



Original article

Serum lysophospholipidome of dietary origin as a suitable susceptibility/risk biomarker of human hypercholesterolemia: A cross-sectional study



Lorena Calderón-Pérez ^{a, b, 1}, Susana Suárez-García ^{c, 1}, Anna Pedret ^{a, b, *}, Manuel Suárez ^{c, **}, Elisabet Llauroadó ^b, Laura Rubió ^d, Josep M. del Bas ^a, Antoni Caimari ^a, Francesc Puiggrós ^a, Lluís Arola ^c, Rosa Solà ^{b, e, 2}, Rosa M. Valls ^{a, b, 2}

^a Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain

^b Universitat Rovira i Virgili, Facultat de Medicina i Ciències de la salut, Functional Nutrition, Oxidation, and Cardiovascular Diseases Group (NFOC-Salut), Reus, Spain

^c Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Nutrigenomics Research Group, Spain

^d Food Technology Department, XaRTA-TPV, Agrotecnio Center, Escola Tècnica Superior d'Enginyeria Agrària, University of Lleida, Lleida, Catalonia, Spain

^e Hospital Universitari Sant Joan de Reus, Reus, Spain

ARTICLE INFO

Article history:

Received 14 September 2021

Accepted 29 November 2021

Keywords:

Lysophospholipids
Lysophosphatidylcholines
Lysophosphatidylethanolamines
Trimethylamine-N-Oxide
Hypercholesterolemia
Polyunsaturated fatty acids

SUMMARY

Background & aims: Whether bioactive lysophospholipids (lyso-PLs) and trimethylamine-N-oxide (TMAO) serve as non-invasive biomarkers in early human hypercholesterolemia (HC) is unknown. This study aimed to assess whether serum lyso-PLs and plasma TMAO may be suitable susceptibility/risk biomarkers of HC in humans. Secondly, we aimed to evaluate the relationships between targeted metabolites, diet composition and circulating liver transaminases, and verify these results in hamsters. **Methods:** A targeted metabolomics and lipidomics approach determined plasma TMAO and serum lysophosphatidylcholines (lyso-PCs) and lysophosphatidylethanolamines (lyso-PEs) in low (L-LDL-c) and moderate to high (MH-LDL-c) LDL-cholesterol subjects. Additionally, the relationships between targeted metabolites, liver transaminases and diet, particularly fatty acid intake, were tested. In parallel, plasma and liver lyso-PL profiles were studied in 16 hamsters fed a moderate high-fat (HFD) or low-fat (LFD) diet for 30 days.

Results: Predictive models identified lyso-PC15:0 and lyso-PE18:2 as the most discriminant lyso-PLs among groups. In MH-LDL-c (n = 48), LDL-cholesterol and saturated FAs were positively associated with lyso-PC15:0, whereas in L-LDL-c (n = 70), LDL-cholesterol and polyunsaturated fatty acids (PUFAs) were negatively and positively related to lyso-PE18:2, respectively. Interestingly, in MH-LDL-c, the lower lyso-PE 18:2 concentrations were indicative of higher LDL-cholesterol levels. Intrahepatic accumulation of lyso-PLs-containing essential n-6 PUFAs, including lyso-PE18:2, were higher in HFD-fed hamsters than LFD-fed hamsters.

Conclusions: Overall, results revealed a possible hepatic adaptive mechanism to counteract diet-induced steatosis in animal and hypercholesterolemia progression in humans. In particular, low serum lyso-PE18:2 suggests a suitable susceptibility/risk biomarker of HC in humans.

© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

* Corresponding author. Facultat de Medicina, Universitat Rovira i Virgili, C/ Sant Llorenç, 21, 43201, Reus, Spain.

** Corresponding author. Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, C/ Marcel·lí Domingo, 1, 43007, Tarragona, Spain.

E-mail addresses: anna.pedret@urv.cat (A. Pedret), manuel.suarez@urv.cat (M. Suárez).

¹ LC-P and SS-G contributed equally to the study.

² Senior authors.

Abbreviations list

ALA	Alpha-linolenic acid
ALT	Alanine transaminase
AST	Aspartate transaminase
DBP	Diastolic blood pressure
DEI	Daily energy intake
EL	Endothelial lipase
FDR	False discovery rates
GGT	Gamma-glutamyl transferase
HC	Hypercholesterolemia
HFD	High-fat diet
LA	Linoleic acid
L-LDL-c	Low LDL-cholesterol

LFD	Low-fat diet
Lyso-PCs	Lysophosphatidylcholines
Lyso-PEs	Lysophosphatidylethanolamines
Lyso-PLS	Lysophospholipids
MH-LDL-c	Moderate to high LDL-cholesterol
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
PUFAs	Polyunsaturated fatty acids
ROC	Receiver operating characteristic
SFAs	Saturated fatty acids
SBP	Systolic blood pressure
TC	Total cholesterol
TG	Triglycerides
TMAO	Trimethylamine N-oxide

1. Introduction

Hypercholesterolemia (HC), an imbalanced and pathologic state of cholesterol homeostasis, is a major risk factor for cardiovascular disease (CVD) [1]. Severe primary HC alone is characterized by an elevation of low density lipoprotein (LDL)-cholesterol > 190 mg/dL, with normal values of triglycerides (TG) [1]. Elevated plasma LDL-cholesterol levels have been a primary target of therapy for many years to reduce the risk of adverse cardiovascular events [2]. Thus, despite the great efforts and health plans that have been designed to improve the early detection and management of HC subjects, the population remains underdiagnosed in most cases, which translate into late detection and therefore prescription of lipid-lowering agents [3].

Cholesterol metabolism in humans is complex, and several pathways have been identified for the net flow of cholesterol particles through major tissue compartments [4]. Many exogenous and endogenous metabolites act as intermediates in cholesterol metabolism, which suggests the applicability of integrative omics approaches such as nuclear magnetic resonance (NMR)-based metabolomics and lipidomics for profiling novel early metabolic biomarkers [5,6].

Among various cellular metabolites, lysophospholipids (lyso-PLs), which are present in most cellular membranes, constitute a diverse group of bioactive molecules involved in a broad range of physiological and pathological processes in humans [7]. Lyso-PLs activities are primarily mediated by specific G protein-coupled receptors (GPCRs), implicating them in the aetiology of a growing number of disorders, such as inflammation, reproduction, angiogenesis, carcinogenesis, atherosclerosis and obesity [7]. Many lines of evidence have revealed a mechanistic link between both lyso-PLs and TMAO levels and CVD risk [8,9] and the incidence of major adverse cardiovascular events, such as myocardial infarction, stroke, and even death [10]. Lysophosphatidylcholines (lyso-PCs), the most abundant lyso-PLs in human blood have been suggested to promote atherosclerosis by altering the functions of a number of cells eliciting an inflammatory response [11]. However, controversy exists surrounding lyso-PC pro- or anti-atherogenic/inflammatory properties [11,12].

Additionally, lyso-PL simple structures are primarily composed of a hydrophobic tail of fatty acid residue and the hydrophilic head group, as well as the type of fatty acyl chain, that largely determine their health effects [12]. Group 1b phospholipase A₂ (PLA2g1b) is the major enzyme responsible for phospholipid hydrolysis in the intestinal lumen, yielding lyso-PLs and free fatty acids that are

absorbed into enterocytes [13]. Concurrently, free omega 3 (n-3) PUFAs from diet may be able to bind to lyso-PLs, altering their fatty acyl composition, as previously suggested in C57BL/6 mice fed n-3 PUFA-enriched diets [14]. In the intestine, hydrolysed lyso-PLs become a substrate for trimethylamine (TMA) synthesis [13], a direct intestinal precursor of trimethylamine N-oxide (TMAO) [15]. Several gut bacteria belonging to the Firmicutes, Actinobacteria and Proteobacteria phyla, can form TMA through several enzyme complexes involved in anaerobic choline and L-carnitine metabolism [16]. Outside the intestinal tract, TMA is further oxidized to TMAO in the host liver by flavin monooxygenase enzymes, primarily FMO₃ [9].

Dietary factors are important determinants of circulating TMAO and lyso-PL levels. In this sense, Western-like diets, which are characterized by high intake of animal protein and saturated fat and low fibre, have been related with increased plasma TMAO levels in mice [17]. Similarly, a HFD enriched in phosphatidylcholine has demonstrated to increase TMAO levels in plasma and to induce dyslipidemia in rats [18]. On the other hand, in vivo studies have identified altered serum lyso-PLs levels in dyslipidemic hamsters after HFD administration, pointing out the role of lyso-PLs in the management of lipid disorders [19]. Nevertheless, the evidence on the potential relationships between dietary components and lysophospholipidome is scarce in humans. Thus, early identification of novel bioactive molecules is required to predict HC susceptibility and guide preventive strategies in clinical practice.

To address these issues, we performed a cross sectional study in which we carried out targeted metabolomics and lipidomics with special emphasis on identifying circulating metabolites and bioactive lyso-PLs of HC susceptibility. We hypothesize was that particular serum lyso-PLs and plasma TMAO concentrations could be precursors of HC progression because their rise occurs prior to LDL-cholesterol increase. Therefore, the overall goal of this study was to assess whether serum lyso-PLs and plasma TMAO may be suitable susceptibility/risk biomarkers of HC progression in humans. Furthermore, because these metabolites depend highly on diet and dietary animal-based foods, we also aimed to evaluate potential associations with diet composition, with special emphasis on fatty acid intake. Finally, considering that the liver is highly involved in extracellular lyso-PL level regulation by modifying the expression of several enzymes [19], to extend our knowledge on possible hepatic mechanisms involved in lyso-PL metabolism, we examined relationships with serum liver transaminases. In addition, the results were verified in a second *in vivo* study in hamsters with HC induced by chronic intake of a HFD.

2. Materials and methods

2.1. Subjects and study design

A cross sectional study involving male and female subjects undergoing blood testing was performed. All subjects were recruited from March to October 2014 at the *Hospital Universitari Sant Joan de Reus* (HUSJ, Spain) and Eurecat, Reus. Based on the 2019 ESC/EAS Guidelines for the Management of Dyslipidaemias [20], subjects were divided into two groups with low (≤ 115 mg/dL) and moderate to high (116–190 mg/dL) serum LDL-cholesterol, as shown in [Supplemental Fig. 1](#). Subjects were eligible for enrolment in the study if they were over 18 and provided written informed consent, and were excluded if they met any of the following criteria: were diagnosed with any chronic metabolic disorder; had triglycerides levels > 150 mg/dL; had a body mass index (BMI) > 30 kg/m²; used prescribed lipid-lowering treatment or other nutraceutical; had altered renal function; suffering anaemia (hemoglobin ≤ 13 mg/dL in men and ≤ 12 mg/dL in women); suffering chronic gastrointestinal, respiratory or hepatic disorders; were pregnant or breastfeeding; were smoking; or were participating in another study.

The protocol was conducted in accordance to the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (ICH GCP) and was approved by the Ethical Committee of Clinical Research of the HUSJ (with 09-12-17/proj1 reference).

2.2. Clinical and lifestyle data collection

All clinical information was collected according to standard procedures at two different visits performed at the HUSJ and Eurecat, Reus. In the first pre-screening visit, after indicating informed consent, a clinical interview was used to verify that participants met all the eligibility criteria, and a routine blood test was collected under fasting conditions. In addition, clinical information related to a history of disease and use of medications or supplements was obtained, and a physical examination was performed, including blood pressure and anthropometric parameters, such as weight, height, BMI, and waist circumference measures. Accordingly, the following procedures were applied:

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were monitored with participants in a seated position after resting for 2–5 min using a multiple automated sphygmomanometer (OMRON HEM-907; Peroxфарма, Barcelona, Spain). Anthropometric parameters including weight and body composition were measured with a body composition analyser (Tanita SC 330-S; Tanita Corp., Barcelona, Spain). Waist circumference was measured at the umbilicus level using a 150 cm anthropometric steel measuring tape.

After the first pre-screening visit, participants who met all the eligibility criteria were included in the study and scheduled for a second visit in one week. At the second visit, blood pressure and anthropometrical parameters were measured again, and a fasting blood sample was collected for biochemical analysis. No subject was using antibiotics, probiotics or diuretics that could alter the levels of circulating metabolites at the time of inclusion. In addition, lifestyle information including dietary habits and physical activity, was recorded.

Data on diet composition and mean daily energy and nutrients intake were assessed through a 3-day dietary record (2 labour and 1 week-end consecutive days) and calculated by Spanish Food Composition Tables (CESNID) [21]. The percentage (%) of daily energy intake (% DEI) from primary macronutrients (proteins, carbohydrates and fatty acids) was calculated by dividing the calories derived from each by the total calories consumed. In addition, from

the 3-day dietary record, the intake of well-known choline animal-based foods, according to the US Department of Agriculture (USDA) Database for the Choline Content of Common Foods [22], was grouped as follows: red meat, white meat, processed meat, fatty fish, lean fish, shellfish, whole milk, low-fat milk, skim milk, yogurt, cheese, butter and eggs. These foods provided between 250 and 14 mg total choline/100g, with eggs being the largest contributors and dairy products, particularly yogurt, the lowest.

Physical activity was evaluated by completion of the “Physical Activity Questionnaire Class AF” validated questionnaire [23]. There were no subject withdrawals between the first and second visits; thus, data were available for all subjects.

2.3. Determination of biochemical parameters

Blood samples were taken under over-night fasting conditions. Briefly, total cholesterol (TC), LDL-cholesterol, HDL-cholesterol, TG, and glucose and the hepatic enzymes aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transferase (GGT) concentrations were measured in serum by standardized enzymatic automated methods in an autoanalyzer (Beckman Coulter-Synchron, Galway, Ireland).

2.4. Targeted metabolomics and lipidomics analysis of plasma TMAO and serum lysophospholipids

Quantification of TMAO in plasma samples was performed as described previously [24]. Briefly, for sample extraction, 25 μ L of plasma was mixed with 80 μ L of methanol with labelled IS working solution (TMAO-d9; Cambridge Isotope Laboratories, Massachusetts, USA) and mixed 30 s to precipitate proteins. The samples were centrifuged at 9000 rpm for 5 min at room temperature, and the supernatants were diluted with 150 μ L of Milli-Q water. Diluted samples were filtered with PVDF filters 0.22 μ m and transferred into HPLC vials for analysis. The analysis was performed by liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) (Waters, Milford, MA, USA) using a column Acquity UPLC BEH HILIC (1.7 μ m 2.1 $\times$ 100 mm).

Lyso-PLs content was determined in a subsample of 24 L-LDL-c and 26 MH-LDL-c subjects through targeted lipidomic analysis. A previously reported methodology [25] based on protein precipitation with cold methanol assisted by ultrasounds was used. The quantitative lyso-PLs evaluation and analysis is detailed in [Supplemental Information 1](#).

2.5. In vivo study to validate circulating lysophospholipid suitability as susceptibility/risk biomarkers and to inquire into hepatic mechanisms

2.5.1. Animals and diets

A second exploratory in vivo study was performed using 16 male Golden Syrian hamsters (Charles River Laboratories, Barcelona, Spain) fed a moderate HFD (21% energy as fat) or LFD (10% energy as fat). Golden Syrian hamsters were used because they display the greatest similarity to humans with regard to lipoprotein metabolism [26]. The animal protocol was approved by the Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain), and all of procedures have been performed in accordance with the European Communities Council Directive (86/609/EEC). The detailed procedure for sample extraction is described in [Supplemental Information 2](#). Briefly, after 2 weeks of adaptation to a LFD, the animals were randomly distributed into two groups with comparable body weights ($n = 8$ per group) and fed either the LFD or a HFD *ad libitum* for 30 days (the composition of the diets is shown in [Supplemental Information 3](#)). On day 30, blood samples

were withdrawn by cardiac puncture, and the liver was perfused, patted dry and then weighed for the determination of the relative weight. White adipose tissue was also excised for determination of the relative weight. All samples were snap frozen in liquid nitrogen and then stored at -80°C until further analyses.

Targeted lipidomics was used to quantify lyso-PL levels in hamster liver and plasma following the same methodology described above.

2.5.2. Liver histology

The detailed procedure on histological examination is described in [Supplemental Information 2](#). Briefly, frozen liver tissue samples were thawed and fixed by immersion in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin at 52°C . Then, paraffin blocks were sectioned and prepared in slides for automated staining. Morphometric evaluation was conducted by expert pathologists to assess the degree of hepatic steatosis in the biopsies [27]. The steatosis grade was determined estimating the percentage of hepatocytes containing lipid droplets: absent – score 0, $<5\%$; mild – score 1, $5\text{--}33\%$; moderate – score 2, $>33\text{--}66\%$; severe – score 3, $>66\%$ [27]. The diagnosis also included determination of the cytoplasmic configuration (microvesicular and macrovesicular steatotic patterns), tissue distribution and inflammation extent.

2.6. Statistical analysis

Data on clinical, biochemical and dietary parameters in humans are expressed as means $\pm$ SDs, and the normality of variables was assessed using the Kolmogorov–Smirnov test. The differences among groups were assessed using the Mann–Whitney test and the Student's *t*-test for comparison of non-normally and normally distributed variables, respectively. Fisher's exact test was used for categorical variables comparisons. Correlations between circulating lyso-PLs, TMAO, and clinical and dietary variables in humans were evaluated using Spearman's correlation coefficients (*r*) adjusting for the age, sex, BMI and energy intake confounders. To manage false-positive correlations, multiple testing using the Benjamini–Hochberg correction was used, and adjusted *p* values were set [28]. Stepwise multiple linear regression analysis was then used to predict the strength of the associations, setting clinical and dietary variables as predictors, and TMAO and discriminant lyso-PLs as response variables. In hamsters, the results of plasma lipid markers, plasma lyso-PL concentrations and liver histology are expressed as means $\pm$ SEM from the indicated number of hamsters ($n = 8$) in each group. Correlations between plasma and liver lyso-PL levels in hamsters were evaluated using Pearson's correlation coefficient (*r*). A two-tailed value of $p < 0.05$ was considered statistically significant for all human and animal tests. Univariate statistical analysis was performed with Statistical Package for Social Sciences (IBM SPSS Statistics, version 25.0).

Lyso-PL content was also comprehensively examined in both the MH-LDL-c and L-LDL-c groups using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The variable importance in projection (VIP) score from the PLS-DA model was calculated to estimate the importance of lyso-PLs and their contribution to the overall differentiation between groups. The predictive models were further validated by conducting receiver operating characteristic (ROC) curves. The optimal combination of the most important identified lyso-PLs predictive of HC was that associated with the highest predictive accuracy (maximum sensitivity and specificity). All multivariate analysis were performed after data normalization and autoscaling with the use of the software MetaboAnalyst (version 4.0) available online.

Sample size was calculated using the GRANMO freeware based on the number of participants required to detect significant differences in LDL-cholesterol levels at baseline in each of the groups. Based on means $\pm$ SDs for serum concentration of LDL-cholesterol from previous studies in the Spanish population [29,30], it was calculated that a minimum of 27 subjects/group would be required.

3. Results

3.1. Subject characteristics

From 138 subjects who were assessed for eligibility, 20 were excluded due to not fulfilling the inclusion criteria or because their informed consent was withdrawn. 118 subjects, 70 L-LDL-c and 48 MH-LDL-c, were enrolled and analysed. The flowchart of the study according to the STROBE statement is depicted in [Supplemental Fig. 1](#).

Baseline characteristics of the study population are illustrated in [Table 1](#). MH-LDL-c subjects were significantly older than L-LDL-c subjects ($p < 0.001$) and had higher SBP, DBP, BMI and waist circumference ($p < 0.05$). Concerning biochemical parameters, the MH-LDL-c group, had higher serum concentrations of TC and TG than the L-LDL-c group ($p < 0.001$). Despite these differences in lipid markers between groups, it is important to highlight that their baseline levels remained within normal values except for LDL-cholesterol (MH-LDL-c, 144.0 ± 19.9 mg/dL; L-LDL-c, 86.1 ± 18.2 mg/dL; $p < 0.001$).

Daily energy and nutrients intake was similar between groups except for complex carbohydrates, the most consumed macronutrient in both groups (MH-LDL-c, 102.57 ± 39.18 g/day; L-LDL-c, 114.51 ± 44.13 g/day). In the L-LDL-c group, complex carbohydrate intake, which is expressed as the percentage of daily energy intake, was significantly higher than that in the MH-LDL-c group ($p = 0.010$). In the MH-LDL-c group, the mean ethanol intake was significantly higher than that in the L-LDL-c group ($p = 0.006$) ([Supplemental Table 1](#)). Reported data from 3-day dietary records showed significantly greater daily mean intake of lean fish ($p = 0.039$), animal protein ($p = 0.023$) and several amino acids ($p < 0.05$) in MH-LDL-c subjects than in L-LDL-c subjects ([Supplemental Table 1](#)). No significant differences were found in other dietary parameters analysed or in physical activity.

3.2. Identification of circulating metabolites involved in hypercholesterolemia susceptibility

After targeted metabolomics analysis, a total of 27 serum lyso-PLs (19 lyso-PCs and 8 lyso-PEs) were identified and plasma TMAO concentrations were assessed ([Table 2](#)). There were no significant differences in TMAO concentrations between groups, while particular lyso-PLs differed significantly among L-LDL-c and MH-LDL-c groups. The most of the identified lyso-PCs and lyso-PEs were highly concentrated in the MH-LDL-c group compared to L-LDL-c group, primarily lyso-PC 15:0 (1.12 ± 1.40 vs. 1.05 ± 0.26 μM , $p < 0.001$). Exceptionally, lyso-PE 18:2 was significantly higher in L-LDL-c and lower in MH-LDL-c (6.08 ± 1.51 vs. 5.32 ± 1.16 μM , $p = 0.05$). Additionally, in L-LDL-c group, lyso-PC 14:0, 16:1, 18:1, 18:2, 18:3 and 20:4, and lyso-PE 18:1 and 20:4, were more concentrated ([Table 2](#)). The projection scores (VIPs) in the first component of the PLS-DA model indicated seven lyso-PLs, with VIPs > 1.0 contributing to the overall differentiation between groups ([Table 2](#)). In particular, lyso-PC 15:0 and lyso-PE 18:2 showed the highest scores (2.74 and 2.24, respectively). Consistent with this, when the individual scores were hierarchically clustered in a heatmap, two distinct patterns in the serum lyso-PL profile, characterized by higher lyso-PC 15:0 and lower lyso-PE 18:2 in the MH-LDL-c group, and lower

Table 1
Baseline characteristics of study population.

Variables	Low LDL-cholesterol	Moderate to high LDL-cholesterol	P-value
N	70	48	
Age, y	35.4 ± 10.3	46.6 ± 11.9	<0.001
Gender, (F/M)	(36/34)	(25/23)	0.547
Anthropometry			
Weight, kg	65.0 ± 10.4	66.8 ± 11.3	0.380
BMI, kg/m ²	22.4 ± 2.5	24.0 ± 2.8	0.002
Waist circumference, cm	79.1 ± 8.7	83.4 ± 8.8	0.010
Blood chemistry (mg/dL)			
Total cholesterol	166.1 ± 24.1	227.3 ± 25.6	<0.001
LDL-cholesterol	86.1 ± 18.2	144.0 ± 19.9	<0.001
HDL-cholesterol	66.4 ± 15.0	64.8 ± 15.1	0.568
Triglycerides	67.5 ± 26.1	88.0 ± 27.7	<0.001
FBG	84.0 ± 8.9	86.7 ± 9.4	0.125
Blood pressure (mm Hg)			
Systolic BP	114.7 ± 14.3	120.4 ± 15.2	0.038
Diastolic BP	67.9 ± 9.6	72.4 ± 9.3	0.017

Data expressed as mean ± SD. P-value for gender was calculated by Fisher's exact test. P-value for the rest of the variables was calculated by Student's t-test and Mann–Whitney U test. Abbreviations: BMI, body mass index; BP, blood pressure; FBG, fasting blood glucose; HDL, high density lipoproteins; LDL, low density lipoproteins.

Table 2
Mean concentrations (μM) and variable importance in projection (VIP) score of serum lysophospholipid and plasma TMAO in low and moderate to high LDL-cholesterol groups.

Metabolite	Low LDL-cholesterol	Moderate to high LDL-cholesterol	Metabolite VIP score
Lyso-PC^a			
14:0	2.19 ± 0.83	2.18 ± 0.68	0.78
15:0	1.05 ± 0.26	1.12 ± 0.22**	2.74
16:0	63.46 ± 4.87	64.10 ± 3.59	0.09
16:1	4.28 ± 1.44	4.14 ± 0.67	0.65
17:0	4.06 ± 1.03	4.51 ± 0.97	1.40
17:1	4.83 ± 0.31	4.74 ± 0.34	0.44
18:0	60.98 ± 10.44	67.02 ± 11.15*	0.12
18:1	66.06 ± 12.55	65.49 ± 12.70	1.55
18:2	94.84 ± 13.17	89.38 ± 11.67	1.04
18:3	1.84 ± 0.71	1.75 ± 0.69	0.51
20:0	1.12 ± 0.26	1.34 ± 0.34*	0.36
20:1	2.66 ± 0.66	2.79 ± 0.71	0.30
20:2	4.41 ± 0.92	4.58 ± 0.99	0.94
20:3	25.06 ± 9.04	25.27 ± 7.66	0.31
20:4	90.21 ± 19.24	88.27 ± 17.76	1.15
20:5	10.62 ± 7.67	12.67 ± 11.66	0.74
22:4	1.95 ± 0.67	1.79 ± 0.46	0.03
22:5	5.81 ± 1.80	5.90 ± 1.73	0.68
22:6	26.77 ± 6.50	30.56 ± 10.25	0.66
Lyso-PE^a			
16:0	2.89 ± 0.75	3.00 ± 0.64	0.78
16:1	0.27 ± 0.07	0.25 ± 0.05	0.29
18:0	2.41 ± 0.63	2.67 ± 0.49	0.96
18:1	2.64 ± 0.84	2.41 ± 0.73	0.74
18:2	6.08 ± 1.51*	5.32 ± 1.16	2.24
20:4	4.20 ± 0.99	4.09 ± 0.92	0.24
20:5	0.39 ± 0.16	0.41 ± 0.20	0.08
22:6	2.53 ± 0.56	2.83 ± 0.77	1.07
TMAO^b			
	5.83 ± 4.03	6.25 ± 5.12	0.77

Metabolite concentrations expressed as mean ± SD. VIP scores of the first component of the PLS-DA model, VIP score >1.0 depicted in **bold**.

Significance for differences in metabolite concentrations between groups was measured using independent Student's t-test: * $p \leq 0.05$; ** $p < 0.001$. Abbreviations: lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; TMAO, trimethylamine N-oxide.

^a Lysophospholipids available in a sub-group of $n = 50$ subjects.

^b TMAO available in $n = 107$ subjects.

lyso-PC 15:0 and higher lyso-PE 18:2 in the L-LDL-c group, were evidenced (Fig. 1-A).

A PCA model was built including the seven most discriminant lyso-PLs to further test the independent predictive capability of the identified metabolites. As the three-dimensional score plot showed, it was possible to distinguish a wide separation between the MH-LDL-c and L-LDL-c groups (Fig. 1-B). Thus, 62.4% of the variance was explained when the scores of the first three principal components were represented. The PLS-DA model upgraded the differentiation between groups (Fig. 1-C), with lyso-PC 15:0 and lyso-PE 18:2 being the major contributors to the separation. The model reported a good fit of the data ($R^2Y = 0.68$) and an optimal quality assessment statistic ($Q^2 = 0.54$), over the threshold of 0.4 stipulated for biological models [31]. In addition, lyso-PC 15:0 and lyso-PE 18:2 explained 59.3% and 40.7% of the variance when the scores of the two principal components were computed (Fig. 1-D). ROC curves using lyso-PC 15:0 and lyso-PE 18:2 achieved optimal predictive power with an accuracy of 70.4%, and an AUC of 0.777 (95% CI 0.65–0.932) (Fig. 1-E). These models confirmed that two particular lyso-PLs, lyso-PC 15:0 and lyso-PE 18:2, indicated good clinical accuracy and could be suitable susceptibility/risk biomarkers for HC in preliminary stages of the disease.

3.3. Associations of circulating lysophospholipids with TMAO

A correlation study with the 27 serum lyso-PLs identified in the MH-LDL-c and L-LDL-c groups was performed to investigate the contribution of lyso-PLs to TMAO concentrations. As shown in Supplemental Fig. 2, after the multiple linear regression analysis lyso-PC 20:5 and lyso-PE 20:5 were positively associated with TMAO concentrations in MH-LDL-c subjects ($\beta = 0.216$ and $\beta = 0.562$, respectively) ($p < 0.05$), while in L-LDL-c subjects, lyso-PC 14:0 showed a positive contribution to TMAO concentrations ($\beta = 0.382$, $p < 0.05$). These positive associations indicated that particular polyunsaturated and saturated chain lyso-PLs could be precursors for TMA and TMAO synthesis in HC progression.

3.4. Associations of discriminant lysophospholipids and TMAO with classical cardiovascular risk factors and dietary components

3.4.1. Associations with lipid-related markers

No significant correlations were found in either group between serum lipid markers and TMAO concentrations. However, positive correlations were observed for lipid markers with the most discriminant serum lyso-PLs (Table 3). In MH-LDL-c subjects, TC, LDL-cholesterol and the atherogenic ratio LDL-cholesterol/HDL-cholesterol were positively correlated with lyso-PC 15:0 levels ($r > 0.46$, $p < 0.01$ and $p_{\text{adj}} < 0.05$), supporting lyso-PC 15:0 suitability as susceptibility/risk biomarker of HC. In L-LDL-c subjects, an inverse correlation was observed between TC and LDL-cholesterol with lyso-PE 18:2 ($r > -0.30$, $p < 0.05$ and $p_{\text{adj}} = 0.134$).

3.4.2. Associations with dietary components

A correlation study was performed at three different levels: nutrient intake, amino acid intake, and with choline animal-based foods (Supplemental Table 2).

In the MH-LDL-c group, saturated fatty acids (SFAs) were positively associated with lyso-PC 15:0 ($r = 0.52$, $p < 0.001$ and $p_{\text{adj}} = 0.002$) and lyso-PC 17:0 ($r = 0.63$, $p < 0.001$ and $p_{\text{adj}} = 0.002$). In addition, dairy products including yogurt, cheese and whole milk were positively associated with lyso-PC 15:0 ($r > 0.35$, $p < 0.05$ and

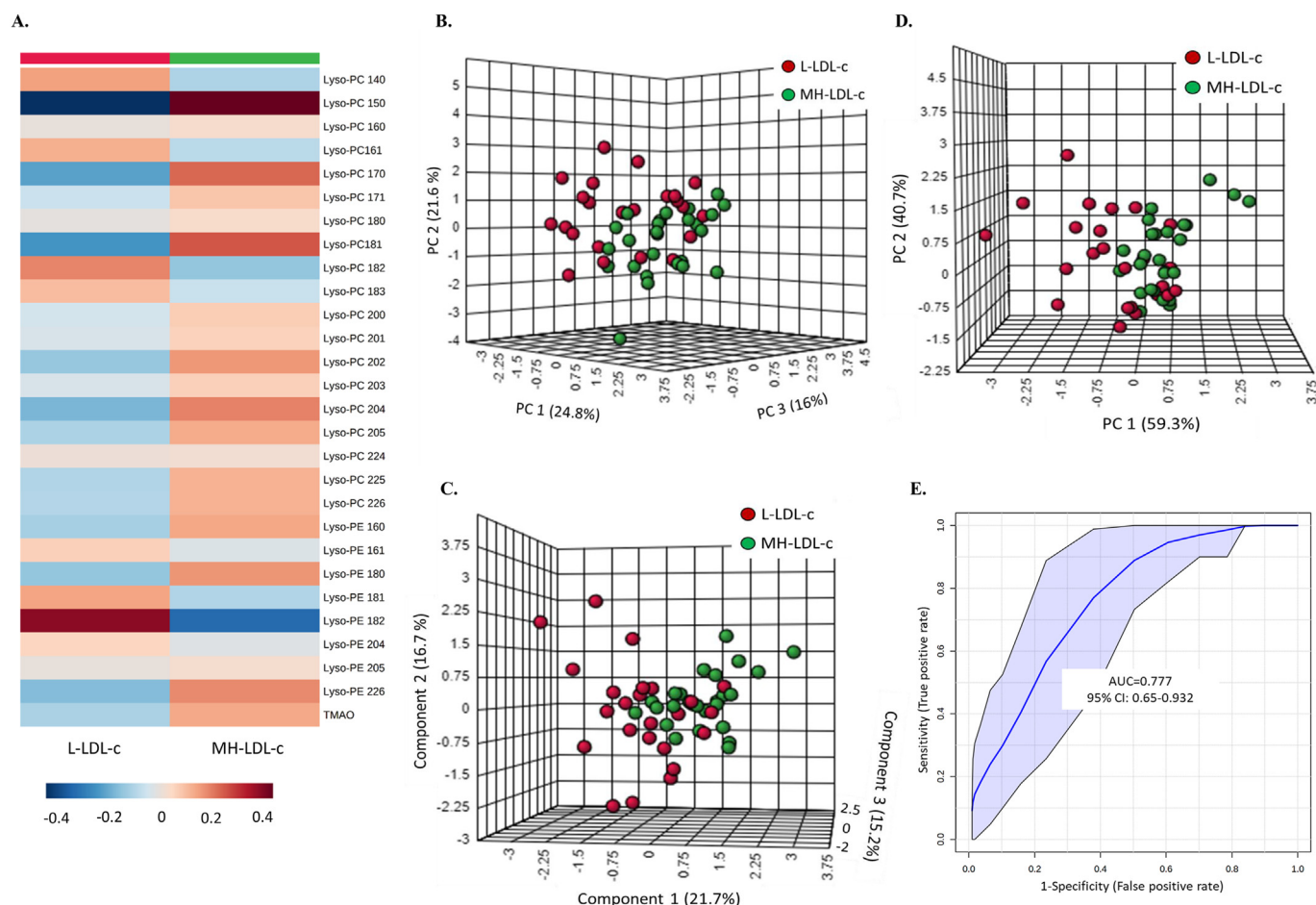


Fig. 1. Multivariate analysis of the circulating lysophospholipid and TMAO levels identified in 24 low LDL-cholesterol and 26 moderate to high LDL-cholesterol subjects. (A) Heatmap plot from hierarchical clustering analysis of lyso-PLs and TMAO levels in low and moderate to high LDL-cholesterol subjects. Each row represents a metabolite colored by its range-scaled abundance intensity. The scale from -0.4 (blue) to +0.4 (red) represents the normalized abundance in arbitrary units. (B) Three-dimensional score plot of the PCA model and (C) PLS-DA predictive model with the use of 7 serum lyso-PLs with the highest variable importance in projection (VIP) scores. (D) Two-dimensional score plot of the PCA model using lyso-PC 15:0 and lyso-PE 18:2. (E) ROC curve analysis of the PLS-DA model using lyso-PC 15:0 and lyso-PE 18:2. The area under the curve (AUC) and corresponding 95% confidence interval (CI) are reported in the inset. PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; ROC, receiver operating characteristic. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3

Spearman correlations between serum lysophospholipid biomarkers, classical lipid-related markers and dietary components in low and moderate to high LDL-cholesterol groups.

Lysophospholipid, μM	Lipid-related marker Dietary component	Spearman correlation index	p.value	Adjusted p. value
Low LDL-cholesterol				
Lyso-PE 18:2	Total cholesterol	-0.309	0.028	0.134
	LDL-cholesterol	-0.318	0.024	0.134
	PUFAs	0.389	0.005	0.033 ^a
Moderate to high LDL-cholesterol				
Lyso-PC 15:0	Total cholesterol	0.462	<0.001	0.003 ^a
	LDL-cholesterol	0.482	<0.001	0.002 ^a
	LDL-cholesterol/HDL-cholesterol ratio	0.543	0.006	0.042 ^a
	SFAs	0.520	<0.001	0.002 ^a
	Glutamic acid	0.432	0.001	0.016 ^a
	Yoghurt	0.532	0.005	0.043 ^a
	Cheese		0.021	0.181
	Whole milk		0.052	0.525

Lysophospholipid available in a sub-group of $n = 50$ subjects. Significant correlations were set at $p < 0.05$.

^a Significant Bonferroni-Hochberg adjusted p value < 0.05. Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; Lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty acid.

p.adj $\leq$ 0.05) and whole milk with lyso-PC 17:0 ($r = 0.35$, $p = 0.058$ and $p.\text{adj} > 0.05$) (Table 3). However, yogurt and whole milk were inversely associated with unsaturated lyso-PCs 20:4 and 20:5 ($r > -0.39$, $p < 0.05$ and $p.\text{adj} > 0.05$) (Supplemental Table 2). Vitamin D and fatty fish were positively associated with lyso-PC 20:5 and lyso-PE 20:5 ($r > 0.33$, $p \leq 0.05$). Concerning the L-LDL-c group, ethanol, total fatty acids, yogurt and cheese were positively correlated with Lyso-PC 14:0 ($r > 0.40$, $p < 0.05$ and $p.\text{adj} < 0.05$). In addition, in L-LDL-c, positive associations were stated between PUFAs and lyso-PE 18:2 ($r = 0.38$, $p = 0.005$ and $p.\text{adj} = 0.033$) (Table 3). Major associations of discriminant lyso-PLs with dietary components are shown in Supplemental Table 3.

A schematic of major correlations between TMAO and lyso-PL biomarkers and all clinical and dietary features in both groups is provided in Supplemental Fig. 3.

3.5. Linking lysophospholipid biomarkers and TMAO with liver transaminases and possible implication for hypercholesterolemia progression

Overall relationships for serum liver transaminases with discriminant lyso-PLs, TMAO and diet are shown in Supplemental Table 4, and significant correlations were included in a multiple linear regression analysis (Supplemental Fig. 4).

Table 4

Lysophospholipid content in plasma and liver of low-fat diet (LFD) or high-fat diet (HFD)-fed hamsters after 30 days.

Metabolite	Plasma (nM)		Liver ($\mu\text{M/g}$)	
	LFD	HFD	LFD	HFD
Lyso-PC				
14:0	35.9 $\pm$ 1.4	30.7 $\pm$ 1.1*	79.8 $\pm$ 9.9	48.1 $\pm$ 1.9*
15:0	38.2 $\pm$ 3.3	29.3 $\pm$ 1.4*	78.4 $\pm$ 7.0	44.4 $\pm$ 2.4**
16:0	5263 $\pm$ 303	5006 $\pm$ 172	9458 $\pm$ 371	8533 $\pm$ 275
16:1	92.2 $\pm$ 5.5	62.3 $\pm$ 2.8***	218.1 $\pm$ 24.1	118.7 $\pm$ 7.6**
17:0	48.8 $\pm$ 5.6	39.1 $\pm$ 2.1*	227.8 $\pm$ 26.3	158.3 $\pm$ 7.3*
17:1	19.2 $\pm$ 1.6	13.6 $\pm$ 0.4*	18.5 $\pm$ 2.5	9.3 $\pm$ 0.9**
18:0	1327 $\pm$ 117	1498 $\pm$ 58	3723 $\pm$ 202	3511 $\pm$ 105
18:1	1470 $\pm$ 120	1365 $\pm$ 46	821.5 $\pm$ 57.4	794.3 $\pm$ 50.2
18:2	1532 $\pm$ 107	1616 $\pm$ 66	364.1 $\pm$ 18.7	533.5 $\pm$ 47.6**
18:3	18.7 $\pm$ 1.0	16.0 $\pm$ 0.4*	65.1 $\pm$ 9.8	75.5 $\pm$ 9.3
20:0	8.7 $\pm$ 0.2	8.9 $\pm$ 0.2	13.9 $\pm$ 0.8	14.4 $\pm$ 0.4
20:1	11.1 $\pm$ 0.2	14.0 $\pm$ 0.5***	13.9 $\pm$ 0.8	30.2 $\pm$ 1.2
20:2	17.1 $\pm$ 0.8	25.5 $\pm$ 1.3	24.3 $\pm$ 1.4	36.6 $\pm$ 1.9***
20:3	44.4 $\pm$ 5.1	33.7 $\pm$ 2.1	56.9 $\pm$ 3.4	139.1 $\pm$ 16.9**
20:4	170.5 $\pm$ 16.1	157.8 $\pm$ 4.9**	78.1 $\pm$ 5.6	161.4 $\pm$ 20.2**
20:5	16.1 $\pm$ 1.3	9.8 $\pm$ 0.3**	5.9 $\pm$ 1.0	6.6 $\pm$ 1.1
22:5	24.4 $\pm$ 2.0	15.6 $\pm$ 0.5**	10.4 $\pm$ 0.8	12.7 $\pm$ 1.4
22:6	66.9 $\pm$ 6.5	41.4 $\pm$ 2.6**	57.7 $\pm$ 4.2	96.2 $\pm$ 11.4**
Lyso-PE				
16:0	73.3 $\pm$ 4.2	61.6 $\pm$ 2.8*	1080 $\pm$ 101	728.8 $\pm$ 44.6**
16:1	7.0 $\pm$ 0.2	6.0 $\pm$ 0.1**	24.3 $\pm$ 2.7	18.4 $\pm$ 3.4
18:0	65.5 $\pm$ 1.6	70.3 $\pm$ 4.2	1671 $\pm$ 104	1347 $\pm$ 57.2*
18:1	34.8 $\pm$ 2.4	35.1 $\pm$ 1.7	666.7 $\pm$ 63.5	646.7 $\pm$ 82.6
18:2	40.4 $\pm$ 2.9	42.0 $\pm$ 2.0	56.5 $\pm$ 5.2	122.6 $\pm$ 14.1***
18:3	4.3 $\pm$ 0.1	4.1 $\pm$ 0.0*	7.0 $\pm$ 0.9	9.9 $\pm$ 1.3
20:1	4.2 $\pm$ 0.1	4.4 $\pm$ 0.1	7.2 $\pm$ 0.4	11.2 $\pm$ 0.5***
20:2	3.7 $\pm$ 0.1	3.9 $\pm$ 0.0	2.2 $\pm$ 0.2	4.1 $\pm$ 0.3***
20:3	5.3 $\pm$ 0.3	4.7 $\pm$ 0.1	3.3 $\pm$ 0.4	9.8 $\pm$ 1.6***
20:4	19.9 $\pm$ 1.4	20.3 $\pm$ 0.6	33.0 $\pm$ 3.6	95.6 $\pm$ 14.2***
22:4	3.7 $\pm$ 0.1	3.7 $\pm$ 0.0	0.4 $\pm$ 0.1	1.6 $\pm$ 0.3**
22:5	6.1 $\pm$ 0.3	4.9 $\pm$ 0.1**	7.5 $\pm$ 1.0	13.0 $\pm$ 1.7*
22:6	14.0 $\pm$ 1.8	9.1 $\pm$ 0.4*	24.4 $\pm$ 3.4	37.3 $\pm$ 4.2*

Hamsters were fed ad libitum either LFD or HFD during 30 days. Samples were obtained on the last day of dietary intervention after a 6 h fast. For each animal group, the data are presented as the mean $\pm$ SEM ($n = 8$). Significance regarding the LFD-fed group (control animals) was measured using independent Student's t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Abbreviations: Lyso-PC, lysophosphatidylcholine; Lyso-PE, lysophosphatidylethanolamine.

MH-LDL-c group showed significantly higher concentrations of AST, ALT and GGT than the L-LDL-c group ($p = 0.013$, $p < 0.001$ and $p = 0.009$, respectively) (Supplemental Fig. 4-A). When correlations were investigated regardless of the group ($n = 118$), TMAO and ethanol intake were positively correlated with GGT levels (Supplemental Table 4). In the L-LDL-c group, Lyso-PC 14:0 was positively associated with GGT levels in L-LDL-c group ($\beta = 0.574$, $p = 0.003$) (Supplemental Fig. 4-B). In addition, a significant positive association was noted between TMAO and ethanol intake with GGT (Supplemental Table 4). In the MH-LDL-c group, lyso-PE 18:2 correlated positively with GGT levels ($\beta = 0.495$, $p = 0.010$) (Supplemental Fig. 4-C). Additionally, a positive association was observed between PUFA intake and GGT ($\beta = 0.630$, $p < 0.001$) (Supplemental Fig. 4-D).

3.6. Validation of the suitability of lysophospholipids as biomarkers involved in hypercholesterolemia progression in HFD-fed hamsters

3.6.1. Changes in circulating lipid markers and identification of plasma and liver lysophospholipids altered after the chronic intake of HFD

After 30 days of chronic treatment with HFD, lipid parameters and traditional indicators of liver impairment were determined in the animal's plasma (Supplemental Table 5). Hamsters fed the HFD exhibited significantly higher amount of total cholesterol than LFD-fed animals ($p = 0.011$), which resulted in a marginal increase (of 13%) of the atherogenic index TC/HDL-cholesterol ($p = 0.046$). An increasing trend of 30% LDL-cholesterol levels was observed in the HFD-fed group compared to the LFD-fed group ($p = 0.008$). No effects were observed on the transaminase levels.

Lyso-PC and lyso-PE levels were markedly modified in liver and plasma after chronic HFD intake (Table 4). A generalized drop (nearly 50%) in the circulating levels of lyso-PLs was noted in HFD-fed animals, which was accompanied by an intrahepatic accumulation of lyso forms, particularly polyunsaturated acyl chain lyso-PEs 18:2 ($p < 0.001$), 22:5 and 22:6 ($p < 0.05$). Conversely, saturated acyl chain lyso-PC 14:0, 15:0 and 17:0, and lyso-PE 16:0, were significantly reduced in both plasma and liver ($p < 0.05$).

An additional correlation analysis was performed between the 31 lyso-PLs identified in plasma and liver of hypercholesterolemic hamsters (Fig. 2-A). The lyso-PL content in both tissues was highly correlated predominantly in a negative sense. Lyso-PC 14:0, 15:0, 16:1, 17:1, 18:3, 20:5, 22:5 and 22:6, as well as Lyso-PE 16:1 and 22:6, were associated with a greater number of negative correlations ($r > -0.5$) and therefore were decreased in circulation.

3.6.2. Effect of the chronic administration of HFD on the hamster liver tissue

Histological analysis of the liver samples showed an increase in the steatotic profile in the liver of hamsters fed the HFD (Fig. 2). As shown in Fig. 2-B, diet-induced steatosis was defined by hepatocytes distended by multiple microvesicles filled of with lipids that did not displace the nucleus of the cell, but no evidences was found of macrovesicular fat accumulation. The relative liver weights of both groups were comparable (Fig. 2-C), and the score of steatosis was measured according to the percentage of hepatic parenchyma compromised, revealing that HFD-fed hamsters had significantly higher scores than LFD-fed animals (Fig. 2-D). Importantly, many of the HFD-fed hamsters that developed steatosis also presented inflammation signs with prevalent moderate intensity and lobular arrangement (Fig. 2-E). None of the biopsies exhibited signs of ballooning degeneration or fibrosis of the hepatocytes.

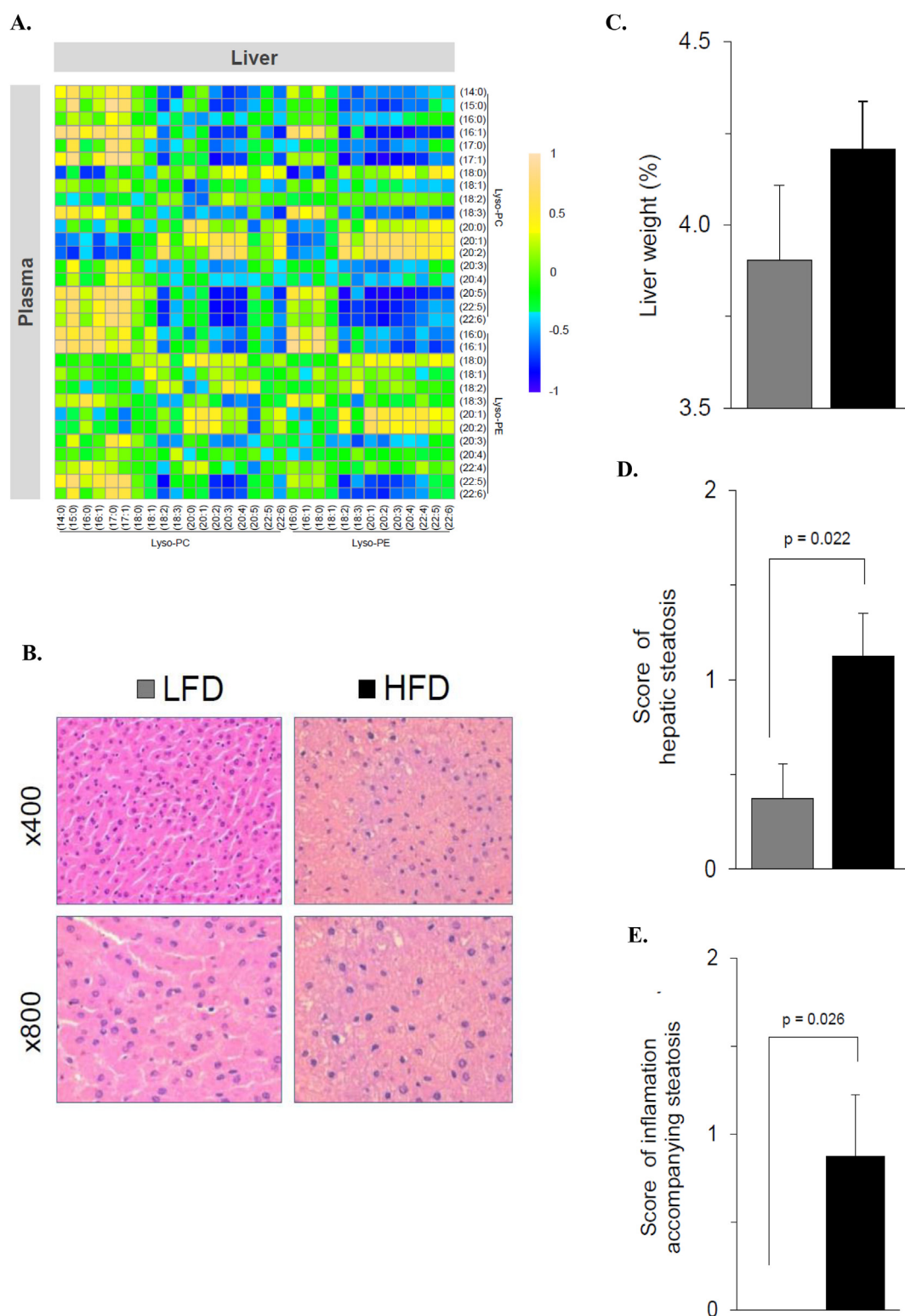


Fig. 2. Metabolic correlations across plasma and liver lysophospholipid contents and the effect of the thirty-day administration of low-fat diet (LFD) and a high-fat diet (HFD) on the hamster liver. (A) The normalized abundance of Lyso-PLs was correlated across both biological compartments using Pearson's correlation testing. The outgoing correlation coefficients (r) are displayed on a color-coded matrix ranging from -1 (blue) to +1 (orange). The bar chart shows the circulating metabolites with greater number of negative ($r < -0.5$) and positive ($r > 0.5$) correlations. (B) Histological analysis of liver tissue showing representative images of the study. Liver sections were stained with hematoxylin-eosin and were examined under a light microscope ($n = 8$ per group). The liver of HFD-fed animals shows hepatocellular vacuolization with microvesicular fat accumulation. (C) Relative liver weight in both animal groups, which was calculated by the formula (liver weight/body weight*100) and expressed as % of the total body weight. Data are expressed as mean $\pm$ SEM. (D) Score of hepatic steatosis. The score ranged from 0 to 3 and was determined by estimating the % of hepatocytes containing lipid droplets. (E) Score of inflammation (0–3) associated with steatosis. The significance was measured by conducting independent Student's t -test ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

The circulating serum lysophospholipidome consists of a niche of bioactive lipids with promising applicability as risk biomarkers in preliminary stages of major lipid disorders. In this study, we confirm the hypothesis that particular lyso-PLs could be precursors of HC progression because their rise occurs prior to serum LDL-cholesterol increase. After targeted lipidomics analysis, a discrete number of lyso-PL species is shown to be suitable for use as susceptibility/risk biomarkers for HC in MH-LDL-c compared to L-LDL-c subjects. Lyso-PC 15:0 and lyso-PE 18:2 were identified as maximal contributors to the differentiation between both groups, showing optimal accuracy. In particular, lyso-PE 18:2 confers protective capabilities against the advancement of diet-induced hepatic steatosis in hamsters, and their low serum levels are suggestive of HC progression in humans. In addition, the strong relationships stated between lyso-PL biomarkers and dietary components, primarily fatty acids, add insights into the dietary origin of serum lyso-PLs and their role in diet-induced HC.

Lyso-PLs act as signalling molecules and are involved in a broad range of physiological and pathological processes [7]. However, whether they exert pro- or anti-atherogenic actions remains controversial. The influence of lyso-PCs in atherosclerosis appears to be due to the chain length and the degree of saturation of the fatty acyl moiety [12]. We found lyso-PC 15:0 to be the most discriminating lyso form in the MH-LDL-c group. This finding was accompanied by strong positive associations with classical lipid-related markers such as TC, LDL-cholesterol and the atherogenic index LDL-cholesterol/HDL-cholesterol, which is similar to previous human studies reporting a link for lyso-PC 15:0 to the incidence of type 2 diabetes [32] and increased diastolic BP [33]. Nonetheless, these results provide an added link between lyso-PC 15:0 to lipid disorders, highlighting its possible role as susceptibility/risk biomarker for HC. Also, the observed moderate positive correlations among lyso-PC 15:0 and dairy foods, such as yogurt, cheese and whole milk, indicated that lyso-PC 15:0 mainly originates from SFAs present in dairy. Conversely, in the L-LDL-c group, lyso-PE 18:2 showed the highest contribution to the differentiation and exhibited inverse associations with TC and LDL-cholesterol, which suggests a possible protective role for human HC in the healthy state. In contrast, in MH-LDL-c, the observed significantly lower concentrations of lyso-PE 18:2 in circulation would be indicative of LDL-cholesterol increase in a preliminary stage of the disease, making it a suitable susceptibility/risk biomarker. Supporting this theory, in the second *in vivo* study, we showed that after chronic treatment with HFD, hamsters experience an overall intrahepatic accumulation of PUFA-containing lyso-PLs, including lyso-PE 18:2, which could explain the existence of a hepatic response mechanism to the excess fat of the diet. This statement agrees with a previous animal study [19], in which lyso-PE 18:2 was among the lyso-PLs that exhibited the earliest alterations in response to induced dyslipidemia in hamsters. Thus, we speculate the existence of a hepatic response mechanism to excess fat in the diet, which is mediated by an accumulation of PUFA-containing lyso-PLs to counteract the increase in LDL-cholesterol. As recently reported in rat models [34], isolated HC induced by diet led to marked alterations in the hepatic lipid profile. These alterations were characterized by significant increases in fatty acid content, predominantly palmitoleic acid (C16:1n-7), oleic acid (C18:1n-9) and ALA (C18:3n-3) in hypercholesterolaemic rats, while higher linoleic acid (LA) (C18:2n-6) was present in normocholesterolaemic rats [34]. Thus, this previous study in animals [34] reinforces the proposed theory that in preliminary stages of HC, the hepatic accumulation of dietary omega (n)-PUFAs, primarily LA, could promote the synthesis of lyso-PE 18:2, which is a mechanism by which the liver protects

itself from HC progression. Nonetheless, further human studies are required.

Also, we have reported that HFD promotes the development of microsteatosis in hamster liver, which is in accordance with other studies conducted in rodents in which the chronic administration of diets rich in fats led to intrahepatic accumulation of lipids [35,36]. However, in the proposed model, histological examination did not reveal hepatocyte ballooning degeneration or fibrosis, and circulating transaminases were unaltered, suggesting that the steatosis degree induced by diet was still incipient and that the liver had not yet experienced substantial injuries. In contrast, when hypercholesterolemia is induced by strategies other than diet, such as chemical agents, steatotic signs are pronounced earlier, as previously reported in hamsters with dyslipidaemia induced by Poloxamer 407 compared to HFD-fed animals [19]. Therefore, the intrahepatic accumulation of polyunsaturated lyso forms could also protect against the benign progression of diet-induced hepatic steatosis in hamsters. In this sense, previous studies have described the ability of biologically active lyso-PLs to restrict fatty acid β -oxidation in rodent liver and thereby reduce energy expenditure in response to the chronic exposure to dietary fats [37].

Interestingly, the results of this study revealed that the polyunsaturated acyl chain of lyso-PLs can be highly influenced by dietary fatty acids. As pointed out the strong association between dietary PUFA intake and lyso-PE 18:2 in both groups, the intake of omega (n)-PUFAs such as LA could modulate the circulating lyso-phospholipidome, conferring lyso-PE 18:2 a protective role for the onset of human hypercholesterolemia and hepatic steatosis in hamsters. In addition to the known hydrolytic action that several secreted enzymes (e.g. LCAT, sPLA2 and lipases) exert on circulating phospholipid to produce lyso-PLs, direct hepatic secretion is an important source of lyso forms, particularly of those that contain PUFAs [38], which reinforces the importance of their dietary origin. Dietary intake of essential PUFAs, such as LA (C18:2n-6) and alpha-linolenic acid (ALA) 18:3 (n-3), in a balanced manner is necessary to maintain proper circulating LDL-cholesterol because they cannot be synthesized by humans [39]; however, whether the changes in circulating LDL-cholesterol are mediated by lyso-PL action deserves further investigation. The protective role of lyso-PE 18:2 in the liver could also be explained by a decrease in endothelial lipase (EL) expression. EL is highly involved in cholesterol removal from peripheral tissues to the liver [40], and lower expression levels have been previously reported in the liver of hamsters after HFD treatment [19].

In parallel to the liver accumulation of polyunsaturated lyso-PEs, from the *in vivo* study, we also revealed an intrahepatic replacement of saturated and monounsaturated acyl lyso forms in HFD-fed hamsters. Although the HFD was primarily enriched in SFAs and monounsaturated fatty acids, it could be explained by sequential processes of desaturation and elongation of saturated and monounsaturated lyso forms in the hamster liver to form polyunsaturated lyso-PL structures, as reported in several studies on free fatty acids [14,41,42]. Therefore, through this process dietary SFAs, such as myristic acid (14:0) or pentadecanoic acid (15:0), would regulate the bioavailability of LA and ALA in hepatocytes, as previously described in rats [42] and human [43]. The improved bioavailability of essential fatty acids in the liver would contribute to the synthesis of omega (n)-PUFA-enriched lyso-PLs as a protective mechanism against the progression of lipoapoptotic incidents. In this context, the strong positive associations found in MH-LDL-c subjects between SFA intake, including whole dairy products, and lyso-PCs 15:0 and 17:0 supports SFA intermediate involvement on the configuration of omega (n)-PUFA-containing lyso-PLs.

Liver transaminases, specifically GGT, has been suggested as a superior marker for predicting CVD risk in adults with mild

dyslipidemia [44]. For the first time, this study provides an integrated view of the interrelationships between liver transaminases, discriminant lyso-PLs and diet in humans. In this manner, the multiple-way positive interactions stated in the MH-LDL-c group for GGT with lyso-PE 18:2 and PUFAs add further insights into the previously speculated hepatic mechanism to mitigate HC progression. Conversely, in the L-LDL-c group, the observed increases in serum lyso-PC 14:0 levels together with the positive association with GGT also indicate this lyso form could be altered in response to higher GGT enzymatic activity. However, the positive association for lyso-PC 14:0 with TMAO indicated that it could be a precursor for TMAO synthesis in the healthy state, and therefore act better as a susceptibility/risk biomarker for HC. Indeed, lyso-PC 14:0 has been previously considered to be a good predictor for other cardiometabolic risk factors, such as obesity [45]. However, given the limited evidence, further studies to clarify lyso-PC 14:0 predictive capabilities are required.

In this study, TMAO was left out the primary susceptibility/risk biomarkers for HC. Despite their consolidated pro-atherogenic properties and the reported close relationships with cardiovascular events [8,10], the results of this study support the notion that in the preliminary stages of disease, certain lyso-PLs are better predictors for HC than TMAO. The multiple positive relationships stated in both groups with dietary components, particularly with lean fish in the MH-LDL-c group, indicate that fasting TMAO levels are strongly regulated by diet [46]. However, we cannot dismiss whether different gut microbial compositions among groups may have influenced the response of TMAO to animal-based foods; therefore, it is important to study this topic in more detail in the future.

Overall, the present results provide novel insights, particularly from a lipidomic angle, into the suitability of particular lyso-PLs as susceptibility/risk biomarkers for human HC progression, specifically in preliminary stages of the pathophysiological process. Based on the relationships of lyso-PL biomarkers with diet and hepatic transaminases in humans, we hypothesize the existence of a liver adaptive mechanism dependent of lyso-PLs to counteract HC progression, which is strengthened by our second *in vivo* study, where a contradictory response of the hamster liver to the long-term administration of HFD is evidenced. However, it cannot be excluded that lyso-PL levels depend largely on the hydrolysis of oxidized phospholipids in LDL and that the pro-hypercholesterolemic state in the MH-LDL-c group could be caused by a direct action of oxidized LDL rather than through the lyso-PL action. Despite this, the comprehensive analysis of dietary components allowed us to demonstrate interesting associations among particular lyso forms and diet, and revealed a more complex clinical presentation of HC than that represented by classical lipid markers. However, these associations should be interpreted with caution due to the cross-sectional design of this study that could limit the establishment of causality. Given this limitation, the results of this study support the idea of resuming this study in the future to obtain a prospective vision of the changes in the lysophospholipidome and better understand their role as prognostic biomarkers for HC.

5. Conclusion

Two distinct patterns in the serum lyso-PL profile, characterized by higher lyso-PC 15:0 and lower lyso-PE 18:2 in the MH-LDL-c group, and lower lyso-PC 15:0 and higher lyso-PE 18:2 in the L-LDL-c group, were evidenced. Therefore, among all the species of lyso-PLs, serum lyso-PC 15:0 and lyso-PE 18:2 constitute promising susceptibility/risk biomarkers for HC. Dietary essential PUFAs, such as LA (C18:2n-6), promote the intrahepatic synthesis of lyso-PE

18:2, being this an adaptive mechanism of liver to protect itself from diet-induced hepatic steatosis in animal and to counteract HC progression in humans. These biomarkers, particularly low serum levels of Lyso-PE 18:2 in humans, could be used in clinical practice to guide novel preventive strategies to tackle HC.

Funding sources and grants

The research leading to these results was 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 (FP7-KBBE-2009-3 BIOCLAIMS) under grant agreement number 244995. Additionally, it was financially supported by the Centre for the Development of Industrial Technology (CDTI) of the Spanish Ministry of Science and Innovation under grant agreement: TECNOMIFOOD project (CER-20191010).

Credit author contributions

The authors' responsibilities were as follows – RS, LA, JMdB, AC and FP: Project administration and Conceptualization. RMV, RS, AP, LC-P and EL: Investigation, Methodology and Validation of the human study. SS-G: Investigation, Methodology and Formal analysis of the animal study. LC-P and LR: Data curation. LC-P: Formal analysis, Writing - original draft and Visualization. RS, MS, RMV and AP: Writing – Reviewing and Editing, Supervision. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

We would like to thank Miguel Querol, Lorena Torrado and Lluïsa Iniesta, who were nurses ascribed to the Technological Unit of Nutrition and Health (Eurecat, Reus), for their enthusiastic support administering this study. Additionally, we gratefully acknowledge Niurka Llópiz (URV, Tarragona, Spain) for the valuable technical assistance provided in the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.clnu.2021.11.033>.

References

- [1] Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, et al. ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). *Eur Heart J* 2019;41:111–88. <https://doi.org/10.1093/eurheartj/ehz455>. 2020.
- [2] Grundy SM, Cleeman JJ, Merz CNB, Brewer HBJ, Clark LT, Hunninghake DB, et al. Implications of recent clinical trials for the national cholesterol education program adult treatment panel III guidelines. *J Am Coll Cardiol* 2004;44: 720–32. <https://doi.org/10.1016/j.jacc.2004.07.001>.
- [3] Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, et al. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *Eur Heart J* 2013;34:3478–3490a. <https://doi.org/10.1093/eurheartj/ehz455>.
- [4] Wang HH, Garruti G, Liu M, Portincasa P, Wang DQ-H. Cholesterol and lipoprotein metabolism and atherosclerosis: recent advances in reverse

- cholesterol transport. *Ann Hepatol* 2017;16(Suppl 1):S27–42. <https://doi.org/10.5604/01.3001.0010.5495>.
- [5] Song Z, Wang H, Yin X, Deng P, Jiang W. Application of NMR metabolomics to search for human disease biomarkers in blood. *Clin Chem Lab Med* 2019;57: 417–41. <https://doi.org/10.1515/cclm-2018-0380>.
 - [6] Wang R, Li B, Lam SM, Shui G. Integration of lipidomics and metabolomics for in-depth understanding of cellular mechanism and disease progression. *J Genet Genomics* 2020;47:69–83. <https://doi.org/10.1016/j.jgg.2019.11.009>.
 - [7] Grzelczyk A, Gendaszewska-Darmach E. Novel bioactive glycerol-based lysophospholipids: new data – new insight into their function. *Biochimie* 2013;95:667–79. <https://doi.org/10.1016/j.biochi.2012.10.009>.
 - [8] Schiattarella GG, Sannino A, Toscano E, Giugliano G, Gargiulo G, Franzone A, et al. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. 2018. p. 2948–56. <https://doi.org/10.1093/eurheartj/ehx342>.
 - [9] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011;472:57–63. <https://doi.org/10.1038/nature09922>.
 - [10] Haghikia A, Li XS, Liman TG, Bledau N, Schmidt D, Zimmermann F, et al. Gut microbiota-dependent trimethylamine N-oxide predicts risk of cardiovascular events in patients with stroke and is related to proinflammatory monocytes. *Arterioscler Thromb Vasc Biol* 2018;38:2225–35. <https://doi.org/10.1161/ATVBAHA.118.311023>.
 - [11] Knuplez E, Marsche G. An updated review of pro- and anti-inflammatory properties of plasma lysophosphatidylcholines in the vascular system. *Int J Mol Sci* 2020;21:4501. <https://doi.org/10.3390/ijms21124501>.
 - [12] Akerele OA, Cheema SK. Fatty acyl composition of lysophosphatidylcholine is important in atherosclerosis. *Med Hypotheses* 2015;85:754–60. <https://doi.org/10.1016/j.mehy.2015.10.013>.
 - [13] Hui DY. Intestinal phospholipid and lysophospholipid metabolism in cardiometabolic disease. *Curr Opin Lipidol* 2016;27:507–12. <https://doi.org/10.1097/MOL.0000000000000334>.
 - [14] Balogun KA, Albert CJ, Ford DA, Brown RJ, Cheema SK. Dietary omega-3 polyunsaturated fatty acids alter the fatty acid composition of hepatic and plasma bioactive lipids in C57BL/6 mice: a lipidomic approach. *PLoS One* 2013;8:e82399. <https://doi.org/10.1371/journal.pone.0082399>.
 - [15] Wang Z, Zhao Y. Gut microbiota derived metabolites in cardiovascular health and disease. *Protein Cell* 2018;9:416–31. <https://doi.org/10.1007/s13238-018-0549-0>.
 - [16] Rath S, Heidrich B, Pieper DH, Vital M. Uncovering the trimethylamine-producing bacteria of the human gut microbiota. *Microbiome* 2017;5:54. <https://doi.org/10.1186/s40168-017-0271-9>.
 - [17] Chen K, Zheng X, Feng M, Li D, Zhang H. Gut microbiota-dependent metabolite trimethylamine N-oxide contributes to cardiac dysfunction in western diet-induced obese mice. *Front Physiol* 2017;8:139. <https://doi.org/10.3389/fphys.2017.00139>.
 - [18] Wang M, Wang F, Wang Y, Ma X, Zhao M, Zhao C. Metabonomics study of the therapeutic mechanism of *Gynostemma pentaphyllum* and atorvastatin for hyperlipidemia in rats. *PLoS One* 2013;8:e78731. <https://doi.org/10.1371/journal.pone.0078731>.
 - [19] Suárez-García S, Caimari A, Del Bas JM, Suárez M, Arola L. Serum lysophospholipid levels are altered in dyslipidemic hamsters. *Sci Rep* 2017;7:10431. <https://doi.org/10.1038/s41598-017-10651-0>.
 - [20] Guideline E. 2019 ESC/EAS guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *Atherosclerosis* 2019;290: 140–205. <https://doi.org/10.1016/j.atherosclerosis.2019.08.014>.
 - [21] CESNID. Tablas de composición de alimentos del Cesnid. *Rev Esp Salud Publica* 2004. <https://doi.org/10.1590/s1135-57272004000300011>.
 - [22] Patterson KY, Bhagwat S, Williams JR, Howe JC, Holden JM, Zeisel SH, et al. USDA database for the choline content of common foods. *Nutr Data Lab* 2008: 1–37. <https://doi.org/10.15482/USDA.ADC/1178141>.
 - [23] Vallbona Calbó C, Roure Cuspinera E, Violan Fors M, Alegre Martín J. *Guia de prescripció d'exercici físic per a la salut (PEFS)*. 2007. Barcelona.
 - [24] Zhao X, Zeisel SH, Zhang S. Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. *Electrophoresis* 2015;36: 2207–14. <https://doi.org/10.1002/elps.201500055>.
 - [25] Suárez-García S, Arola L, Pascual-Serrano A, Arola-Arnal A, Aragonès G, Bladé C, et al. Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum. *J Chromatogr B, Anal Technol Biomed Life Sci* 2017;1055–1056:86–97. <https://doi.org/10.1016/j.jchromb.2017.04.028>.
 - [26] Russell JC, Proctor SD. Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis. *Cardiovasc Pathol Off J Soc Cardiovasc Pathol* 2006;15:318–30. <https://doi.org/10.1016/j.carpath.2006.09.001>.
 - [27] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;41:1313–21. <https://doi.org/10.1002/hep.20701>.
 - [28] Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 1995;57:289–300.
 - [29] Guallar-Castillón P, Gil-Montero M, León-Muñoz LM, Graciani A, Bayán-Bravo A, Taboada JM, et al. Magnitude and management of hypercholesterolemia in the adult population of Spain, 2008–2010: the ENRICA Study. *Rev Esp Cardiol (Engl Ed)* 2012;65:551–8. <https://doi.org/10.1016/j.recresp.2012.02.005>.
 - [30] Ibarrola-Jurado N, Bulló M, Guasch-Ferré M, Ros E, Martínez-González MA, Corella D, et al. Cross-sectional assessment of nut consumption and obesity, metabolic syndrome and other cardiometabolic risk factors: the PREDIMED study. *PLoS One* 2013;8:e57367. <https://doi.org/10.1371/journal.pone.0057367>.
 - [31] Worley B, Powers R. Multivariate analysis in metabolomics. *Curr Metabolomics* 2013;1:92–107. <https://doi.org/10.2174/2213235X11301010092>.
 - [32] Kröger J, Zietemann V, Enzenbach C, Weikert C, Jansen EH, Döring F, et al. Erythrocyte membrane phospholipid fatty acids, desaturase activity, and dietary fatty acids in relation to risk of type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Am J Clin Nutr* 2011;93:127–42. <https://doi.org/10.3945/ajcn.110.005447>.
 - [33] Petersen KS, Keogh JB, Lister N, Weir JM, Meikle PJ, Clifton PM. Association between dairy intake, lipids and vascular structure and function in diabetes. *World J Diabetes* 2017;8:202–12. <https://doi.org/10.4239/wjdv8.i5.202>.
 - [34] Csonka C, Baranyai T, Tiszlavicz L, Félbél H, Szűcs G, Varga ZV, et al. Isolated hypercholesterolemia leads to steatosis in the liver without affecting the pancreas. *Lipids Health Dis* 2017;16:144. <https://doi.org/10.1186/s12944-017-0537-z>.
 - [35] Sampey BP, Vanhoose AM, Winfield HM, Freerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. *Obesity (Silver Spring)* 2011;19:1109–17. <https://doi.org/10.1038/oby.2011.18>.
 - [36] Nam M, Choi M-S, Jung S, Jung Y, Choi J-Y, Ryu DH, et al. Lipidomic profiling of liver tissue from obesity-prone and obesity-resistant mice fed a high fat diet. *Sci Rep* 2015;5:16984. <https://doi.org/10.1038/srep16984>.
 - [37] Labonté ED, Pfluger PT, Cash JC, Kuhel DG, Roja JC, Magness DP, et al. Postprandial lysophospholipid suppresses hepatic fatty acid oxidation: the molecular link between group 1B phospholipase A2 and diet-induced obesity. *FASEB J Off Publ Fed Am Soc Exp Biol* 2010;24:2516–24. <https://doi.org/10.1096/fj.09-144436>.
 - [38] Brindley DN. Hepatic secretion of lysophosphatidylcholine: a novel transport system for polyunsaturated fatty acids and choline. *J Nutr Biochem* 1993;4: 442–9. [https://doi.org/10.1016/0955-2863\(93\)90061-Z](https://doi.org/10.1016/0955-2863(93)90061-Z).
 - [39] Saini RK, Keum Y-S. Omega-3 and omega-6 polyunsaturated fatty acids: dietary sources, metabolism, and significance – a review. *Life Sci* 2018;203: 255–67. <https://doi.org/10.1016/j.lfs.2018.04.049>.
 - [40] Annema W, Tietge UJF. Role of hepatic lipase and endothelial lipase in high-density lipoprotein-mediated reverse cholesterol transport. *Curr Atheroscler Rep* 2011;13:257–65. <https://doi.org/10.1007/s11883-011-0175-2>.
 - [41] Legrand P, Rioux V. The complex and important cellular and metabolic functions of saturated fatty acids. *Lipids* 2010;45:941–6. <https://doi.org/10.1007/s11745-010-3444-x>.
 - [42] Rioux V, Catheline D, Bouriel M, Legrand P. Dietary myristic acid at physiologically relevant levels increases the tissue content of C20:5 n-3 and C20:3 n-6 in the rat. *Reprod Nutr Dev* 2005;45:599–612. <https://doi.org/10.1051/rnd:2005048>.
 - [43] Dabadie H, Peuchant E, Bernard M, LeRuyet P, Mendy F. Moderate intake of myristic acid in sn-2 position has beneficial lipidic effects and enhances DHA of cholesteryl esters in an interventional study. *J Nutr Biochem* 2005;16: 375–82. <https://doi.org/10.1016/j.jnutbio.2005.01.010>.
 - [44] Park E-O, Bae EJ, Park B-H, Chae S-W. The associations between liver enzymes and cardiovascular risk factors in adults with mild dyslipidemia. *J Clin Med* 2020;9. <https://doi.org/10.3390/jcm9041147>.
 - [45] Kim JY, Park JY, Kim OY, Ham BM, Kim H-J, Kwon DY, et al. Metabolic profiling of plasma in overweight/obese and lean men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF MS). *J Proteome Res* 2010;9:4368–75. <https://doi.org/10.1021/pr100101p>.
 - [46] Canyelles M, Tondo M, Cedó L, Farràs M, Escolà-Gil JC, Blanco-Vaca F. Trimethylamine N-oxide: a link among diet, gut microbiota, gene regulation of liver and intestine cholesterol homeostasis and HDL function. *Int J Mol Sci* 2018;19:3228. <https://doi.org/10.3390/ijms19103228>.