isobaric species. To address these issues, several analytical methods have been proposed including tailored condensed phase separations coupled to MS or tandem mass spectrometry strategies (Han and Gross 2005, Fahy et al. 2009, Sud et al. 2006, Niemela and Sjöström 1986). In this respect ion mobility-mass spectrometry (IM-MS) has been suggested as one such technique that could improve the separation of a wide range of lipid species (Kliman et al. 2011, Hinz et al. 2017). In addition, drift

tube ion mobility (DTIM) allows calculation of the ion's collision cross section (CCS), a physicochemcal parameter, when travelling through an inert gas such as nitrogen. In this study, with the aim to create a metabolomic fingerprinting profile for the diagnosis of IBD, metabolomic variations of the compound's levels in CD, UC and healthy plasma samples were investigated by either ultra high performance liquid chromatography coupled with a DTIM time of flight mass spectrometer for lipidomics, or a high performance liquid chromatography coupled with a triple quadrupole mass spectrometer system for polar metabolomics.

2 Materials and Methods

2.1 Patients

Patients were recruited at the Department of Gastroenterology of the Policlinico Universitario di Monserrato (CA), Italy. All clinical information of patients are reported in supplementary materials (Table S1). Briefly, the 78 UC patients (31 females, 47 males) showed ranges of age and body mass index (BMI) of 20-76 and 15.7-35.6, respectively, while the same ranges for the 50 CD patients (28 females and 22 males) were 21-78 and 16-35, respectively. Furthermore, patients had their diagnosis confirmed by endoscopic, histological and radiographic data (Agouridis 2011, Nikolaus 2007, Schroeder 1987, Harvey 1980) resulting in 21 CD and 39 UC samples to be in a quiescent or inactive phase of the disease, 8 CD and 25 UC samples to have a moderate activity, and 21 CD and 14 UC samples to show a severe activity.

Also, patients were under a different course of therapies: 14 CD and 6 UC were under the Adalimumab treatment, 9 CD and 12 UC were under the Azathioprine treatment, 4 CD and 7 UC were using Infliximab, 16 CD and 48 UC were under a traditional therapy (corticosteroids, 5-aminosalicylates), while 7 CD and 5 UC were not under a therapy.

The healthy volunteers (n=60, 21 females and 39 males) were recruited locally (Sardinia, Italy). Exclusion criteria were age above 80 or below 20 years, recent use of antibiotics or probiotics and pregnancy. After collection, samples were delivered to the laboratory within 3 hours and stored at -80°C until use.

2.2 Materials

Methanol, chloroform, dicloromethane, isopropanol, acetonitrile and analytical standards were purchased from Sigma Aldrich (Milan, Italy).

2.3 Internal Standards

A mixture of 26 deuterated lipids containing phosphatidic acid (PA), phosphatidylcholines (PC), phosphatidylethanolamines (PE), glycerophospholipids, phosphatidylinositols (PI), phosphatidylserines (PS), sphingomyelins (SM), ceramides (Cer), triacylglycerols (TG) and fatty acids (FA) was used for the plasma lipid profile analysis (Avanti Polar Lipids, Inc., Alabama 35007-9105).

A mixture of 7 standards containing D3-proline, D8-valine, D10-leucine, U-^{13}C lysine, U-^{13}C Glutamic acid, D5-phenylalanine and D4-serotonine (2018 Merck KGaA, Darmstadt, Germany; Cambridge Isotope Laboratories, Inc., Cambridge,United Kingdom), at the concentration of 10 µM, dissolved in a 0.1M solution of HCl, was used for the plasma polar profile analysis.

2.4 Sample preparation for untargeted lipidomics

Plasma samples were centrifuged at 5139g for 10 min at 4°C and 400 µL of supernatant was transferred to eppendorf tubes. A modified Folch method was used to extract the lipophilic

compounds. 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 was mixed with 600 µL of methanol, 600 µL of chloroform and 175 µL of Milli-Q water and centrifuged at 5139g for 30 min. at 4°C. The high volume of plasma was chosen with the aim to use a single extraction protocol to analyse also the aqueous metabolic profile designated for the gas chromatography-MS and ^{1}H NMR analysis (experiments from an unpublished study). The lipid chloroform phase was separated and dried under a gentle stream of nitrogen. Samples were suspended in 1 mL of methanol, vortexed for 3 min and sonicated for 5 min. After sonication, 50 µL of the samples was transferred into vials and diluted with 50 µL of internal standard and 75 µL of Chromasol ultrapure water.

2.4.1 Solid Phase Extraction of the lipid fraction

Ten folch extracts from each class (CD, UC and C) were submitted to chromatographic separation by solid phase extraction (SPE) to obtain three different fractions each containing different lipid classes: neutral lipids, fatty acids and phospholipids. Each class was formed of 5 males and 5 females without other pathologies, no smokers and with a normal BMI (18.5-24.9 kg/m^2). The dried organic phases from the Folch extracts were reconstituted in 800 µL of chloroform and subjected to ultrasound for 5 min to dissolve and mix the lipids. After sonication, 20 µL of samples was transferred into a vial and diluted with 100 µL of internal standard. The solution was sequentially dried under a gentle stream of nitrogen and then suspended in 800 µL of chloroform. Chromabond aminopropyl-modified silica NH_2 (1mL/100mg) columns from Macherey-nagel were conditioned with 3 mL of hexane. After conditioning, 800 µL of samples were added and eluted using: 1 mL of chloroform: isopropanol 2:1 (v/v) solution for the neutral lipids extraction; 1 mL of diethyl ether: acetic acid 98:2 (%) solution for the fatty acids and 1 mL of methanol for the phospholipids. All the samples were then dried and suspended with 50 uL of a mixture of isopropanol: acetonitrile: water (2:1:1 v/v).

147 **2.5 Sample preparation for the polar phase analysis of plasma samples**

148 Twenty µL of plasma was transferred into an Eppendorf tube and 100 µL of an internal standard mix
149 solution was added. Samples were sonicated for 5 min and centrifuged for 5 min at 5139g. The
150 obtained surfactant was dried under a gentle stream of nitrogen. Samples were then suspended in 100
151 µL of a 10 mM ammonium acetate solution, vortexed, sonicated for 5 min and centrifuged 5 min at
152 5139g. Samples were then transferred to a 96 multi-well plate for LC/MS analysis.

153

154 **2.6 LC/DTIM-QTOF analysis**

155 An Agilent Infinity II UPLC coupled to a 6560 Ion mobility QTOF was used with an Agilent
156 Jetstream source, operated in the positive and negative ion mode while, the capillary potential was
157 set to 60 V and the needle at 20 kV. High purity Nitrogen (99.999%) was used as a drift gas with a
158 trap fill time and a trap release time of 2000 and 500 µs, respectivelly. Before the analysis, the
159 instrument was calibrate using an Agilent tuning solution at the mass range of m/z 100-1700. Samples
160 were evaporated with Nitrogen at the pressure of 48 mTorr and at the temperature of 375 °C, while
161 an Agilent reference mass mix for mass re-calibration was continuously injected during the run
162 schedule. An Agilent tuning mix solution was injected every ten samples to perform $^{DT}CCS_{N2}$ re-
163 calibration. Full-scan spectra were obtained in the ranges of 100-1700 amu for the lipid plasma
164 extracts, setting the detector at 1500 V. The organic fractions were analysed using a Waters Acquity
165 CSH C18 column (100 x2.1 mm 1.7 µm). The mobile phase for positive ionization mode consisted
166 of (A) 10 mM ammonium formate solution in 60% of milliQ water and 40% of acetonitrile and (B)
167 10 mM ammonium formate solution containing 90% of isopropanol, 10% of acetonitrile. The mobile
168 phase was pumped at a flow rate of 400 µL/min as follows: initially 60% of A, then a linear decrease
169 from 60% to 50% of A in 2 min then at 1% in 5 minutes staying at this percentage for 1.9 minutes
170 and then brought back to the initial conditions in 1 min. The Agilent MassHunter LC/MS Acquisition
171 console from The MassHunter suite was used for data acquisition. The mobile phase for negative

172 ionization mode differed only for the use of 10 mM ammonium acetate instead of ammonium
173 formate. Identical DTIM parameters were used for the SPE extracts analysis, while the mobile phase
174 was pumped at a flow rate of 400 µL/min using the following gradient: initially 60% of A, then a
175 linear decrease to 50% of A in 2.10 min and decreased to 30% of A in 10 min and to 1% of A in 6
176 min maintaining these condition for 0.1 min, back to the initial conditions in 2 min. Identifications of
177 the most important compounds were confirmed by the use of a targeted MS/MS using different
178 collision energy: 20, 30 and 40 V. An overlay of the extracted ion chromatograms of all the identified
179 species is shown in Fig 1, while their retention times, $^{DT}CCS_{N2}$ and fragmentations are reported in
180 Table 1.

181

182 **2.7 LC/MS/MS QqQ analysis of plasma samples.**

183 Samples were analysed by a Thermo scientific UHPLC+ series coupled with a TSQ Quantiva mass
184 spectrometer (Thermos fisher scientific, Waltham, Massachusetts, United States). The electrospray
185 voltage was set to 3500 V for positive ionisation mode and to 2500 V for the negative ionisation
186 mode. The organic phases were analysed with an ACE Excel 2 C18 PFP (100A. 150 x 2.1 mm 5µ)
187 column. The mobile phase contained 0.1% of formic acid water solution as solvent A and 0.1% of A
188 formic acid acetonitrile solution as solvent B. The mobile phase was pumped at a flow rate of 500
189 µL/min initially at 100% of A for 1.60 min, then a linear decrease from 100% to 70% of A in 2.4 min
190 and to 10% in 0.5 min, then constant for 0.5 min and brought back to initial condition after 0.1 min.
191 The Xcalibur software (Thermos fisher scientific, Waltham, Massachusetts, United States) was used
192 for data acquisition. Putative recognition of all detected metabolites was performed using a targeted
193 MS/MS analysis. Calculated masses and mass fragments of the measured compounds are reported in
194 supplementary materials (Table S2).

195

196 **2.8 Data processing**

197 Data acquired with the Agilent 6560 DTIM Q-TOF LC-MS was pre-processed with a set of software
198 from the MassHunter Workstation suite (Agilent Technologies, Santa Clara, USA) to perform mass
199 re-calibration, $^{DT}CCS_{N2}$ re-calibration, and deconvolution, yielding a matrix containing all features
200 present across all samples. This matrix was further processed with KniMet (Liggi et al. 2017, Liggi.
201 S., 2018), a pipeline based on the KNIME analytic platform (Berthold et al. 2007) for the post-
202 processing of metabolomics MS-based data. Features were filtered based on their presence in blanks
203 and QC samples (threshold = 40%) (Dunn et al. 2011, Dunn et al., 2012). The remaining features
204 were subjected to missing values imputation with Random Forest and Probabilistic Quotient
205 Normalisation (Dieterle et al. 2006) based on QCs. Finally, data was annotated based on accurate
206 mass match with the LIPID MAPS database (Fahy et al. 2007, Schmelzer et al. 2007).

207

208 **2.9 Multivariate statistical data analysis**.

209 All the multivariate statistical analysis (MVA) were performed using the SIMCA software 14.0
210 (Umetrics, Umeå, Sweden). Principal component analysis (PCA) was used along Hotelling's T^2 and
211 DmodX tests to evaluate distribution of the samples and evaluate the presence of any possible outliers.
212 This was followed by partial least square-discriminant analysis (PLS-DA) with its orthogonal
213 extension (OPLS-DA), which was used as classificatory model to visualize and evaluate the
214 differences between samples. Statistical power of the models, as well as the possible presence of
215 overfitting, were evaluated by considering the R^2X, R^2Y and Q^2 parameters along with permutation
216 test and cross validation analysis. From the PLS-DA, the variable importance in the project (VIP) and
217 the coefficient analysis were used to provide, respectively, the contribution of the components to the
218 separation of sample groups and the expression of how strongly the variable class is correlated to the
219 systematic part of each of the metabolites. Further, the discriminant compounds highlighted by the

220 VIP and loadings analyses were compared with those found in the S-plot from the OPLS-DA that
221 combines the modelled covariance and correlation in a scatter plot (Erikson 2013).

222

223 **2.10 Univariate statistical analysis.**

224 GraphPad Prism software (version 7.01, GraphPad Software, Inc., CA, USA) was used to perform
225 univariate statistical analysis of the data. To verify the significance of metabolites obtained using
226 multivariate statistical analysis, a Mann-Whitney U test was performed (Ruxton 2006). For the
227 analysis of the samples subjected to SPE extraction, all the univariate statistical results were obtained
228 using a one way non parametric test with the Bonferroni correction (Steinbach et al. 1994). Variables
229 with a p value lower than 0.05 were considered statistically significant.

230

231 **3 Results and discussion**

232

233 **3.1 Untargeted lipid analysis of IBD samples revealed changes in the levels of phospholipids**

234 The lipid profile of IBD and healthy samples was studied by LC/DTIM-QTOF. Data processing
235 yielded 1038 (498 annotated as lipids) and 607 (104 annotated as lipids) features for positive (PIA)
236 and negative ionization analysis (NIA), respectively, which were subjected to MVA. The extract
237 composition consists of lipids from the following classes: 7 free FA, 23 lysophosphatidylcholines
238 (LPC), 106 PC, 45 PS, 10 PE, 92 diacylglycerols (DAG), 26 cholesteryl esters (CE), 49 SM, 10 Cer
239 and 234 TG. The unsupervised analysis of both (PIA) (PCA R^2X=0.69, Q^2=0.44) and (NIA) (PCA
240 R^2X=0.5 and Q^2=0.4) ion mode features did not indicate any clusters related to gender, age, BMI,
241 therapies, state of disease and localization of inflammation associated with the samples (data not
242 shown). DmodX and Hotelling's T^2 analysis showed 7 outliers for PIA, which were removed from

243 the subsequent analysis, while no outliers were identified for NIA. Three different OPLS-DA were
244 performed: CD *vs* C, UC *vs* C and CD *vs* C. Models displayed a clear separation between healthy
245 subjects and both pathological classes in the two-way comparisons (Figure 2, OPLS-DA model for
246 PIA data of CD and healthy patients, $R^2Y=0.67$, $Q^2=0.51$ (A); OPLS-DA model for PIA data of UC
247 and healthy patients $R^2Y=0.71$, $Q^2=0.33$ (B); both models passed permutation test with Q^2 of -0.48
248 and -0.14, respectively). For the NIA, OPLS-DA also discriminated between the healthy controls and
249 both disease groups (Figure 2, OPLS-DA model for NIA of CD and healthy patients, $R^2Y=0.7$,
250 $Q^2=0.7$ (C), OPLS-DA model of NIA of UC and healthy patients, $R^2Y=0.7$, $Q^2=0.5$ (D); both models
251 passed permutation test with $Q^2 = -0.20$ and -0.31, respectively). In contrast, the comparison between
252 the two pathological classes did not show separation between groups for both ionization modes (data
253 not shown). The most discriminant compounds highlighted by the VIP list, the loadings and the S-
254 plot where subjected to MS/MS analysis and cross-referenced to the LIPID MAPS and METLIN (The
255 Scripps Research Institute, La Jolla, CA) libraries to further confirm their identities (Table 1, Fig 3.)
256 Additional confidence in these identifications was obtained by comparing the $^{DT}CCS_{N2}$ values of
257 these compounds with those available in recent literature (Paglia et al., 2015, Stow et al., 2017, Zohu
258 et al., 2017, Kyle et al., 2018), resulting in an average relative standard deviation of 0.6% (Table S3).
259 The statistical significance of the discriminant compounds from the MVA analyses were confirmed
260 by the Mann-Whitney test. Regarding the results obtained for CD samples in PIA, LysoPC 18:0,
261 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
262 decreased in CD samples, while PC16:0/22:6, PC18:0/22:6, PC 18:3/20:3, SM 16:1/20:1, TG
263 18:1/18:2/20:4 and TG 16:0/18:1/18:2 were increased in CD samples. Similar results were obtained
264 for UC samples in PIA where LysoPC 18:1, LysoPC 18:2, PC 18:0/18:1, PC 18:1/18:1, PC 18:2/18:2,
265 PC 18:3/20:4, TG 14:0/16:0/18:2 and TG 16:0/18:0/18:1 were decreased for UC samples, while PC
266 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. On the
267 other hand, analysis of the NIA data showed that PC 18:1/18:2, PC 16:0/22:6 and PC 18:0/18:2 were
268 decreased in CD samples, while free fatty acid including oleic acid, linoleic acid, palmitic acid and

269 palmitoleic acid were increased in CD and UC samples. Only PC 18:1/18:2 was decreased in UC
270 samples (Fig. 4, Table S4).

271 Among the most discriminant compounds, LysoPC (18:0, 18:1 and 18:2) were found to be decreased
272 in pathological samples. The major pathway for the production of LysoPC, such as 18:0, 18:1 and
273 18:2, all of which decreased in the disease state, is the hydrolysis of fatty acids from PCs by
274 phospholipases such as phospholipase A_2 (PLA_2). PLA_2 has been reported to be relevant during
275 different inflammatory processes, including IBD pathogenesis (Schaloske et al. 2006), and the
276 expression of its activator antigen was found to be increased in the monocytes and granulocytes of
277 IBD intestinal sections (Peterson et al. 1996). In addition, one of our previous studies showed a
278 modification of the PLA_2 metabolic pathways in IBD faecal samples (Santoru et al. 2017). Therefore,
279 the increase in LysoPCin plasma derived from IBD patients observed in this study may suggest a
280 higher PLA_2 activity. In addition, levels of saturated and monounsaturated PCs containing 18:0, 18:1
281 and 18:2 fatty acids were decreased in plasma samples of UC and CD patients, where increased levels
282 of PCs containing polyunsaturated fatty acids such as arachidonic and docosahexaenoic acid were
283 also found. As these fatty acids represent substrates for the generation of eicosanoids and other
284 oxylipins, both of which are important during inflammatory responses, this result may indicate a
285 higher demand for these lipids in IBD patients. In support, Ehehalt et al., (2004) also highlighted an
286 increase of PC and LysoPC (PC 16:0/18:1, PC 16:0/18:2, PC 18:0/18:1, PC 18:0/18:2, LysoPC 16:0
287 and LysoPC 18:0) in the rectal mucus of UC patients compared to CD and healthy individuals. On
288 the other hand, some PC levels were found decreased in the pathological samples when compared
289 with controls, and this trend can be explained by a change of the lipid composition in the intestinal
290 mucosa as already suggested by Bischoff et al. (2014).

291

292 **3.2 Class-specific analysis of lipids showed changes in the lipid profile**

293 Analysis of the lipidome did not highlight any difference between CD and UC samples, which could
294 be related to the high intra-class variability as well as the similarity between these two pathological
295 conditions which could reflect also in their lipid profile. To increase the class specificity of our
296 untargeted approach and provide a more in-depth analysis of potential differences between the UC
297 and CD, we performed SPE on a sub-set of the samples matched for age, gender, BMI and therapy.
298 This separated lipids into three fractions, fatty acids, neutral lipids and phospholipids prior to LC-MS
299 analysis.

300 The LIPID MAPS database was used to annotate compounds based on accurate mass match, resulting
301 in 98 TG, 44 glycerophosphocolines, 43 free FA, 28 SM, 42 DAG, 32 Cer, 11 LysoPC and 9 CE.
302 One-way ANOVA test was performed for the 3 classes of samples CD, UC and C by expressing each
303 lipid as the ration of the total signal for that particular class of lipids and applying a Bonferroni
304 correction. Statistical analysis of the results from SPE of the phospholipid fraction showed an increase
305 in PCs in UC samples when compared with CD samples, while unsaturated LPCs were decreased in
306 CD samples. Furthermore, saturated DAGs were increased in samples from CD patients compared to
307 UC. MVA of this fraction did not show any outliers or clusters (PCA $R^2X=0.50$, $Q^2=0.3$), and OPLS-
308 DA comparing IBD samples *vs* healthy individuals reported the same results of the untargeted
309 analysis described above (data not shown). However, unlike the analysis of the untargeted lipidomics
310 data, the model comparing CD *vs* UC for the phospholipid fraction reported good validation
311 parameters ($R^2Y=0.7$, $Q^2=0.3$, and passed permutation test). Twelve lipids were identified as
312 significantly increased by both multivariate statistics and the univariate Mann-Whitney test for UC
313 samples when compared to healthy individuals, while only one was increased in CD samples. For the
314 UC group the identified compounds were: PC18:2/18:0, PC18:1/18:0, PC22:5/16:0, PC20:3/18:0,
315 PC16:0/18:2, PC16:0/18:1, PC 18:1/18:1, PC16:0/20:3 and PC16:1/16:0 (Table 2). From the analysis
316 of the neutral lipid fraction, the only category that was found significantly altered was CE, which was
317 increased in UC compared with CD. At last, the analysis of the fatty acid fraction showed a significant
318 increase of unsaturated FA in CD compared to controls (Fig. 5). MVA was performed also for the

319 neutral lipid and FA fractions, however, no significant differences were found comparing CD vs UC
320 (data not shown). The variations found between CD and UC in the levels of saturated diacylglycerols
321 and CE confirm our original hypothesis, suggesting that intra-class variability and similarity between
322 the two diseases could lead to loss of information in the analysis of a more complex lipid extract in
323 the larger human cohort. Among these findings, variations in CE levels can be correlated with a
324 change in the lipoprotein system (Bruce,et al., 1998). In fact, lipoprotein levels are known to be
325 affected by a range of physiological and pathological conditions (Ansell et al., 2003), and these
326 findings were confirmed by a study of Biyyani et al. (2010) where, in a lipoprotein profile comparison
327 between IBD patients and controls, lower total cholesterol and high-density lipoprotein cholesterol
328 levels were detected in IBD patients. Furthermore hypocholesterolaemia is a common symptom in
329 patients with various types of acute disease, including surgery, trauma, burn injury and sepsis, with
330 it suggested that chemokine action might drive this variation (Fraunberger et al. 2000). Chemokines
331 are chemotactic cytokines that stimulate leukocytes migration to areas of inflammation and lead cell
332 activation events (Banks et al. 2003). It has been reported that the local inflammation and tissue
333 damage in UC and CD is caused by a local expression of specific chemokines in IBD tissues (Banks
334 et al. 2003). Further experiments are recommended to understand why the levels of CEs were
335 increased in UC when compared with CD.

336

337 **3.3 Alterations in the amino acid metabolism.**

338 Fifty-five polar compounds from 166 plasma samples were identified and measured from the
339 LC/MS/MS analysis. Data was processed by first dividing the detected area of the most abundant ion
340 by that of the related ion from the internal standard. Group means for these ratios were compared
341 using the Mann-Whitney U test. Overall, 16 and 21 compounds were found to be significantly
342 changed when samples from the CD *vs* healthy groups and UC *vs* healthy groups were compared,
343 respectively. β-amino isobutyric acid, α-hydroxy butyric acid, histidine, creatine, isocitric acid and

344 citric acid levels were increased in CD samples, while alanine, betaine, citrulline, threonine,
345 hydroxyproline, proline, valine, methionine, tyrosine, methionine sulfoxide where decreased in CD
346 when compared with healthy samples(Fig. 1S). For the UC group cysteine, β-amino isobutyric acid,
347 AMP, α-hydroxybutyric, cAMP, creatine, isocitric acid, GMP, citric acid, uridine, GABA, β-hydroxy
348 isovaleric acid and ketoleucine were increased, while arginine, alanine, betaine, citrulline, threonine,
349 proline, methionine, tyrosine and tryptophan were decreased when compared with healthy samples
350 (Fig. 2S and Fig. 4). The levels of cAMP, found to be increased in UC are consistent with the results
351 of Moore et al. (1995), who described how the production of cAMP in leucocytes is due to 3-
352 adrenergic catecholamines, histamine and the E series prostaglandins by a receptor coupled activation
353 of adenylate cyclase. In vitro studies have also reported that the agents responsible for the increase of
354 cAMP levels were implicated in the reduction of several inflammatory metabolic pathways, including
355 the release of histamine as well as leukotrienes and the proliferation of lymphocytes (Harvart 1991,
356 Rivkin et al. 1977). Moreover, a profound decrease of amino acid levels was found in IBD patients,
357 confirming the results already reported by Hisamatsu et al. (2012) that suggested a multivariate
358 analysis comprising plasma amino acid profiles as a novel, non-invasive approach for the diagnosis
359 and monitoring of IBD. Similarly, Scoville et al (2018) reported alteration on the levels of several
360 amino acids in the serum of CD subjects compared to both control and UC samples, while no variation
361 occurred when comparing UC with control samples. These amino acid changes in IBD samples could
362 be linked to the mucosa damage, characteristics of these disease, and is corroborated by the decreased
363 plasma levels of citrulline, a marker of extensive mucosal barrier injury of the small intestine
364 (Blijlevens et al. 2004). Moreover, these amino acid variations could reflect the use of proteins as a
365 catabolic energy source in the inflammatory state (Hong et al., 2009). In fact, T and B cells utilize
366 amino acids, glucose and lipids as energy sources during oxidative phosphorylation and mitogenic
367 stimulation (Kominsky, et al., 2010.). In addition, it has been noticed how a specific protein intake in
368 IBD patients can cause changes in the intestinal health by modulating the quantity and the nature of

369 both amino acids absorbed and undigested proteins delivered to the large intestine (Vidal-Lletjós et
370 al. 2017).

371

372 **4 Conclusions.**

373 In this study, both metabolomics and lipidomics provided good classification not only between
374 healthy and diseased samples but also in separating samples from UC and CD patients. The
375 remodelling of PCs detected in both disease groups may implicate an inflammatory process mediated
376 through PLA_2 activity although further work would be required, for example examining the
377 production of pro-inflammatory eicosanoids. Reductions in many amino acids were also detected
378 which may relate to mucosal damage in IBD. Further investigations are strongly recommended to
379 understand the two most important related metabolic pathways: PC remodelling and the inflammatory
380 response and amino acid metabolism and mucosa damage. Add a sentence about biomarkers

381

382 **Author contribution**

383 P.C., L.A., J.L.G., A.M. and P.U. conceived the study, directed the project and designed the
384 experiments.

385 A.M., M.L.S., C.P. and S.L., performed the lipid metabolite profile extraction of the plasma samples.

386 A.M. and C.M. performed the polar metabolite profile extraction of plasma samples

387 A.M., C.H., J.W., J.D. and S.L. performed metabolomics and lipidomics experiments and data
388 analysis.

389 A.M., C.H. and Z.H., contributed on the lipid targeted analysis

390 A.M. wrote the first draft of the manuscript, P.C., L.A., S.L., C.H., and J.L.G. contributed to the final
391 version.

392 A.M., S.L., C. H., J.L.G., P.C. and L.A., critically reviewed the data and the manuscript.

393 All authors read and approved the final version of the manuscript.

394

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400
401 **Compliance with ethical standards**

402 **Conflict of interest** The authors declare no conflict of interest.

403 **Ethical approval** All procedures performed in studies involving human participants were in
404 accordance with the ethical standards of the institutional and/or national research committee and with
405 the 1964 Helsinki declaration and its later amendments or comparable ethical standards
406 (PG/2014/11480).

407 **Informed consent** Informed consent was obtained from all individual participants included in the
408 study.

409

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588

589 **Fig 1 A)** Extracted ion chromatogram of the most discriminant compounds from the positive
590 ionization analysis **B)** Extracted ion chromatogram of the most discriminant compounds from the
591 negative ionization analysis.

592 **Fig 2** Scores plot from the LC/DTIM-QTOF analysis. **A)** Scores plot from the PIA OPLS-DA of CD
593 samples (red filled circles) vs control samples (grey filled circles): R^2Y=0.7, Q^2=0.5; **B)** Scores plot
594 from the PIA OPLS-DA of UC samples (black filled circles) vs healthy patient samples (grey filled
595 circles). R^2Y=0.7 Q^2=0.3. **C)** Scores plot from the NIA OPLS-DA of CD samples (red filled circles)
596 vs control samples (grey filled circles): R2Y=0.70 Q2=0.5; **D)** Scores plot from the NIA OPLS-DA
597 of UC samples (black filled circles) vs control samples (grey filled circles): R^2Y=0.7 Q^2=0.5.

598 **Fig 3** Example of MS/MS fragmentation patterns for different lipid categories: **A)** LysoPC 18:2, **B)**
599 PC 36:4, **C)** SM 42:2, **D)**. TG 52:1. Rn= carboxylic chain

600 **Fig 4** Venn diagram related to the most discriminant compounds of both untargeted and targeted
601 analysis for the main two pathological classes: Crohn's disease and ulcerative colitis.

602 **Fig 5** Graphs indicating the levels of lipid categories from the SPE plasma samples examined by one-
603 way ANOVA test between CD vs UC vs C samples with a Bonferroni correction. Variable intensities
604 are shown in the y-axis. *, **, and *** indicates levels of significance with a p value <0.05, <0.01,
605 <0.001, respectively.

Lipid	Adduct	m/z experimental	m/z theoretical	Δppm	Fatty acid composition	t_R (min)	$^{DT}CCS_{N2}$ (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	16:0, 22:5	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	18:0, 18:2	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	18:0, 18:1	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	16:0, 18:1	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	18:0, 20:3	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	18:1, 18:2, 20:4	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	16:0, 18:1, 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	16:0,18:0, 18:1	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	165.3	-
FA 18:2	$-H^-$	279.232	279.2330	5	-	1.6	171.9	-
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	864.5760	0	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	173.3	-
PC 36:3	$+OAc^-$	842.592	842.5917	0	18:1, 18:2	4.9	294.1	768.5, 281.3, 279.2

Table 1 Discriminant lipids of IBD plasma samples identified by DTIM-C-MS/MS. * = Retention time related to the SPE LC/MS method.

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

Table 2 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.