The American Journal of Clinical Nutrition
Official publication of the American Society for Nutrition

The American Journal of Clinical Nutrition
AJCN/2016/130872
Version 3

Impairment of lysophospholipid metabolism in obesity: altered plasma profile and desensitization to the modulatory properties of n-3 PUFAs in a randomized controlled trial

Corresponding Author: Antoni Caimari
Additional Authors: Josep Maria del Bas, Maria Isabel Rodriguez-Naranjo, Caroline E Childs, Carolina Paras Chavez, Annette L West, Elizabeth A Miles, LLuís Arola, Philip C Calder

Trial registered: ISRCTN.org Reg No. ISRCTN96712688

Impairment of lysophospholipid metabolism in obesity: altered plasma profile and desensitization to the modulatory properties of n-3 PUFAs in a randomized controlled trial. [1-3]

Josep M. del Bas, Antoni Caimari, Maria Isabel Rodriguez-Naranjo, Caroline E. Childs, Carolina Paras Chavez, Annette L. West, Elizabeth A. Miles, Lluis Arola, and Philip C. Calder

[1]From Nutrition and Health Research Group, Technological Center for Nutrition and Health, Tecnio, Campus de Excelencia Internacional Cataluña Sur (CEICS), Reus, Spain (JMdB, AC, MIRN, LA); Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton, United Kingdom (CEC, CPC, ALW, EAM, PCC); National Institute for Health Research Southampton Biomedical Research Centre, University Hospital Southampton National Health Service Foundation Trust and University of Southampton, Southampton, United Kingdom (PCC); and Nutrigenomics Research Group, Department of Biochemistry and Biotechology, Universitat Rovira i Virgili, Tarragona, Spain (LA).

[2]Supported by the European Commission through its Seventh Framework Programme "BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food" project (BIOCLAIMS, Grant agreement no. 244995). CPC was supported by a scholarship from CONACYT.

[3]Address correspondence to Dr Antoni Caimari, Nutrition and Health Research Group, Technological Center for Nutrition and Health, Avinguda Universitat 1, Reus 43204, Spain. Email: antoni.caimari@ctns.cat. Telephone 0034977752965.

Authors last names: del Bas, Caimari, Rodriguez-Naranjo, Childs, Paras Chavez, West, Miles, Arola, Calder.

Short running head: Lysophospholipid metabolism in obesity.

[4]**Abbreviations used**: ANCOVA, analysis of covariance; BMI, body mass index; CVD , cardiovascular disease; DHA, docosahexaenoic acid ; ETA, eicosatetraenoic acid; HFM, high fat meal; HOMA, homeostasis model assessment; IR, insulin resistance; LCAT, lecitin cholesterol acyl transferase; LP, lysophospholipid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; MetS, metabolic syndrome; MTT, thiazolyl blue tetrazolium bromide ; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ORO, Oil red O; PC, principal component; PCA, principal component analysis; PLS-DA, partial least square discriminant analysis; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acid; ROC, receiver operating characteristics.

Clinical Trial registration number: ISRCTN96712688 (www.controlled-trials.com)

ABSTRACT

Background: Plasma lysophospholipids (LPs) have emerged as signalling molecules with important effects in inflammation, insulin resistance and fatty liver disease, each of which is closely linked to obesity. Dietary n-3 PUFAs may be able to improve these conditions.

Objective: The objective was to assess the response of the plasma LPs to obesity, n-3 PUFA consumption and a high fat meal challenge in order to better understand the role of LP metabolism in the progression of obesity related disorders.

Study design: We determined the concentrations of 8 lysophosphatidylcholines (LPCs), 11 lysophosphatidylethanolamines and 7 lysophosphatidylinositols in plasma of 34 normoweight and 38 obese subjects randomized to corn oil (as control) or n-3 PUFA-rich fish oil (3 g/day, n=15-19 per group) for 90 days. Blood samples were collected on the last day of the study under fasting conditions and 6 h after a high fat meal (1135 kcal, 86 g fat) challenge. The profile of secreted LPs was studied in HepG2 cells under palmitate induced steatosis.

Results: Obese and normoweight subjects had different profiles of plasma LPs. Multivariate combination of the 26 LPs could discriminate between normoweight and obese subjects with an accuracy of 98%. The high fat meal challenge altered the concentration of plasma LPCs in an oil treatment-dependent manner in normoweight, but not in obese, subjects, suggesting that obesity impairs the sensitivity of LP metabolism to n-3 PUFAs. Non-cytotoxic steatosis in HepG2 cells affected the secretion pattern of LPs partially resembling the changes observed in plasma of obese subjects.

Conclusions: Obesity has a significant impact on LP metabolism, altering plasma LP profile and abolishing its sensitivity to dietary n-3 PUFAs. These effects could contribute to the onset or progression of alterations associated with obesity such as inflammation, insulin resistance and fatty liver disease. This trial was registered at www.controlled-trials.com as ISRCTN96712688.

Keywords: lysophospholipid metabolism, lysophosphatidylcholine, lysophosphatidyletanolamine, obesity, insulin resistance, inflammation, fatty liver disease, polyunsaturated fatty acids, omega-3, fish oil

INTRODUCTION

Obesity is linked to chronic low-grade inflammation, insulin resistance (IR), non-alcoholic fatty liver disease (NAFLD), metabolic syndrome (MetS) and cardiovascular disease (CVD) among other conditions (1,2). Recent advances in the field of metabolomics have allowed untargeted exploration of the metabolic changes induced by obesity or obesity-related complications. Using this approach different lysophospholipid (LP) species, mainly lysophosphatidylcholines (LPCs), have been identified as being differentially changed in the plasma of subjects with obesity (3–6), non-alcoholic steatohepatitis (NASH) (7–9) or NAFLD (10), and to accompany the amelioration of different features associated to obesity and MetS (11,12). These findings suggest that obesity and obesity-related conditions are linked to altered plasma LPs. LPs act as signalling molecules, modulating processes such as inflammation, insulin production and insulin sensitivity through their interaction with G-protein coupled receptors (13). Thus, LPs may be important molecules in obesity and its related disorders. Nevertheless, the effects of obesity on LP metabolism and the changes that the obese phenotype can induce in the plasma LP profile remain unexplored.

There is increasing evidence that increased intake of n-3 polyunsaturated fatty acids (n-3 PUFAs) can partly ameliorate some obesity-associated conditions. Thus, different meta-analyses show that n-3 PUFAs have beneficial effects on obesity and insulin resistance (14–17) and exert anti-inflammatory actions at the local and systemic level (14,18,19). These effects are proposed to take place through different mechanisms such as modulation of the activity of the peroxisome proliferator activated receptor (PPAR) family of nuclear receptors or of the production of eicosanoids and other lipid mediators (16). Little is known regarding the actions of

n-3 PUFAs on the metabolism of LPs, although it has been shown that LPC containing n-3 polyunsaturated acyl chains (i.e. docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) exert anti-inflammatory actions *in vitro* and in mice by affecting prostaglandin formation (20,21). Therefore, incorporation into glycerophospholipids represents a mechanism by which n-3 PUFAs can interplay with LP metabolism.

Because fatty acid handling and hepatic lipid metabolism are dysregulated in obesity, we hypothesized that LP metabolism, which is highly interconnected with these processes, is also altered, resulting in measurable changes in the concentrations of plasma LP species that have been related with the progression of alterations linked to obesity. Therefore, our objective was to assess the effects of obesity on LP metabolism and how different factors that either ameliorate this condition, such as n-3 PUFA consumption, or induce metabolic stress, such as a high fat meal (HFM) challenge, can modulate the plasma LP profile. Due to the emerging role of LPs as metabolic signals, our aim is to provide new evidence to support the role of LP metabolism as a key factor in the onset and progression of obesity-related diseases.

SUBJECTS, MATERIALS AND METHODS

Subjects and intervention

All procedures involving human subjects were approved by the NRES South Central–Berkshire Research Ethics Committee (submission number 11/SC/0384). Normoweight (n = 50) and obese (n = 50) male and female subjects were recruited from February 2012 to October 2013 at the University of Southampton. The primary aim of the study was to assess the effect of n-3 PUFA consumption on blood inflammatory markers in normoweight and obese subjects in the fasting state and in response to a standard HFM challenge. Secondary outcomes include the examination of the effects of obesity, n-3 PUFAs and the HFM on blood lipids and related metabolites, which was the focus of the present study. Subjects were eligible for enrolment into the study if they were men or women aged 18-65 y, had a BMI 18.5-25 kg/m^2 (normoweight) or BMI 30-40 kg/m^2 with a waist circumference > 94 cm for men or > 80 cm for women (obese), did not eat more than one oily fish meal per week, and if they provided written informed consent. Subjects were excluded if they met any of the following criteria: diagnosed with diabetes; use of prescribed medicine to control inflammation, hypertension or dyslipidaemia; use of fish oil or other oil supplements; having chronic gastrointestinal problems; being pregnant or planning to become pregnant within the study period; participation within another clinical trial. The trial was registered at www.controlled-trials.com as ISRCTN96712688.

The trial was designed in two separated phases (**supplementary Figure 1**). The first one was a crossover study designed to determine the effects of including n-3 PUFAs or corn oil (as control) with a single HFM on post-prandial changes in metabolites and inflammatory markers in both normoweight and obese subjects

(supplementary Figure 1). The HFM consisted of two croissants served with 28 g of butter and 18 g of jam, 3 x 1 g capsules containing corn oil (or n-3 PUFAs) and a milkshake made with 250 mL of semi-skimmed milk, 32 g of Nesquik™ powder and 75 g of double cream. The HFM provided 85.8 g fat, 76.9 g carbohydrate, 18.6 g protein and 1134 kcal. An abdominal adipose tissue biopsy was collected during the first phase of the trial. The current work is based on the second phase of the trial, which was focused on the effects of daily intake of n-3 PUFAs or corn oil (as control) for 90 days on blood lipids and other metabolites and inflammatory markers (supplementary Figure 1). Sample size for the trial was calculated based upon anticipated findings from the second phase of the trial: a 20% reduction in plasma concentration of interleukin-6 with 90 days n-3 PUFA treatment was anticipated. Based upon mean and SD for plasma interleukin-6 concentration from previous studies it was calculated that 20 subjects per group (i.e. corn oil; n-3 PUFA) would be needed to detect a 20% difference with 80% power at the 5% significance level ($p < 0.05$). Thus, to allow for a 20% drop out rate, 50 normoweight and 50 obese subjects needed to be recruited. 50 normoweight and 50 obese subjects were initially recruited and participated in the first phase of the trial (supplementary Figure 1). Before starting this second phase, 16 normoweight and 12 obese subjects withdrew from the study; this was mainly because of the requirement to provide a second abdominal adipose tissue biopsy at the end of the second phase. The remaining cohort (34 normoweight and 38 obese subjects) was randomly allocated, in a double blinded fashion, to 3 g/day corn oil as control or to 3 g/day EPAX6000 TG (EPAX, Oslo, Norway), a source of eicosapentaenoic acid (EPA; 1.1 g/day) and DHA (0.8 g/day) for 90 days (supplementary Figure 1). EPAX6000 TG is referred to as “fish oil” throughout the rest of the manuscript. Random allocation of subject study code to

treatment (blinded as A or B) was performed using an online random number generator. Corn oil and fish oil were provided as one g gelatine coated capsules. Capsules were provided to subjects in sealed containers. The appearance of the capsules and containers and the labelling on containers were identical for both capsule types. Subjects and all researchers were blinded to allocation until after statistical analysis was complete. After 90 days subjects attended the National Institute for Health Research Wellcome Trust Clinical Research Facility at Southampton General Hospital in the morning after an overnight fast (> 10 hours without food or drink except water). A blood sample was collected into heparin as anticoagulant and then subjects consumed the same HFM described in phase one with placebo capsules. All subjects consumed the same meal and placebo capsules. Blood was collected into heparin as anticoagulant 1, 2, 3, 4 and 6 h after finishing the meal. Plasma was prepared by centrifugation and stored at -80^{o}C until analysis. Here data for the fasting and 6 h plasma samples are presented. There were no subject withdrawals in the second phase of the trial and so data are available for all subjects.

Determination of plasma lipid, glucose and insulin concentrations

Plasma triglyceride, cholesterol, HDL-cholesterol, non-esterified fatty acid (NEFA) and glucose concentrations were measured using an iLAB 600 clinical chemistry analyser and software (Instrumentation Laboratories, Warrington, UK) and enzyme-based kits provided by Wako and Instrumentation laboratories. LDL-cholesterol concentrations were estimated using the Friedwald equation. Plasma insulin concentrations were measured using a quantitative sandwich ELISA kit from Dako. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as:

[Glucose in mmol/L] x [(Insulin in μU/L)/22.5]

Sample preparation for determination of LPs

For the extraction of metabolites, 100 μL of human plasma were added to 900 μL of methanol:water (8:1 vol/vol) containing 0.5 mg/L of LPC (13:0) and 0.1 mg/L deuterated taurocholic acid-D5 as internal standard. The mixture was homogenized by ultrasonication (30 s) and vortexing (20 s). After that, samples were incubated on ice for 10 min, and then centrifuged (16000 x g, 5 min). The supernatant was dried under nitrogen flow to eliminate the solvent. Finally, it was re-dissolved in 200 μL of methanol:water (1:1 vol/vol) to obtain a 2-fold dilution of the initial plasma concentration.

Separation of LPs by reverse phase liquid chromatography

LPs were separated by reverse phase (RP) liquid chromatography performed using an Agilent ZORBAX C18 SB-Aq (Agilent Technologies, Santa Clara, CA, USA) 2.1 mm × 50 mm, 1.8 μm particle analytical column. An Agilent ZORBAX C-8 (Agilent Technologies, Santa Clara, CA, USA), 2.1 mm × 30 mm, 3.5-μm particle guard column was placed in series in front of the analytical column. An Agilent 1290 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a binary pump and degasser, thermostated well plate autosampler and column compartment were used. The autosampler temperature was 4 °C, the injection volume was 2 μL, column temperature was 60 °C, and the flow rate was 0.6 mL/min. A 2%–98% linear gradient of solvent A (0.2% acetic acid in water) to B (0.2% acetic acid in methanol (Honeywell, Morristown, NJ, USA)) was employed over 16 min followed by a solvent B hold of 2 min and a 5 min post-time for both positive and negative ion polarity analysis.

Identification of LP species by mass spectrometry

LP species were identified by mass spectrometry. An Agilent 6550 Accurate-Mass Quadrupole-Time of Flight (Q-TOF) mass spectrometer (MS) (Agilent Technologies, Santa Clara, CA, USA) was operated in ESI+ and ESI- modes. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution (121.050873 and 922.009798 for positive polarity, and 119.03632 and 980.016375 for negative polarity). Scanning conditions were as follows: drying gas temperature of 325 °C and flow rate of 10 L/min; vaporizer temperature of 350 °C; nebulizer pressure of 45 psi; capillary voltage 4,000 V. MS data acquired in positive mode were used to quantitative analysis of LPC species and negative mode MS data were used to quantify LPE and LPI species. Quantitative analysis was performed as described previously (22). Calibration curves were constructed using 1 to 1500 μg/L LPC (16:0), LPC (18:0), LPC (20:0), LPE (18:1), and LPI (18:1) as standards. LPC (16:0) calibration curve was used to quantify LPC (14:0), LPC (16:0) and LPC (16:1); LPC (18:0) calibration curve was used to quantify LPC (18:0), LPC (18:1), LPC (18:2), LPC (18:3) and LPC (18:4); and LPC (20:0) was used to quantify LPC (20:0), LPC (20:1), LPC (20:2), LPC (20:3), LPC (20:4), and LPC (22:5). All LPE and LPI species were quantified using the calibration curves of LPE (18:1) and LPI (18:1), respectively. The limit of detection was 0.04 μM.

Cell culture experiments

HepG2 cells were maintained in complete cell culture medium obtained by supplementing DMEM (Lonza Ibérica, Barcelona, Spain) with 1% L-Glutamine (Lonza Ibérica, Barcelona, Spain), 1% penicillin/streptomycin-EDTA (Sigma, Madrid, Spain), 1% non-essential amino acids (Sigma, Madrid, Spain) and 10% fetal

bovine serum (FBS, Sigma, Madrid, Spain). Cells were seeded either in 12 or 48 well plates at a concentration of 120,000 cells/mL in complete cell culture medium. Twelve hours after seeding, media were replaced with complete serum free media containing 1% of bovine serum albumin (BSA, Sigma, Madrid, Spain) bound to sodium palmitate (Sigma, Madrid, Spain) at 0.2, 0.5 and 0.75 mmol/L or without sodium palmitate as control for 24 h. Treatment media preparation has been described previously (23). After 24 h treatment, cell media were collected, centrifuged at 1000 g for 10 min at 4 ºC to discard cell debris and stored at -80 ºC until extraction and quantification of LPs. For neutral lipid staining, cells were extensively washed with PBS, fixed with 4% paraformaldehyde and subsequently incubated with oil red O (ORO; Sigma, Madrid, Spain). After microscopic analysis and micrography (Nikon Eclipse Ti-S, Izasa, Barcelona, Spain), ORO was eluted with isopropyl alcohol (Sigma, Madrid, Spain) and the absorbance read at 510 nm. For cytotoxicity assays, after 24 h of treatment, media were replaced with complete serum free media containing a 1% BSA and thiazolyl blue tetrazolium bromide (MTT; Sigma, Madrid, Spain) as previously described (23). After 4.5 h incubation, cells were washed with PBS, micrographed and intracellular MTT was extracted with DMSO (Sigma, Madrid, Spain) and quantified at 570 and 660 nm. Cell viability was quantified as Viability (%) = [A570 –A660 (palmitate)/A570-A660 (control)]×100.

Statistical analyses

Data were available for 15 normoweight subjects in the control group, 19 obese subjects in the control group, 19 normoweight subjects in the n-3 PUFA group and 19 obese subjects in the n-3 PUFA group. Data are expressed as means ± SEM. Differences in baseline characteristics between subject groups were analysed by analysis of covariance (ANCOVA), in which treatment group assignment (G) was

used as a fixed factor and the variables age and sex were included as covariates. An ANCOVA model was also used to analyse the data at the end point, in both fasting and postprandial conditions. The circulating levels of LPs or their delta values (changes between the pre- and post-high fat challenge) and the delta values of anthropometric and biochemical parameters (changes from baseline) were used as dependent variables, obesity (O) and n-3 PUFA treatment (T) were included as fixed factors and the variables age and sex were included as covariates. When the interaction between obesity and treatment was statistically significant under the ANCOVA, a Bonferroni post hoc test was used to compute pairwise comparisons between groups (i.e. the effect of treatment within obesity groups and the effect of obesity within treatment groups). Grubbs' test was used to detect outliers, which were discarded for subsequent analyses. All statistical analyses were performed with SPSS Statistics 18 (SPSS, Inc., Chicago, IL), setting the level of statistical significance at bilateral 5%. Principal component analyses (PCA), partial least squares discriminant analyses (PLS-DA), multivariate biomarker validation using receiver operating characteristics (ROC) curves and hierarchical clustering analyses were performed after data normalization and autoscaling using the software Metaboanalyst 3.0 (24).

RESULTS

Subject characteristics

Data were collected between February 2012 and October 2013 from 34 normoweight subjects treated daily with 3 g of either corn oil (N-CO group, n=15) or fish oil (N-FO group, n=19) for 90 days and from 38 obese subjects who received the same treatments for the same duration (O-CO group, n=19; O-FO, n=19). At baseline, obese subjects were significantly older than the normoweight subjects and had higher body weight, BMI, waist circumference and body fat mass (**Table 1**). Obese subjects had higher plasma concentrations of total cholesterol, LDL-cholesterol, triglycerides and non-esterified fatty acids than normoweight subjects (Table 1), although the mean levels of these parameters in the obese subjects were typically within the acceptable concentration range, according to the American Association of Clinical Endocrinologist's Medical (AACE) Guidelines for management of dyslipidaemia and prevention of atherosclerosis (25). Obese subjects also had higher plasma glucose and insulin concentrations (Table 1) and the mean HOMA-IR value above 2.5 indicates insulin resistance (26).

There was a significant effect of treatment on fasting HDL-cholesterol and triglyceride concentrations (Table 1). N-3 PUFAs lowered triglycerides and elevated HDL-cholesterol concentrations compared with corn oil, the control (Table 1). No significant changes were found in other anthropometric and biochemical parameters analysed (Table 1).

Obesity and n-3 PUFAs induce changes in plasma LP concentrations in the fasted state

Obesity was associated with significant differences in plasma LP concentrations in the fasting state compared with those seen in normoweight individuals (**Table 2**). Obese subjects independent of treatment group, had lower concentrations of most of the identified LPCs, with the exception of LPC 20:2, which was higher in the obese subjects (Table 2). Obese subjects had lower concentrations of 5 out of 11 LPEs (LPE 14:1, LPE 18:1, LPE 18:2, LPE 20:0 and LPE 20:2) than seen in normoweight subjects (Table 2). The interaction between obesity and treatment was significant for the plasma concentration of LPE 18:0, which was lower in the obese subjects that received the n-3 PUFAs compared to the normoweight participants submitted to the same treatment (Table 2).Treatment with n-3 PUFAs affected the concentration of 8 out of 26 LPs. Thus, n-3 PUFA groups had lower concentrations of LPC 18:2, LPC 20:3, LPE 18:1, LPE 18:2 and LPE 20:4 and higher concentrations of LPE 20:5, LPE 22:6 and LPI 18:0.

PCA analysis including all the LPs revealed a clear phenotype-dependent clustering of the subjects when the scores of the 3 first principal components, explaining ~56 % of the variance, were represented (**Figure 1A**). Consistent with the ANCOVA and post-hoc analyses, when the individual scores were colored depending on both the phenotype and the oil treatment no clear treatment-dependent clusters were identified (**Figure 1B**).

The 26 LPs were used to set up a PLS-DA predictive model for discriminating between normoweight and obese subjects (**Figure 1C**). The quality parameters associated to the model were excellent. The degree of fit of the model to the data, represented by R2 was 0.86, the quality assessment statistic (Q2), which reports the result of crossvalidation of the model was 0.78, while a threshold of > 0.4 has been proposed as an acceptable value for a biological model (27), and the prediction

accuracy of the crossvalidation process was 97.7%. The cross-validation revealed that the accuracy of the model was maximal (97.7%) when all 26 LPs were used, presenting an area under the ROC curve of 0.996 with a narrow confidence interval ranging from 0.982 to 1 (**Figure 1D**). These results suggest that the profile of LP abundance clearly differs between normoweight and obese subjects, and that the more LP species used to characterize the subject the better the discrimination between the two phenotypes.

A high fat meal challenge magnifies the differences in plasma LPs between normoweight and obese subjects and modifies the long-term effects of n-3 PUFA treatment

The abundance of the 26 LPs was determined in the same subjects 6 h after the intake of a HFM (**Table 3**). The HFM challenge accentuated the differences between normoweight and obese groups, producing the same differences in the plasma concentrations of LPCs and LPEs reported in the fasted state and revealing additional differences in the concentrations of LPEs (18:0, 20:4, 22:5 and 22:6) and LPIs (18:1 and 22:6) (Table 3). These results suggest that the obesity related differences in LPC profile are robust regardless of the absorptive state of the subject while the profile of LPE and LPI species is sensitive to short-term high fat loads. By contrast, the HFM challenge produced, in general, different changes in the circulating levels of LPs to those seen in the fasting state in the subjects chronically treated with n-3 PUFAs. Thus, significant interactions between obesity and treatment were found after the HFM intake for the plasma concentrations of LPCs (18:0, 20:1 and 20:3), which were significantly lower in the normoweight subjects that consumed the fish oil (N-FO group) compared to the normoweight participants that were treated with the corn oil (N-CO group) (Table 3). In addition, although the treatment effect was maintained for

LPEs (18:2, 20:4 and 22:6), it was abolished for LPCs (18:2 and 20:3), LPEs (18:1 and 20:5) and LPI 18:0 (Table 3).

The post-HFM challenge concentrations of the 26 LPs were used for unsupervised classification using the PLS-DA model obtained in the fasted state. As a result (**Figure 2A**) 67 out of 72 subjects were correctly classified (93% accuracy), obtaining an area under the ROC curve of 0.991 for the post-HFM challenge values (**Figure 2B**). Since the plasma LP profile 6 h after the HFM challenge can be used to discriminate between normoweight and obese subjects by applying a model that was obtained with the plasma LP profile in fasting conditions, these results reinforce the conclusion that the changes on plasma LP profile induced by obesity are robust and maintained even after refeeding.

The response of LPC metabolism to a high fat meal challenge is modulated by n-3 PUFAs in normoweight but not in obese subjects

Different studies have shown that the obese state is associated with a decreased sensitivity to fasting and refeeding conditions in terms of regulation of genes and proteins involved in key metabolic processes (28,29). To further characterize the effects of long term n-3 PUFA intake on the postprandial response to a HFM challenge we tested whether the plasma LP profile is also sensitive to refeeding conditions. When the differences between the pre- and post-high fat challenge plasma concentrations of LPs were analysed, an interaction between the oil treatment and the obese phenotype was found for all the LPC species, revealing that n-3 PUFA treatment affected the response of LPCs to the HFM challenge only in normoweight subjects (**Figure 3** and **supplementary Table 1**). Thus, in normoweight subjects the concentrations of LPC 16:0, LPC 18:0 and LPC 20:1 decreased in

response to the HFM in normoweight subjects who had consumed n-3 PUFAs (N-FO group) compared to the normoweight subjects who had consumed corn oil (N-CO group). In addition, N-CO subjects showed increased circulating concentrations of LPC 18:1, LPC 18:2, LPC 20:2, LPC 20:3 and LPC 20:4 after the HFM challenge compared to the N-FO group (Figure 3 and supplementary Table 1). These treatment-dependent responses found in normoweight subjects were not observed in obese subjects (Figure 3 and supplementary Table 1). Treatment- and phenotype-dependent changes were not observed in LPE or LPI species, with the exception of LPE 20:4, which significantly increased in response to the HFM in the N-FO group but not in the O-FO group (supplementary Table 1). These results indicate an insensitivity to the effects of high fat feeding on LPC species in obese subjects.

An in vitro model of steatosis partially reproduces the plasma LP profile of obese subjects

Due to the key contribution of the liver to the pool of plasma LPs (30), we hypothesized that the changes in the profile of LPs associated with obesity could be, at least in part, a response of hepatocytes to the increasing load of fatty acids that occurs in obesity (1). To test this hypothesis we established a model of mild steatosis in HepG2 cells. Cells cultured in serum depleted media were exposed to one of three concentrations of palmitate. All three concentrations resulted in statistically significant intracellular neutral lipid accumulation (**Figures 4A** and **4B**). Nevertheless, only 0.2 mmol/L palmitate maintained full viability of the cells, while 0.5 mmol/L and 0.75 mmol/L palmitate significantly decreased cell viability by 50% and 60% respectively (**Figures 4C** and **4D**). The concentrations of all the LPs that could be detected (compiled in **Table 4**) were determined in the preconditioned media of HepG2 cells. PCA (80.9% of the variance explained by PC1, PC2 and PC3, **Figure**

360 **4E**) and hierarchical clustering analysis (**Figure 4F**) using all the detected LPs
361 revealed that the three doses of palmitate resulted in very different LP profiles in the
362 media. Thus, the cluster of cells treated with 0.2 mmol/L palmitate was clearly
363 differentiated from the cluster of cells cultured without palmitate and from cells
364 treated with 0.5 or 0.75 mmol/L palmitate. In turn, the clusters formed by the cells
365 treated with 0.5 and 0.75 mmol/L palmitate overlapped and were clearly differentiated
366 from the other two treatments. These results suggest that both steatosis and cell
367 viability highly affect the profile of LPs produced by HepG2 cells.

368 Analysis of individual LPs (Table 4) revealed exacerbated changes for cells treated
369 with 0.5 or 0.75 mmol/L palmitate, likely due to the release of intracellular contents
370 into the medium upon apoptosis of the cells. Nevertheless, comparison of the cells
371 treated with the vehicle versus cells treated with the 0.2 mmol/L palmitate revealed
372 differences similar to those found in human plasma. Thus, 6 out of 9 LPCs were
373 changed. Although most of the LPC species detected in HepG2 media were different
374 from those detected in human plasma, two of the common species (LPC18:1 and
375 LPC20:2) changed in the same direction in HepG2 cell media and human plasma. The
376 exception to this was LPC16:0, which contains palmitic acid, the fatty acid used to
377 establish the model. LPEs and LPIs followed a similar pattern in HepG2 media and
378 human plasma, with 6 out of 7 common forms unchanged and only one, LPE 18:1,
379 differing in both models. These results suggest that, despite the evident differences
380 between the HepG2 and human models, induction of non-cytotoxic steatosis in
381 HepG2 cells partially reproduces the changes of LP profiles found in human plasma
382 when obese subjects are compared to normoweight subjects.

DISCUSSION

Our results agree with previous research showing decreased levels of different species of LPCs in obesity or insulin resistance in humans and mice (3,4,10,31) that persist even after weight loss (3). In contrast, increased levels of LPC species associated to the onset of type 1 diabetes, IR and obesity have been described as well (5,6,32–35), suggesting that the onset of these alterations might be associated to transient increases of LPC. Beside LPC species, it has been shown that plasma LPEs negatively correlate with BMI (36) and increase as obesity is ameliorated by diet (11). In agreement with these results, our analyses performed on plasma collected in fasting conditions revealed lower circulating concentrations of some LPEs in obese subjects compared to normoweight subjects. Beyond the changes found in individual LP species, it is remarkable that, with our data, the performance of the PLS-DA predictive model was maximal when all the analysed LP species were used. These results highlight that the impact of obesity on plasma LPs is not limited to a discrete number of species but to the whole profile of plasma LPs, including LPCs, LPEs or LPIs, suggesting that obesity widely affects the metabolism of LPs.

We have found that the metabolism of plasma LPCs is sensitive to long-term intake of n-3 PUFAs. Thus, after the HFM challenge, the concentrations of saturated LPCs were sharply decreased and those of unsaturated LPCs remained unchanged in normoweight subjects treated with n-3 PUFAs. Circulating LPCs have been related to inflammation, although whether they exert pro- or anti-inflammatory actions is still under debate. In fact the role of LPCs in inflammation might depend on their fatty acyl chain (13,37). Thus, unsaturated LPCs (20:4 and 22:6) counteract the pro-inflammatory actions of saturated (16:0) LPCs (20,21). Therefore, it could be suggested that the changes exerted by the n-3 PUFA-rich oil are positive, reflecting a

lower inflammatory state after the HFM. This interpretation is consistent with the anti-inflammatory properties attributed to n-3 PUFAs (14,15,38,39). In contrast, long-term intake of corn oil, rich in the n-6 PUFA linoleic acid, had no effects on the response of saturated LPCs to the HFM challenge although the concentration of LPC 20:4 was increased. It is believed that the postprandial state is a period of transient acute inflammation that contributes to increasing CVD risk (40). Therefore, it could be hypothesized that, together with other mechanisms (19,38), n-3 PUFAs might attenuate postprandial inflammation by modulating the metabolism of LPs. Furthermore, in contrast to the findings in normoweight subjects, obesity impaired the sensitivity of LPC metabolism to the HFM. Recently Kardinaal *et al.* have shown that the response of plasma inflammatory markers, such as interleukin-6 and different pro-inflammatory lipids, to a high fat challenge is blunted in obese subjects with MetS compared to healthy subjects (41). These differences in the response to a high fat challenge were attributed to the lack of phenotypic flexibility that might lead to MetS. Our results support this explanation. Nevertheless, it has to be considered that most of the LPCs, including the pro-inflammatory LPC16:0, were lower in obese subjects than normoweight subjects in the fasting state. These findings might seem paradoxical at first, since obesity is associated to chronic low-grade inflammation (42). However, it is possible that the impaired sensitivity of LPC metabolism to pro-inflammatory cues in obese subjects is responsible for this finding. Other possible causes for these differences, such as age differences between groups, need further research.

Although our results highlight the clear effects of obesity and, to a lesser extent, n-3 PUFAs on LPs metabolism, a potential limitation of the present study is that the lack of adjustment for multiple testing might have increased the number of false positives. Therefore, results concerning individual LPs species should be

interpreted with caution, although these represent a starting point to assess the effects of obesity and n-3 PUFA consumption on individual LPs.

The changes observed in the profile of LPs could be related to other processes that are also affected by obesity. For example, it has been shown that LPCs might have an important role in insulin sensitivity. Thus, LPC12:0, 14:0 and 16:0 but not LPCs of 18 and 20 carbons induce the uptake of glucose by cultured adipocytes (43), LPC18:1 induces pancreatic insulin release (44) and circulating levels of LPC16:0 are lower in insulin resistant than in insulin sensitive subjects with NAFLD (10). In fact, lysophosphatidic acid, a product of LPC hydrolysis, has been suggested as a promising agent to treat insulin resistance (13). Therefore, the lower plasma levels of these metabolites observed in obese subjects could be related to their lower insulin sensitivity compared with normoweight subjects, as suggested by the differences found in HOMA-IR indices. This possibility is reinforced by the greater decrease of LPC16:0 observed in normoweight subjects treated with n-3 PUFAs than in the other groups after the HFM challenge, which might indicate transient adverse effects on insulin sensitivity. Indeed, a recent systematic review of meta-analyses has revealed that n-3 PUFAs have unfavourable effects on type 2 diabetes in Caucasians (14). Therefore, the interactions of obesity with LP metabolism could also contribute to or result from the onset of obesity-associated IR.

The differences observed in plasma LPs between normoweight and obese subjects prompted us to further explore the origin of these changes. Plasma LPs might be formed in blood by the actions of LCAT and secretory phospholipases, but a direct hepatic origin has been also demonstrated in rats and proposed as a quantitatively relevant source of LPCs (30). In fact, it has been shown that hepatic alterations such as NAFLD and NASH alter the metabolism of phospholipids and LPs in mice and

458 humans (7–10). Overall, the effects of NAFLD on circulating levels of LPCs are very
459 similar to those described by us and others in obese subjects. In view of this evidence,
460 it is plausible to hypothesize that these common changes have a common origin. It is
461 well known that increasing BMI is associated with increased risk of NAFLD (45) and
462 adipocyte cell size is associated to liver injury (46). The main underlying mechanism
463 relates to the increased flux of fatty acids from the adipose tissue to the liver, together
464 with altered hepatic metabolism of fatty acids and lipoproteins (1,45). Therefore, we
465 hypothesized that accumulation of lipids in hepatocytes might alter the profile of
466 secreted LPs. Our experiment in the *in vitro* model of steatosis obtained by
467 challenging HepG2 with palmitate supports this hypothesis. What is more, despite the
468 obvious differences between the *in vitro* model and human subjects, the changes
469 induced in the profile of LPs in HepG2 media by non-cytotoxic steatosis and in
470 plasma by obesity are remarkably similar. Therefore, it could be hypothesized that the
471 changes induced by obesity in the LP profile are partially due to the metabolic stress
472 that obesity induces in the liver, and place these molecules as promising biomarkers
473 for studying alterations of liver homeostasis. More research is still needed in order to
474 confirm this hypothesis.

475 Overall, our results suggest that obesity has a profound impact in the
476 metabolism of LPs, modifying the profile of plasma LPs in the long term and
477 affecting the sensitivity of these metabolites to dietary fatty acids. These effects could
478 be mediated, at least partially, by the influence of obesity in the metabolism of
479 hepatocytes. Due to the role of LPs as signalling molecules in processes that are
480 usually altered in obesity, these findings provide more evidence to understand the
481 mechanisms that favour the progression of alterations such as insulin resistance,
482 inflammation, NAFLD or MetS. More research is needed in order to better understand

the exact role of plasma LPs in disease progression and, therefore, to assess whether LP metabolism represents a promising target for the prevention and treatment of obesity associated diseases as well as being a source of biomarkers for the early and non-invasive detection of metabolic alterations.

Acknowledgements

We gratefully acknowledge the help of Silvia Pijuan and Yaiza Tobajas, laboratory technicians in CTNS and the Centre for Omics Sciences (COS, Reus, Spain) for the support with HPLC-MS analysis.

Conflict of interests

Authors have no potential conflicts of interest.

Authors' contributions

JdB, AC, LA, EAM, PCC designed research; JdB, MIRN, CEC, CPC, ALW conducted research; JdB, AC, MIRN analyzed data; JdB, AC, PCC drafted the manuscript; JdB, AC, PCC had primary responsibility for final content. All authors read and approved the final manuscript.

References

1. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology (Baltimore, Md). 2010;51:679–89.

2. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 2007;132:2169–80.

3. Heimerl S, Fischer M, Baessler A, Liebisch G, Sigruener A, Wallner S, Schmitz G. Alterations of plasma lysophosphatidylcholine species in obesity and weight loss. PloS One. 2014;9:e111348.

4. Barber MN, Risis S, Yang C, Meikle PJ, Staples M, Febbraio M a, Bruce CR. Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes. PloS One. 2012;7:e41456.

5. Pietiläinen KH, Sysi-Aho M, Rissanen A, Seppänen-Laakso T, Yki-Järvinen H, Kaprio J, Oresic M. Acquired obesity is associated with changes in the serum lipidomic profile independent of genetic effects--a monozygotic twin study. PloS One. 2007;2:e218.

6. Graessler J, Schwudke D, Schwarz PEH, Herzog R, Shevchenko A, Bornstein SR. Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients. PloS One. 2009;4:e6261.

7. Neuschwander-tetri BA. Hepatic Lipotoxicity and the Pathogenesis of Nonalcoholic Steatohepatitis: the Central Role of Nontriglyceride Fatty Acid Metabolites. Hepatology. 2010; 52(2):774-88 .

8. Wattacheril J, Seeley EH, Angel P, Chen H, Bowen BP, Lanciault C, Caprioli RM, Abumrad N, Flynn CR. Differential intrahepatic phospholipid zonation in simple steatosis and nonalcoholic steatohepatitis. PloS One. 2013;8:e57165.

9. Tanaka N, Matsubara T, Krausz KW, Patterson AD, Gonzalez FJ. Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. Hepatology (Baltimore, Md). 2012;56:118–29.

10. Lehmann R, Franken H, Dammeier S, Rosenbaum L, Kantartzis K, Peter A, Zell A, Adam P, Li J, Xu G, et al. Circulating lysophosphatidylcholines are markers of a metabolically benign nonalcoholic fatty liver. Diabetes Care. 2013;36:2331–8.

11. Bondia-Pons I, Martinez JA, de la Iglesia R, Lopez-Legarrea P, Poutanen K, Hanhineva K, Zulet M de los Á. Effects of short- and long-term Mediterranean-based dietary treatment on plasma LC-QTOF/MS metabolic profiling of subjects with metabolic syndrome features: The Metabolic Syndrome Reduction in Navarra (RESMENA) randomized controlled trial. Molecular Nutrition & Food Research. 2015;59:711–28.

12. de la Iglesia R, Lopez-Legarrea P, Abete I, Bondia-Pons I, Navas-Carretero S, Forga L, Martinez JA, Zulet MA. A new dietary strategy for long-term

treatment of the metabolic syndrome is compared with the American Heart Association (AHA) guidelines: the MEtabolic Syndrome REduction in NAvarra (RESMENA) project. British Journal of Nutrition. 2013;111:643–52.

13. Grzelczyk A, Gendaszewska-Darmach E. Novel bioactive glycerol-based lysophospholipids: new data -- new insight into their function. Biochimie. Elsevier Masson SAS; 2013;95:667–79.

14. Li D. Omega-3 polyunsaturated fatty acids and non-communicable diseases: meta-analysis based systematic review. Asia Pacific Journal of Clinical Nutrition. 2015;24:10–5.

15. Monteiro J, Leslie M, Moghadasian MH, Arendt BM, Allard JP, Ma DWL. The role of n - 6 and n - 3 polyunsaturated fatty acids in the manifestation of the metabolic syndrome in cardiovascular disease and non-alcoholic fatty liver disease. Food & Function. 2014;5:426–35.

16. Kim J, Li Y, Watkins B a. Fat to treat fat: Emerging relationship between dietary PUFA, endocannabinoids, and obesity. Prostaglandins & Other Lipid Mediators. Elsevier Inc.; 2013;104-105:32–41.

17. Bender N, Portmann M, Heg Z, Hofmann K, Zwahlen M, Egger M. Fish or n3-PUFA intake and body composition: a systematic review and meta-analysis. Obesity Reviews. 2014;15:657–65.

18. Masoodi M, Kuda O, Rossmeisl M, Flachs P, Kopecky J. Lipid signaling in adipose tissue: Connecting inflammation & metabolism. Biochimica et Biophysica Acta. 2015;1851:503–18.

19. Wang Y, Huang F. N-3 Polyunsaturated Fatty Acids and Inflammation in Obesity: Local Effect and Systemic Benefit. BioMed Research International. 2015;2015:581469.

20. Rao SP, Riederer M, Lechleitner M, Hermansson M, Desoye G, Hallström S, Graier WF, Frank S. Acyl chain-dependent effect of lysophosphatidylcholine on endothelium-dependent vasorelaxation. PloS One. 2013;8:e65155.

21. Riederer M, Ojala PJ, Hrzenjak A, Graier WF, Malli R, Tritscher M, Hermansson M, Watzer B, Schweer H, Desoye G, et al. Acyl chain-dependent effect of lysophosphatidylcholine on endothelial prostacyclin production. Journal of Lipid Research. 2010;51:2957–66.

22. Liebisch G, Schmitz G. Quantification of lysophosphatidylcholine species by high-throughput electrospray ionization tandem mass spectrometry (ESI-MS/MS). Methods in Molecular Biology (Clifton, NJ). 2009;580:29–37.

23. Rojas C, Pan-Castillo B, Valls C, Pujadas G, Garcia-Vallve S, Arola L, Mulero M. Resveratrol enhances palmitate-induced ER stress and apoptosis in cancer cells. PloS One. 2014;9:e113929.

24. Xia J, Sinelnikov I V., Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. Nucleic Acids Research. 2015;gkv380 – .

25. Handelsman Y, Mechanick JI, Dagogo-jack S, Davidson J a. American association of clinical endocrinologists' guidelines for management of dyslipidemia and prevention of atherosclerosis. Endocrine Practice. 2012;18.

26. Son JW, Park C, Kim S, Lee H, Lee Y, Resistance I. Changing Clinical Characteristics according to Insulin Resistance and Insulin Secretion in Newly Diagnosed Type 2 Diabetic Patients in Korea. Diabetes & Metabolism Journal. 2015;39:387–94.

27. Worley B, Powers R. Multivariate Analysis in Metabolomics. Current Metabolomics. 2013;1(1):92-107. [

28. Caimari A, Oliver P, Keijer J, Palou A. Peripheral blood mononuclear cells as a model to study the response of energy homeostasis-related genes to acute changes in feeding conditions. Omics□: a journal of integrative biology. 2010;14(2):129-41.

29. Caimari A, Oliver P, Palou A. Regulation of adiponutrin expression by feeding conditions in rats is altered in the obese state. Obesity (Silver Spring, Md). 2007;15:591–9.

30. Sekas G, Patton GM, Lincoln EC, Robins SJ. Origin of plasma lysophosphatidylcholine: evidence for direct hepatic secretion in the rat. The Journal of Laboratory and Clinical Medicine. 1985;105:190–4.

31. Wallace M, Morris C, O'Grada CM, Ryan M, Dillon ET, Coleman E, Gibney ER, Gibney MJ, Roche HM, Brennan L. Relationship between the lipidome, inflammatory markers and insulin resistance. Molecular BioSystems. 2014;10(6):1586-95.

32. Rauschert S, Uhl O, Koletzko B, Kirchberg F, Mori TA, Huang R-C, Beilin LJ, Hellmuth C, Oddy WH. Lipidomics Reveals Associations of Phospholipids With Obesity and Insulin Resistance in Young Adults. The Journal of Clinical Endocrinology and Metabolism. 2016;101(3):871-9

33. Kim JY, Park JY, Kim OY, Ham BM, Kim H-J, Kwon DY, Jang Y, Lee JH. Metabolic profiling of plasma in overweight/obese and lean men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF MS). Journal of Proteome Research. 2010;9(9):4368-75.

34. Lee SY, Kim M, Jung S, Lee S-H, Lee JH. Altered plasma lysophosphatidylcholines and amides in non-obese and non-diabetic subjects with borderline-to-moderate hypertriglyceridemia: a case-control study. PloS One. 2015;10(4):e0123306

35. Oresic M, Simell S, Sysi-Aho M, Näntö-Salonen K, Seppänen-Laakso T, Parikka V, Katajamaa M, Hekkala A, Mattila I, Keskinen P, et al. Dysregulation of lipid and amino acid metabolism precedes islet autoimmunity in children who later progress to type 1 diabetes. The Journal of Experimental Medicine. 2008; 205(13):2975-84.

36. Weir JM, Wong G, Barlow CK, Greeve M a, Kowalczyk A, Almasy L, Comuzzie AG, Mahaney MC, Jowett JBM, Shaw J, et al. Plasma lipid profiling in a large population-based cohort. Journal of Lipid Research. 2013;54:2898–908.

37. Sevastou I, Kaffe E, Mouratis M-A, Aidinis V. Lysoglycerophospholipids in chronic inflammatory disorders: the PLA(2)/LPC and ATX/LPA axes. Biochimica et Biophysica Acta. 2013;1831:42–60.

38. Calder PC. n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. The American Journal of Clinical Nutrition. 2006;83:S1505–19.

39. Todoric J, Löffler M, Huber J, Bilban M, Reimers M, Kadl A, Zeyda M, Waldhäusl W, Stulnig TM. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. Diabetologia. 2006;49:2109–19.

40. de Vries MA, Klop B, Janssen HW, Njo TL, Westerman EM, Castro Cabezas M. Postprandial inflammation: targeting glucose and lipids. Advances in Experimental Medicine and Biology. 2014;824:161–70.

41. Kardinaal AFM, van Erk MJ, Dutman AE, Stroeve JHM, van de Steeg E, Bijlsma S, Kooistra T, van Ommen B, Wopereis S. Quantifying phenotypic flexibility as the response to a high-fat challenge test in different states of metabolic health. The FASEB Journal. 2015;29:4600–13.

42. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444:860–7.

43. Yea K, Kim J, Yoon JH, Kwon T, Kim JH, Lee BD, Lee H-J, Lee SJ, Kim J-I, Lee TG, et al. Lysophosphatidylcholine activates adipocyte glucose uptake and lowers blood glucose levels in murine models of diabetes. The Journal of Biological Chemistry. 2009;284:33833–40.

44. Soga T, Ohishi T, Matsui T, Saito T, Matsumoto M, Takasaki J, Matsumoto S-I, Kamohara M, Hiyama H, Yoshida S, et al. Lysophosphatidylcholine enhances glucose-dependent insulin secretion via an orphan G-protein-coupled receptor. Biochemical and Biophysical Research Communications. 2005;326:744–51.

45. Ruhl CE, Everhart JE. Determinants of the association of overweight with elevated serum alanine aminotransferase activity in the United States. Gastroenterology. 2003;124:71–9.

46. Wree A, Schlattjan M, Bechmann LP, Claudel T, Sowa J-P, Stojakovic T, Scharnagl H, Köfeler H, Baba HA, Gerken G, et al. Adipocyte cell size, free fatty acids and apolipoproteins are associated with non-alcoholic liver injury progression in severely obese patients. Metabolism: Clinical and Experimental. 2014;63:1542–52.

Table 1. Anthropometric and biochemical characteristics of normoweight and obese subjects at baseline and changes from baseline after the treatment with 3 g/day corn oil or n-3 PUFA rich oil for 90 days.

	Before treatment (absolute values)					After treatment (Δ values)				
	N-FO	**O-FO**	**N-CO**	**O-CO**		**N-FO**	**O-FO**	**N-CO**	**O-CO**	
N	19	19	15	19						
Males, N (%)	8 (42.1)	5 (26.3)	2 (13.3)	5 (26.3)						
Age (y)	30.8 ± 3.5	47.6 ± 2.7	31.9 ± 3.5	40.5 ± 2.9	*O*					
Weight (kg)	63.2 ± 2.1	94.9 ± 2.7	61.3 ± 2.4	101.7 ± 3.2	*O*	0.38 ± 0.37	0.86 ± 0.68	0.25 ± 0.46	0.74 ± 0.53	
BMI (kg/m^2)	22.2 ± 0.4	34.4 ± 0.5	22.5 ± 0.5	35.5 ± 0.7	*O*	0.14 ± 0.13	0.34 ± 0.26	0.09 ± 0.16	0.24 ± 0.19	
Waist (cm)	75.6 ± 1.2	106.8 ± 2.5	75.3 ± 1.7	108.9 ± 2.9	*O*	0.04 ± 0.70	-0.01 ± 1.35	-0.92 ± 0.44	-0.57 ± 0.75	
Fat mass (kg)	12.4 ± 1.1	38.0 ± 1.4	14.4 ± 1.1	41.6 ± 1.7	*O*	0.20 ± 0.30	0.47 ± 0.38	0.14 ± 0.35	0.82 ± 0.46	
TC (mmol/L)	4.38 ± 0.21	5.56 ± 0.27	4.37 ± 0.22	4.87 ± 0.14	*O*	0.01 ± 0.11	-0.07 ± 0.15	0.03 ± 0.11	0.07 ± 0.08	
HDL-C (mmol/L)	1.50 ± 0.08	1.51 ± 0.07	1.58 ± 0.08	1.41 ± 0.07		0.12 ± 0.06	0.05 ± 0.05	-0.06 ± 0.04	-0.01 ± 0.03	*T*
LDL-C (mmol/L)	2.64 ± 0.18	3.69 ± 0.22	2.63 ± 0.21	3.23 ± 0.12	*O*	-0.09 ± 0.09	-0.15 ± 0.12	0.09 ± 0.09	0.04 ± 0.05	
TG (mmol/L)	0.85 ± 0.08	1.43 ± 0.19	0.79 ± 0.06	1.15 ± 0.12	*O*	-0.16 ± 0.08	-0.10 ± 0.08	-0.03 ± 0.04	0.17 ± 0.13	*T*
NEFAs (μmol/L)	423 ± 45	630 ± 37	486 ± 56	575 ± 46	*O*	46.4 ± 57.2	-95.3 ± 50.5	37.6 ± 67.1	-26.9 ± 32.3	

The American Journal of Clinical Nutrition AJCN/2016/130872 Version 3

Glucose (mmol/L)	4.79 ± 0.09	5.49 ± 0.18	4.73 ± 0.07	5.48 ± 0.07	*O*	0.00 ± 0.10	0.16 ± 0.16	0.14 ± 0.11	-0.04 ± 0.10
Insulin (µIU/mL)	5.19 ± 0.61	12.7 ± 1.6	6.00 ± 0.56	16.0 ± 1.9	*O*	-0.63 ± 0.80	0.68 ± 1.21	0.44 ± 0.59	-0.41 ± 1.26
HOMA-IR	1.11 ± 0.12	3.33 ± 0.48	1.28 ± 0.12	3.74 ± 0.47	*O*	-0.12 ± 0.18	0.54 ± 0.32	0.12 ± 0.14	-0.01 ± 0.31

Blood was collected after overnight fasting at study entry and on the last day of the 90 day intervention. The data are given as the mean ± SEM (n = 15-19). Baseline and post-treatment delta values (changes from baseline) are shown. Differences in anthropometric and biochemical characteristics (dependent variables) among groups were evaluated by ANCOVA, in which the variables age and sex were included as covariates. At baseline, treatment group assignment (G) was used as a fixed factor, whereas at the endpoint obesity (O) and n-3 PUFA treatment (T) were included as fixed factors. *O*: significant effect of obesity, *T*: significant effect of treatment (ANCOVA, $p<0.05$). N-FO: normoweight subjects treated with fish oil; O-FO: obese subjects treated with fish oil; N-CO: normoweight subjects treated with corn oil; O-CO: obese subjects treated with corn oil. TC: total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG: triglycerides; NEFAs: non-esterified fatty acids; HOMA-IR: homeostatic model assessment of insulin resistance.

Table 2. Lysophospholipid concentrations in plasma of normoweight and obese subjects treated with 3 g/day corn oil or and n-3 PUFA-rich oil for 90 days and then submitted to overnight fasting

Metabolite (µmol/L)	N-FO	O-FO	N-CO	O-CO	ANCOVA
LPC					
LPC 16:0	266.5 ± 23.6	125.7 ± 10.2	253.3 ± 23.9	131.3 ± 16.6	*O*
LPC 18:0	84.0 ± 12.0	22.2 ± 3.1	85.0 ± 10.1	24.8 ± 3.8	*O*
LPC 18:1	45.6± 5.2	11.9 ± 1.3	49.7 ± 6.9	14.4 ± 1.9	*O*
LPC 18:2	3.25 ± 0.39	0.771 ± 0.09	3.73 ± 0.56	1.06 ± 0.11	*O, T*
LPC 20:1	0.409 ± 0.039	0.177 ± 0.010	0.409 ± 0.038	0.169 ± 0.015	*O*
LPC 20:2	0.297 ± 0.021	0.469 ± 0.036	0.299 ± 0.027	0.524 ± 0.038	*O*
LPC 20:3	2.15 ± 0.23	0.952 ± 0.07	2.95 ± 0.28	1.24 ± 0.15	*O, T*
LPC 20:4	6.32 ± 0.62	2.87 ± 0.26	7.01 ± 0.82	3.40 ± 0.41	*O*
LPE					
LPE 14:1	0.854 ± 0.028	0.791 ± 0.013	0.858 ± 0.023	0.786 ± 0.014	*O*
LPE 16:0	0.096 ± 0.004	0.093 ± 0.005	0.096 ± 0.006	0.088 ± 0.003	
LPE 18:0	0.374 ± 0.025	0.310 ± 0.021*	0.316 ± 0.014	0.322 ± 0.021	*O, OxT*
LPE 18:1	0.241 ± 0.014	0.212 ± 0.014	0.292 ± 0.029	0.256 ± 0.017	*O, T*
LPE 18:2	0.496 ± 0.038	0.394 ± 0.035	0.585 ± 0.051	0.516 ± 0.046	*O, T*
LPE 20:0	0.468 ± 0.034	0.433 ± 0.021	0.443 ± 0.026	0.423 ± 0.031	*O*
LPE 20:2	1.014 ± 0.060	0.766 ± 0.042	1.118 ± 0.117	0.853 ± 0.047	*O*
LPE 20:4	0.298 ± 0.016	0.293 ± 0.023	0.393 ± 0.028	0.356 ± 0.027	*T*
LPE 20:5	0.097 ± 0.019	0.109 ± 0.009	0.065 ± 0.005	0.086 ± 0.011	*T*
LPE 22:5	0.085 ± 0.004	0.087 ± 0.004	0.080 ± 0.005	0.090 ± 0.006	
LPE 22:6	0.410 ± 0.028	0.381 ± 0.023	0.305 ± 0.016	0.275 ± 0.017	*T*
LPI					
LPI 16:1	0.431 ± 0.014	0.405 ± 0.005	0.428 ± 0.013	0.403 ± 0.006	*O*
LPI 18:0	0.097 ± 0.005	0.105 ± 0.005	0.085 ± 0.005	0.094 ± 0.004	*T*
LPI 18:1	0.076 ± 0.004	0.081 ± 0.004	0.077 ± 0.003	0.078 ± 0.004	
LPI 18:2	0.091 ± 0.006	0.091 ± 0.004	0.091 ± 0.006	0.094 ± 0.005	
LPI 20:3	0.062 ± 0.002	0.064 ± 0.002	0.063 ± 0.002	0.065 ± 0.002	
LPI 20:4	0.149 ± 0.008	0.157 ± 0.006	0.150 ± 0.012	0.159 ± 0.010	
LPI 22:6	0.081 ± 0.004	0.086 ± 0.004	0.082 ± 0.004	0.086 ± 0.005	

Blood was collected after overnight fasting on the last day of the 90 day intervention. Data are mean ± SEM (n = 15-19). The effects of treatment, obesity and their interaction were evaluated by ANCOVA. Lysophospholipid concentrations in plasma

were used as dependent variables, obesity (O) and n-3 PUFA treatment (T) were included as fixed factors and the variables age and sex were included as covariates. *O*: significant effect of obesity; *T*: significant effect of treatment; *O*x*T*, significant interaction between obesity and treatment (ANCOVA, $p<0.05$). * Effect of obesity within FO groups (Bonferroni *post hoc* comparisons, $p<0.05$). N-FO: normoweight subjects treated with fish oil; O-FO: obese subjects treated with fish oil. N-CO: normoweight subjects treated with corn oil; O-CO: obese subjects treated with corn oil.

Table 3. Lysophospholipid concentrations in plasma of normoweight and obese subjects treated with 3 g/day of corn oil or n-3 PUFA-rich oil for 90 days and then submitted to a high-fat meal challenge

Metabolite (µmol/L)	N-FO	O-FO	N-CO	O-CO	ANCOVA
LPC					
LPC 16:0	199.2 ± 17.2	131.9 ± 17.9	244.2 ± 15.9	113.3 ± 15.5	*O*
LPC 18:0	60.2 ± 8.1$	25.5 ± 4.4*	88.9 ± 9.8	21.3 ± 4.3#	*O, T, OxT*
LPC 18:1	51.7 ± 5.7	19.9 ± 2.8	72.3 ± 7.8	18.0 ± 2.7	*O*
LPC 18:2	4.28 ± 0.47	1.79 ± 0.21	5.89 ± 0.54	1.76 ± 0.21	*O*
LPC 20:1	0.319 ± 0.028$	0.186 ± 0.019*	0.411 ± 0.031	0.166 ± 0.015#	*O, OxT*
LPC 20:2	0.315 ± 0.025	0.524 ± 0.048	0.437 ± 0.036	0.550 ± 0.045	*O, T*
LPC 20:3	2.66 ± 0.28$	1.50 ± 0.16*	4.61 ± 0.30	1.76 ± 0.20#	*O, T, OxT*
LPC 20:4	6.55 ± 0.62	4.07 ± 0.46	10.51 ± 1.14	4.19 ± 0.52	*O, T*
LPE					
LPE 14:1	0.854 ± 0.005	0.787 ± 0.012	0.899 ± 0.026	0.807 ± 0.009	*O*
LPE 16:0	0.103 ± 0.005	0.089 ± 0.004	0.098 ± 0.005	0.098 ± 0.004	
LPE 18:0	0.393 ± 0.030	0.342 ± 0.024	0.433 ± 0.039	0.308 ± 0.018	*O*
LPE 18:1	0.634 ± 0.057	0.421 ± 0.027	0.723 ± 0.064	0.402 ± 0.029	*O*
LPE 18:2	1.277 ± 0.147	0.802 ± 0.054	1.528 ± 0.139	0.866 ± 0.065	*O, T*
LPE 20:0	0.454 ± 0.041	0.434 ± 0.029	0.547 ± 0.053	0.413 ± 0.028	*O*
LPE 20:2	1.64 ± 0.15	1.13 ± 0.07	1.83 ± 0.15	1.12 ± 0.05	*O*
LPE 20:4	0.517 ± 0.033	0.421 ± 0.022	0.740 ± 0.069	0.467 ± 0.037	*O, T*
LPE 20:5	0.205 ± 0.037	0.135 ± 0.015	0.164 ± 0.032	0.134 ± 0.019	
LPE 22:5	0.108 ± 0.004	0.097 ± 0.005	0.119 ± 0.011	0.099 ± 0.006	*O*
LPE 22:6	0.455 ± 0.034	0.379 ± 0.019	0.408 ± 0.031	0.292 ± 0.013	*O, T*
LPI					
LPI 16:1	0.442 ± 0.018	0.399 ± 0.006	0.437 ± 0.013	0.410 ± 0.008	*O*
LPI 18:0	0.105 ± 0.005	0.109 ± 0.003	0.104 ± 0.005	0.103 ± 0.005	
LPI 18:1	0.133 ± 0.010	0.109 ± 0.004	0.133 ± 0.012	0.109 ± 0.005	*O*
LPI 18:2	0.142 ± 0.013	0.129 ± 0.005	0.138 ± 0.012	0.123 ± 0.008	
LPI 20:3	0.068 ± 0.003	0.068 ± 0.002	0.072 ± 0.004	0.068 ± 0.002	
LPI 20:4	0.234 ± 0.020	0.224 ± 0.010	0.243 ± 0.024	0.220 ± 0.013	
LPI 22:6	0.141 ± 0.011	0.116 ± 0.004	0.141 ± 0.012	0.116 ± 0.005	*O*

Blood was collected 6 h after the administration of a high-fat meal (1135 kcal, 86 g fat, of which 59 g saturated fat). Data are mean ± SEM (n = 15-19). The effects of treatment, obesity and their interaction were evaluated by ANCOVA.

Lysophospholipid concentrations in plasma were used as dependent variables, obesity (O) and n-3 PUFA treatment (T) were included as fixed factors and the variables age and sex were included as covariates. *O*: significant effect of obesity; *T*: significant effect of treatment; *O*x*T*, significant interaction between obesity and treatment (ANCOVA, $p<0.05$). * Effect of obesity within FO groups; # effect of obesity within CO groups; $ effect of treatment within normoweight groups (Bonferroni *post hoc* comparisons, $p<0.05$). N-FO: normoweight subjects treated with fish oil; O-FO: obese subjects treated with fish oil. N-CO: normoweight subjects treated with corn oil; O-CO: obese subjects treated with corn oil.

Table 4. Concentrations of lysophospholipids in media of HepG2 cells treated with vehicle or with different concentrations of palmitate during 24 hours to induce steatosis.

Metabolite (µmol/L)	**vehicle**	**0.2 mmol/L**	**Change in Obese humans** [1]	**0.5 mmol/L**	**0.75 mmol/L**	**ANOVA**
LPC						
LPC 14:0	9±1 [a]	0.3±0.2 [b, *]	N.D.	15±2 [a]	26±4 [c]	**
LPC 16:0	172±7 [a]	173±10 [a]	Decreased	1017±127 [b]	1455±159 [c]	**
LPC 16:1	1876±30	1841±34	N.D.	1862±20	1 976±48	
LPC 18:1	185±8 [a]	146±8 [b, *]	Decreased	245±17 [c]	330±31 [d]	**
LPC 18:3	246±8 [a]	250±12 [a]	N.D.	1220±304 [b]	2042±336 [c]	**
LPC 18:4	54±3 [a]	71±6 [b, *]	N.D.	137±7 [c]	186±16 [d]	**
LPC 20:0	6±1 [a]	10±2 [ab, *]	N.D.	21±6 [b]	26±3 [b]	**
LPC 20:2	130±5 [a]	170±8 [b, *]	Increased	210±4 [c]	200±14 [c]	**
LPC 22:5	306±9	266±11 *	N.D.	328±46	269±61	
LPE						
LPE 18:0	11±1 [a]	12±1 [a]	Unchanged in CO group	29±3 [b]	36±3 [b]	**
LPE 18:1	61±3 [a]	58±6 [a]	Decreased	98±6 [b]	133±9 [c]	**
LPE 18:4	1.0±0.1 [a]	3.2±0.1 [b, *]	N.D.	14±0.4 [c]	15±1 [c]	**
LPE 20:4	9±1 [a]	9±1 [a]	Unchanged	22±2 [b]	33±3 [c]	**
LPI						
LPI 18:1	43±2	40±5	Unchanged	60±3	84±5	
LPI 18:2	12±1	13±2	Unchanged	12±1	16±1	
LPI 20:3	32±2 [a]	27±2 [bc]	Unchanged	24±1 [b]	30±1 [ac]	**
LPI 20:4	48±3	44±4	Unchanged	41±1	54±4	

Cells were maintained for 24 hours with vehicle (media supplemented with BSA) or increasing concentrations of BSA-bound palmitate (0.2, 0.5 and 0.75 mmol/L). Mean ± SEM of culture medium LPCs, LPEs and LPIs of two different experiments run in duplicate. [1] Observed change in overweight compared with normoweight subjects as reported in table 2. N.D. denotes that the metabolite was not detected in human plasma. *significantly different from vehicle ($p<0.05$; Student's t-test). **denotes p

<0.05 for the one-way ANOVA. Superscript letters denote significantly different groups as identified by Fisher's LSD post-hoc test.

Figure Legends

Figure 1.

Multivariate analyses of 26 lysophospholipids detected in plasma of 34 normoweight and 38 obese subjects. (A) Scores plot of principal components 1, 2 and 3 after a principal component analysis. Subjects are colored as normoweight or obese. (B) Same plot shown on panel A with subjects colored as normoweight (N) or obese (O) treated with corn oil (CO) or n-3 PUFA rich oil (FO) for 90 days. (C) Scores plot of components 1, 2 and 3 after a partial least squares discriminant analysis (PLS-DA) using phenotype, i.e. normoweight and obese, as discriminant factor. (D) Receiver Operating Characteristics (ROC) curve analysis of the PLS-DA model using the 2, 3, 5, 10, 20 and 26 lysophospholipids with the highest variable importance in the projection score. The area under the ROC curve and the corresponding 95% confidence interval are reported in the inset.

Figure 2.

Projection in the PLS-DA model shown in figure 1 of lysophospholipid profiles measured 6 hours after a high fat meal challenge in normoweight and obese subjects. (A) Classification of subjects by their lysophospholipid profile as normoweight (open circles and squares) or obese (filled circles and squares) during the cross-validation of the PLS-DA model set up (training group) and classification of subjects by their lysophospholipid profile measured 6 hours after the challenge (validation group). (B) Receiver Operating Characteristics (ROC) curves calculated during the crossvalidation of the PLS-DA model (darker line and shadowed 95% confidence interval of 0.982 to 1) and ROC curve obtained with the lysophospholipid profile determined 6 hours after the challenge as a holdout group validation (lighter line).

The areas under the ROC curve for the cross-validation (CV) and the holdout groups are reported in the inset.

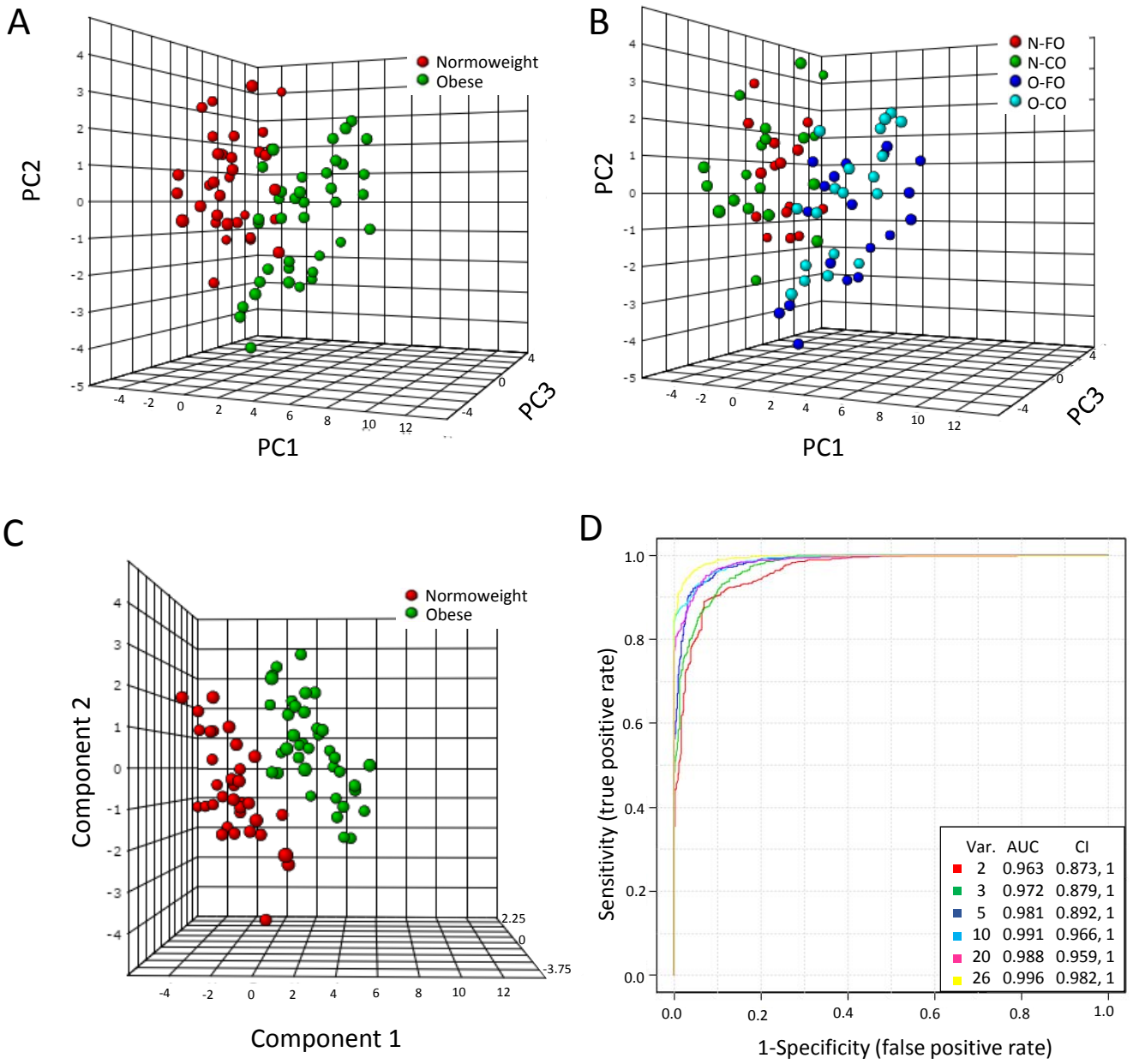
Figure 3.

Differences in lysophosphatidylcholines concentrations between fasting conditions and 6 hours after a high fat meal challenge in normoweight (N) and obese (O) subjects treated with corn oil (CO) or n-3 PUFA rich oil (FO) for 90 days (n=15-19).. Data are mean ± SEM. The effects of treatment, obesity and their interaction were evaluated by ANCOVA. Delta values (changes between the pre- and post-high fat challenge) of lysophospholipid concentrations in plasma were used as dependent variables, obesity (O) and n-3 PUFA treatment (T) were included as fixed factors and the variables age and sex were included as covariates. *O*: significant effect of obesity; *T*: significant effect of treatment; *O*x*T*, significant interaction between obesity and treatment (ANCOVA, $p<0.05$). * Effect of obesity within FO groups; # effect of obesity within CO groups; $ effect of treatment within normoweight groups (Bonferroni *post hoc* comparison, $p<0.05$).

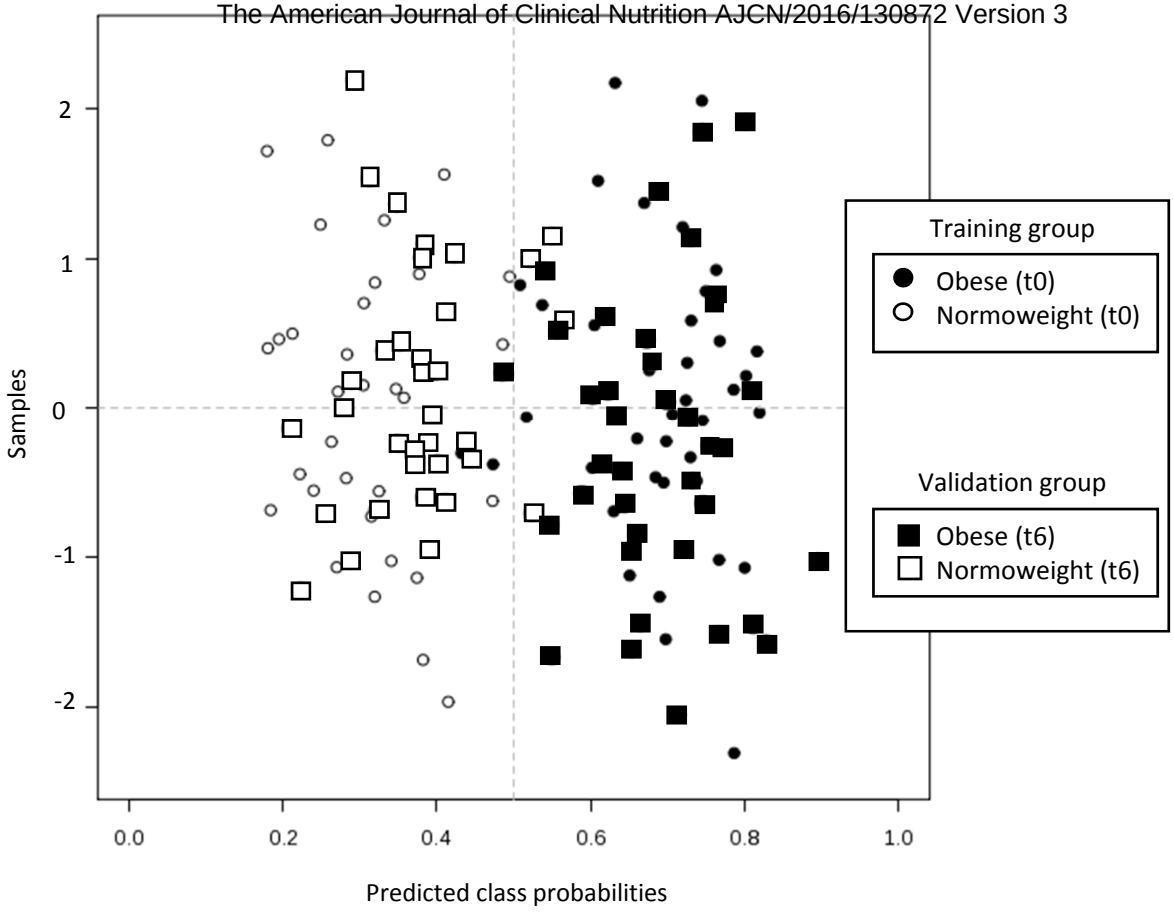
Figure 4.

Changes in the profile of secreted lysophospholipids induced by steatosis in HepG2 cells. Cells were cultured with vehicle or the indicated doses of palmitate during 24 hours. (A) Intracellular accumulation of neutral lipids was evaluated by Oil Red O (ORO) staining. (B) Spectrophotometric quantification after ORO elution from cells. (C) Cell viability was determined by incubating cells with methyl-thiazolyl-tetrazolium (MTT) after 24 hours of treatment with vehicle or palmitate at different doses. (D) Reduced MTT was eluted, quantified spectrophotometrically and cell viability was determined as % of the vehicle signal. (E) The concentrations of 17

lysophospholipids detected in media of vehicle or palmitate treated HepG2 cells were subjected to a principal component analysis and scores of principal components 1,2 and 3 are represented for each replicate. (F) Clustering trends of the different experimental groups were further confirmed by hierarchical clustering analysis. Data in columns are mean ± SEM of two independent experiments run in triplicate. ** $p<0.01$ respect the vehicle group by Student's T test.


A
Normoweight
Obese
PC2
PC1
PC3
B
N-FO
N-CO
O-FO
O-CO
PC2
PC1
PC3
C
Normoweight
Obese
Component 2
Component 1
D
Sensitivity (true positive rate)
1-Specificity (false positive rate)
Var. AUC CI
2 0.963 0.873, 1
3 0.972 0.879, 1
5 0.981 0.892, 1
10 0.991 0.966, 1
20 0.988 0.959, 1
26 0.996 0.982, 1

A


Samples
2
1
0
-1
-2
0.0
0.2
0.4
0.6
0.8
1.0
Predicted class probabilities
Training group
Obese (t0)
Normoweight (t0)
Validation group
Obese (t6)
Normoweight (t6)

B

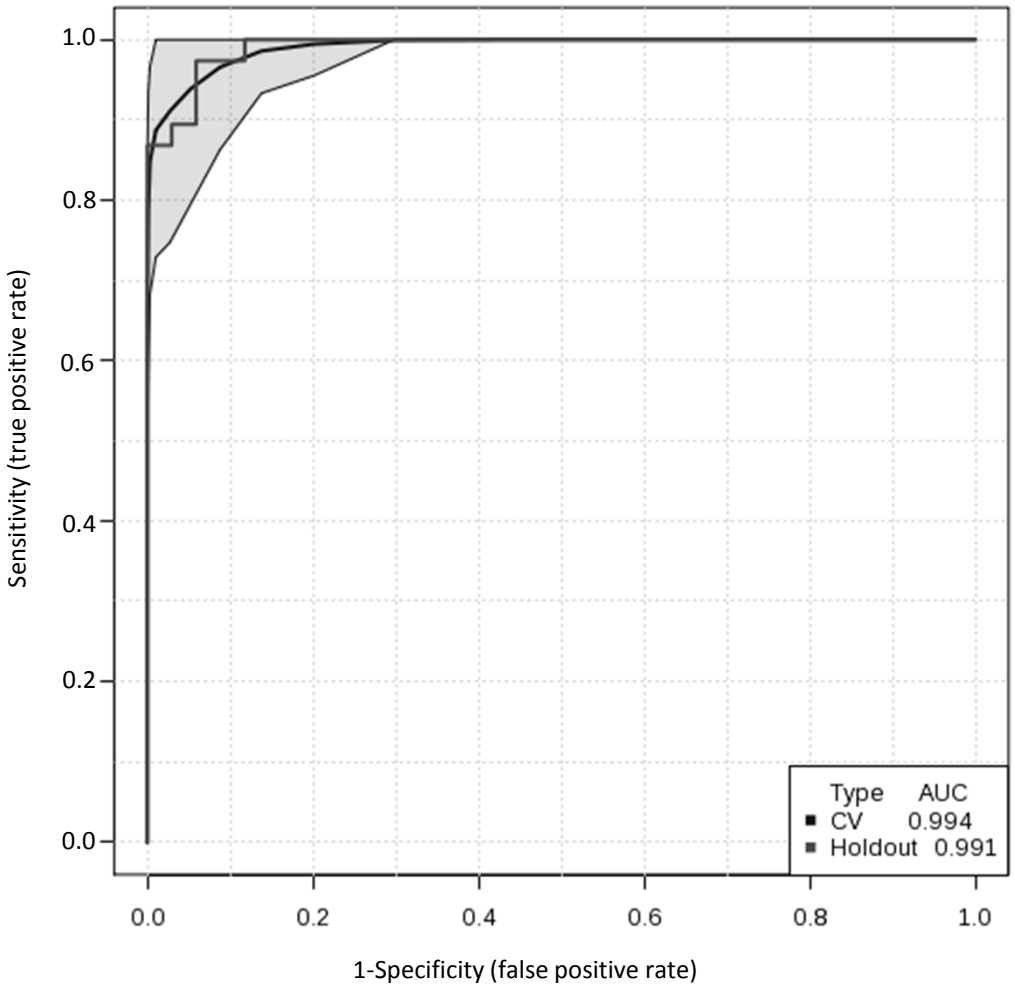
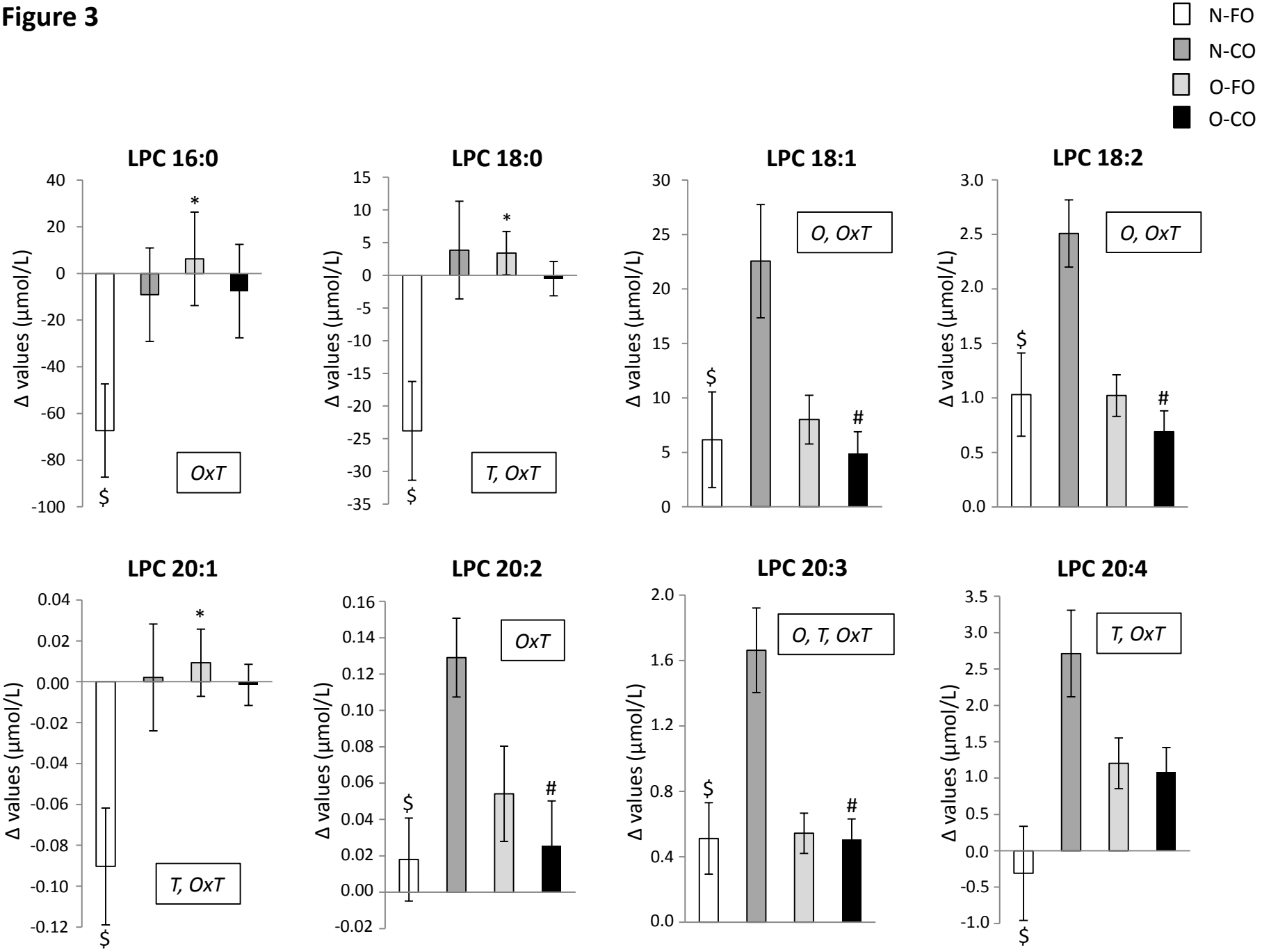
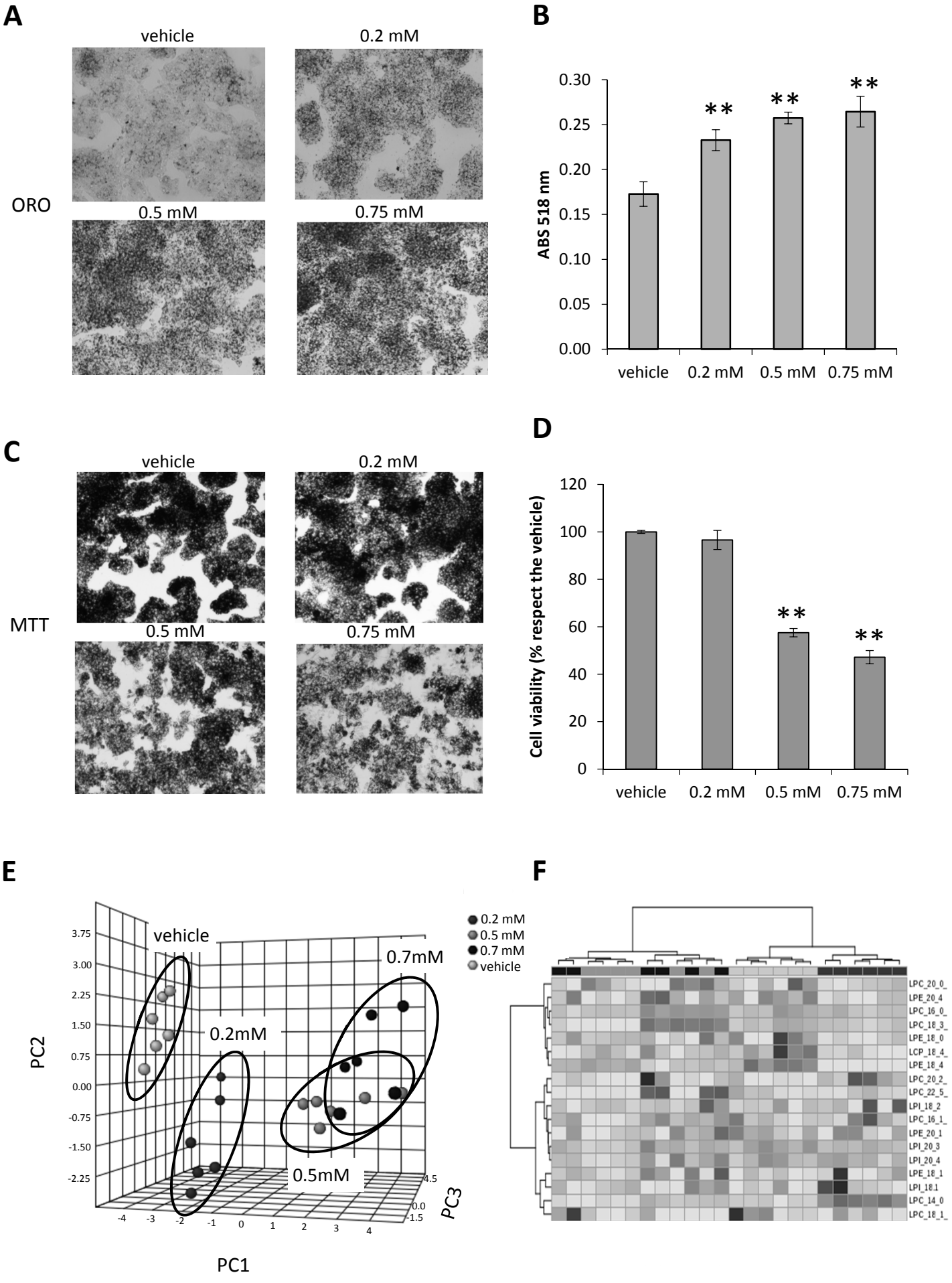

Sensitivity (true positive rate)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1-Specificity (false positive rate)
Type AUC
CV 0.994
Holdout 0.991

Figure 3


N-FO
N-CO
O-FO
O-CO
LPC 16:0
Δ values (µmol/L)
OxT
LPC 18:0
T, OxT
LPC 18:1
O, OxT
LPC 18:2
O, OxT
LPC 20:1
T, OxT
LPC 20:2
OxT
LPC 20:3
O, T, OxT
LPC 20:4
T, OxT


A
vehicle
0.2 mM
ORO
0.5 mM
0.75 mM
B
ABS 518 nm
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
**
**
vehicle
0.2 mM
0.5 mM
0.75 mM
C
vehicle
0.2 mM
MTT
0.5 mM
0.75 mM
D
Cell viability (% respect the vehicle)
120
100
80
60
40
20
0
**
**
vehicle
0.2 mM
0.5 mM
0.75 mM
E
vehicle
0.7mM
0.2mM
0.5mM
PC2
PC1
PC3
3.75
3.00
2.25
1.50
0.75
0.00
-0.75
-1.50
-2.25
-4
-3
-2
-1
0
1
2
3
4
4.5
0.0
-1.5
F
0.2 mM
0.5 mM
0.7 mM
vehicle
LPC_20_0_
LPE_20_4
LPC_16_0_
LPC_18_3_
LPE_18_0
LCP_18_4_
LPE_18_4
LPC_20_2_
LPC_22_5_
LPI_18_2
LPC_16_1_
LPE_20_1
LPI_20_3
LPI_20_4
LPE_18_1
LPI_18.1
LPC_14_0
LPC_18_1_