UNIVERSITAT ROVIRA I VIRGILI

IDENTIFICATION OF NOVEL BIOMARKERS OF ALTERED HOMEÒSTASIS

Susana Suárez García

Identification of novel biomarkers of altered homeostasis

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DOCTORAL THESIS
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supervised by Dr. Lluís Arola Ferrer

and Dr. Manuel Suárez Recio

Department of Biochemistry and Biotechnology

(Nutrigenomics Research Group)

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Tarragona 2017

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FAIG CONSTAR que aquest treball, titulat "Identification of novel biomarkers of altered homostasis", que presenta Susana Suárez García per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "Identification of novel biomarkers of altered homostasis", que presenta Susana Suárez García para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de esta universidad.

I STATE that the present study, entitled "Identification of novel biomarkers of altered homostasis", presented by Susana Suárez García for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this university.

Tarragona, 29 Juny 2017

El/s director/s de la tesi doctoral
El/los director/es de la tesis doctoral
Doctoral Thesis Supervisor/s

Dr. Lluís Arola Ferrer   Dr. Manuel Suárez Recio

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A mis padres…

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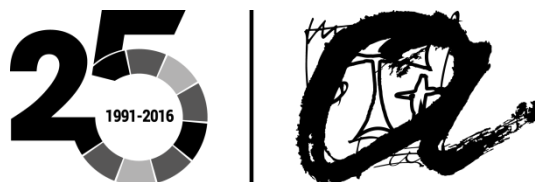Susana Suárez García

"Si buscas resultados distintos,

no hagas siempre lo mismo"

Albert Einstein

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CONTENT

SUMMARY

Poor eating habits exert a direct influence on the incidence of chronic diseases of high global prevalence, such as cardiovascular and fatty liver diseases. Nevertheless, the beneficial effects of a correct nutrition are principally effective in the opening phase of the metabolic disorder, being able to prevent and even reverse its development. By contrast, current biomarkers have a delayed response, contributing to the late diagnosis of the disease and encouraging the use of pharmacological therapies. These treatments can also be expensive and lead to serious side effects for the patients. Therefore, it is essential to find new risk markers that assist on early diagnosis. Only with these novel biomarkers able to determine the primarily metabolic disturbances that lead into a plethora of diseases, nutrition can be efficient in disease therapy. The recent emergence of the omics sciences along with the improvement of the equipment and tools for the processing of large volumes of data, provide the adequate support for biomarker search.

In this context, the present doctoral thesis was aimed to identify novel early biomarkers useful in the diagnosis of metabolic disorders derived for altered nutrition and lifestyle. For this purpose, non-targeted metabolomics was applied to the analysis of plasma samples from rodents fed different diets and subject to behavioural intervention. Once the biomarkers were selected, their distribution and metabolism were further studied. Finally, the adequacy of the putative biomarkers was determined in humans. Our results revealed circulating lysophospholipids as early predictors of chronic disorder. The metabolic assessment of the lysophospholipidome was relevant for the subclinical non-invasive diagnosis of fatty liver. Furthermore, the findings in humans seemed to confirm this assumption. As a conclusion, we propose lysophospholipids, including lysophosphatidylcholines and lysophosphatidylethanolamines, as good candidates for biomarkers of risk of fatty liver in humans.

LIST OF ABBREVIATIONS

Abhd3,   abhydrolase domain-containing 3 gene.

Actb,   beta-actin gene.

ALT,   alanine transaminase.

ANOVA,   analysis of variance.

AJS,   Agilent Jeat Stream technology.

AST,   aspartate transaminase.

AUC,   area under the curve.

BHT,   butylated hydroxytoluene.

BUME,   butanol-methanol extraction.

CAF,   cafeteria diet.

cDNA,   complementary deoxyribonucleic acid.

CEHC,   tocopheronolactone.

CE-MS,   capillary electrophoresis coupled to mass spectrometry.

CI,   confidence interval.

CON,   sedentary animals.

CRP,   C-reactive protein.

CV,   coefficient of variation.

CVD,   cardiovascular disease.

CYP7A1,   cytochrome P450 family 7 subfamily A member 1 (C7αH gene).

C7αH,   cholesterol 7α-hydroxylase.

DHA,   docosahexaenoic acid.

DiHETE,   dihydroxy-eicosatetraenoic acid.

DiHOME,   dihydroxy-octadecenoic acid.

DNA,   deoxyribonucleic acid.

EL,   endothelial lipase.

ELISA,   enzyme-linked immunosorbent assay.

EPA,   eicosapentaenoic acid.

ER,   endoplasmic reticulum.

ESI,   electrospray ionization.

EWAT,   epididymal white adipose tissue.

FXR,   farnesoid X receptor.

GC,   gas chromatography.

GPCR,   G-protein-coupled receptor.

HCA,   hierarchical clustering analysis.

HDLc,   high-density lipoprotein cholesterol.

HFD,   high-fat diet.

HMDB,   Human Metabolome Database.

HMG-CoA,   3-hydroxy-3-methylglutaryl coenzyme A reductase.

HPLC,   high-performance liquid chromatography.

HSV,   high sample volume extraction.

IS,   internal standard.

KEGG,   Kyoto Encyclopedia of Genes and Genomes.

LAR,   leptin/adiponectin ratio.

LC,   liquid chromatography.

LCAT,   lecithin-cholesterol acyltransferase.

LC-MS/MS,   liquid chromatography coupled to tandem mass spectrometry.

LDLc,   low-density lipoprotein cholesterol.

LFD,   low-fat diet.

Lipc,   hepatic lipase gene.

Lipg,   endothelial lipase gene.

LIPID MAPS,   Lipid Metabolites and Pathways Strategy.

LC-PUFAs,   long chain polyunsaturated fatty acids.

LPCAT3,   lysophosphatidylcholine acyltransferase 3.

Lpcat3,   lysophosphatidylcholine acyltransferase 3 gene.

LPL,   lipoprotein lipase.

LSV,   low sample volume extraction.

LXR,   liver X receptor.

Lyso-PC,   lysophosphatidylcholine.

Lyso-PE,   lysophosphatidylethanolamine.

Lyso-PI,   lysophosphatidylinositol.

Lyso-PL,   lysoglycerophospholipid.

MCP-1,   monocyte chemoattractant protein-1.

MDL,   method detection limit.

MedDiet,   Mediterranean diet.

MeOH,   methanol.

MQL,   method quantification limit.

MRM,   multiple reaction monitoring.

METLIN,   Metabolite Link.

MetS,   metabolic syndrome.

MFE,   molecular feature extractor algorithm.

MG,   monoacylglycerol.

MPP,   Mass Profiler Professional software.

mRNA,   messenger ribonucleic acid.

MS,   mass spectrometry.

MS/MS,   tandem mass spectrometry.

MTBE,   methyl-*tert*-butyl ether extraction.

MUFA,   monounsaturated fatty acid.

MWAT,   mesenteric white adipose tissue.

NAFLD,   nonalcoholic fatty liver disease.

NASH,   nonalcoholic steatohepatitis.

NCD,   noncommunicable disease.

NEFA,   non-esterified free fatty acid.

NMR,   nuclear magnetic resonance spectroscopy.

PAF,   platelet activating factor.

PC,   phosphatidylcholine.

PCA,   principal component analysis.

PEMT,   phosphatidylethanolamine N-methyltransferase.

PG,   phosphatidylglycerol.

PLA_1,   phospholipase activity of type 1.

PLA2G1B,   phospholipase A_2 group IB (pancreatic phospholipase).

Pla2g2a,   phospholipase A_2 group IIA.

PLS-DA,   partial least squares for discriminant analysis.

PON1,   paraoxonase-1.

PUFA,   polyunsaturated fatty acid.

P407,   Poloxamer 407.

Q^2,   quality assesment statistic.

QC,   quality control.

QqQ,   triple quadrupole mass spectrometer.

qRT-PCR,   quantitative real-time reverse transcriptase-polymerase chain reaction.

Q-TOF,   quadrupole time-of-flight mass spectrometer.

RNA,   ribonucleic acid.

ROC,   receiver operating characteristic.

RSD,   relative standard deviation.

RT,   retention time.

RWAT,   retroperitoneal white adipose tissue.

SCD1,   stearoyl-CoA desaturase 1.

SEM,   standard error of the mean.

SFA,   saturated fatty acid.

sPLA$_2$–IIA,   secreted phospholipase A$_2$ group IIA.

SREBP2,   sterol regulatory element-binding protein 2.

ST,   standard chow.

TC,   total cholesterol.

TIC,   total ion chromatogram.

TLC,   thin-layer chromatography.

TMH,   treadmill-high intensity group (17 m/min).

TML,   treadmill-low intensity group (12 m/min).

TOF,   time-of-flight mass spectrometer.

UHPLC,   ultra-high-performance liquid chromatography.

UV,   ultraviolet.

VIP,   Variable importance in projection.

VLDL,   very low-density lipoproteins.

1. INTRODUCTION

1.1. Impact of nutrition on health and well-being

The role of nutrition and diet in the promotion and maintenance of health is well known. In fact, it has been confirmed that both of them play an essential paper in the onset and evolution of the so-called "noncommunicable diseases". This group of pathologies, also known as chronic diseases, includes cardiovascular diseases (such as stroke and heart attacks), cancer, chronic respiratory diseases (like asthma and pulmonary disease) and diabetes, among others. To realize about the importance of this group of pathologies it is only necessary to look at the annual statistics published by the International Organisms where it can be seen that noncommunicable diseases (NCDs) are responsible for the death of 40 million people every year (**Fig. I**), representing the 70% of the total deaths worldwide in a year [1].
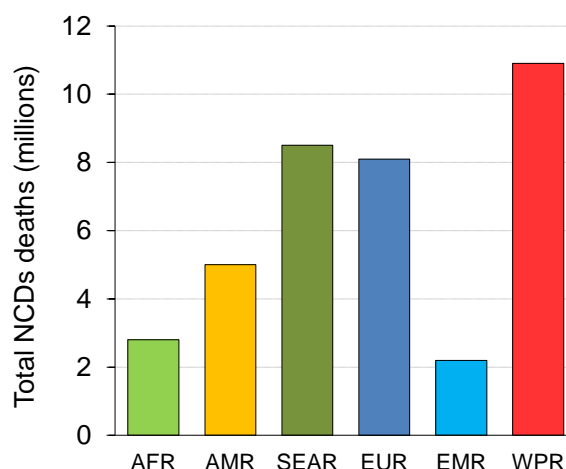
Figure I. Number of annual deaths caused by NCDs. AFR: African Region; AMR: Region of the Americas; SEAR: South-East Asia Region; EUR: European Region; EMR: Eastern Mediterranean Region; WPR: Western Pacific Region. The figure was adapted from [1].

Obesity, hypertension, dyslipidemia and hyperglycemia, all of them highly dependent of nutrition and lifestyle habits (such as physical inactivity, drugs, tobacco and excessive alcohol consumption), are some of the most important metabolic risk factors for the onset of NCDs. Although still it is necessary more research in order to completely understand the mechanisms that link diet with the development of these pathologies, it is known that NCDs are, at some extent, preventable by means of an adequate diet. In fact, this is considered to be the most affordable, cost-effective and sustainable strategy to deal with this worldwide steadily increasing epidemic.

Thus, currently, there are a huge number of research projects and scientific publications that are focused on study the mechanisms by which certain diets and food bioactive compounds are able to counteract and prevent the onset and progression of these undesired metabolic disturbances. As an example, since decades it has been widely accepted the impact of Mediterranean diet (MedDiet) in the prevention of cardiovascular disease (CVD) [2]. However, it has been not up to recent years when scientists have gained knowledge about the mechanisms and pathways by which MedDiet exerts its beneficial properties. In this search of answers, an important step forward was the obtained with the PREDIMED study (*PREvención con DIeta MEDiterránea*). This study was conducted using a high cohort of volunteers (approximately 7,500 people) and evaluated the efficacy of two sets of MedDiet, one supplemented with nuts and the other with extra-virgin olive oil, on primary cardiovascular prevention. In addition these two diets were compared with a low-fat diet. Results confirmed that, in people at high risk of CVD, both of the MedDiet under study reduced the incidence of major cardiovascular events [3].

It has also been demonstrated the relationship between CVD and dietary fatty acids. Studies have shown that, while saturated fatty acids (SFAs), such as palmitic and myristic acids, are associated with an increase in total and low-density lipoprotein (LDL) cholesterol, both mono- and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) lower their circulating levels [4]. Therefore, a simple shift in the daily consumption of fatty acids would have a profound impact in the development of these cardiovascular problems.

Continuing with dietary fatty acids, it has also been confirmed their role as key factors for the development of liver disease. Among other hepatic problems, nonalcoholic fatty liver disease (NAFLD) is characterized by an excess in fat deposition in the hepatocytes without relation with excessive alcohol consumption. The severity of NAFLD is related with its capacity to cause progressive damage in the hepatocytes, from simple steatosis to nonalcoholic steatohepatitis (NASH) which involves several inflammation processes and, finally developing fibrosis, cirrhosis and hepatocellular carcinoma [5]. Several studies have shown that dietary fatty acids are involved with the development and progression of NAFLD. As can be seen in **Fig. II**, several PUFAs have exhibited antiobesity, anti-steatotic and anti-inflammatory effects thus inhibiting the onset of NAFLD. On the other side, results from MUFAs have showed a dual behavior, both inductor and inhibitor of liver disease, in different murine and human models [6].
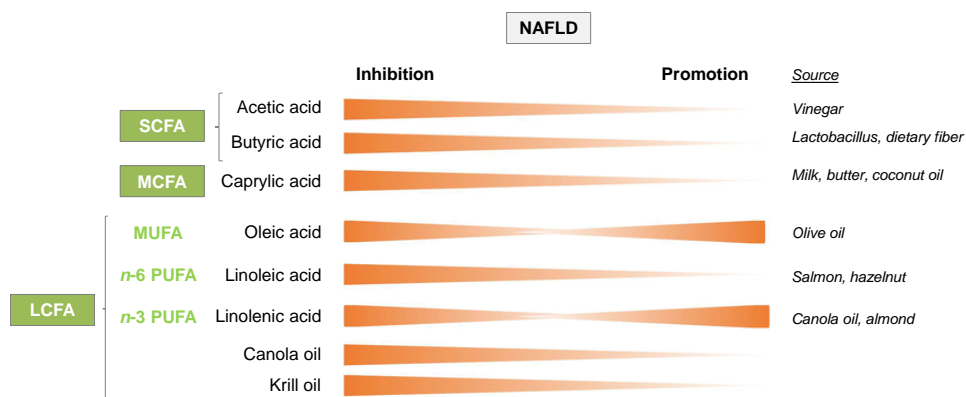
Figure II. Influence of dietary fatty acids in the onset of nonalcoholic fatty liver disease (NAFLD). SCFA: short-chain fatty acid; MCFA: medium-chain fatty acid; LCFA: long-chain fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid. The figure was adapted from [6].

In the same line, epidemiologic studies have shown that the diet of people with NASH is characterized by a high intake of fatty acids (mainly saturated and monounsaturated) and simple sugars and low percentages of complex carbohydrates and protein [7,8]. Therefore, it seems that one effective treatment in order to counteract the progression of fatty liver is to reduce the ingestion of the dietary compounds that induce this disease (for instance, SFAs and free sugars) while incrementing those that is proven to reduce and even revert it (such as PUFAs). The *n*-6 / *n*-3 ratio is also involved in the development of NAFLD since *n*-6 PUFAs are precursors of pro-inflammatory compounds, while *n*-3 PUFAs are anti-inflammatory. Thus, diets with excessive amount of -6 fatty acids were common in patients with NASH. Aside from PUFAs, other food bioactive compounds that have been demonstrated to act against NAFLD are phenolic compounds, melatonin, vitamin E and antioxidants in addition to probiotics [9]. For that reason, one way to reduce the incidence of this serious problem is a substantial change in the dietary habits complemented with a supplementation with those food bioactives that are described to prevent its development.

1.2. Strategies for preventing or reducing the onset of noncommunicable diseases

Within this framework, taking into consideration the amount of evidence that claim the beneficial effect of nutrition and dietary food bioactive compounds against the progression of NCDs, Organisms from several countries have dedicated great efforts to develop strategies and policies that include nutrition and nutritional therapies as means to

allow a reduction of this problem. In 2013, the World Health Organization (WHO) developed a procedure entitled "*Global action plan for the prevention and control of NCDs 2013-2020*" that included the nine global targets that are thought to have the greatest impact on the mortality due to NCDs and addressed its prevention and management [10]. Thus, applying these policies, it is expected that by 2030 the premature mortality from NCDs would be reduced by one-third. However, although the role of nutrition is clear, to obtain the maximum benefit from its management it is still necessary more research that allows a better understanding of the mechanisms underlying its beneficial effects, for example modulating the expression of genes and proteins which finally influences the metabolism and its response.

In this context, one market that has evolved rapidly since the latter half of the 90s is the formed by the functional foods. Functional foods are food products that are claimed to exert a beneficial healthy effect in the people upon its ingestion. Although there is still not a clear consensus about its definition [11], one of the most accepted is the proposed by the FUFOSE which says that functional foods are "*those that it is satisfactorily shown that beneficially affects one or more body functions, improving the health and welfare and/or reducing the risk of disease*" [12]. Consequently, attending at this definition, it is clear that functional foods are very promising tools to be used in the management of NCDs under the nutritional perspective. In Europe, a step forward in the control and regulation of functional food was done in 2002 with the creation of the European Food Safety Authority (EFSA). This organization evaluates all the aspects related with food in Europe and provides scientific support in the legislative process. Regarding functional foods, the EFSA defined a consensus criterion to be applied when assessing the validity of health claims made on foods. However, from thousands of proposals submitted to the EFSA, only very few have reached this goal (**Fig. III**), mainly due to the high scientific standards of the process of health-claim assessment [13].
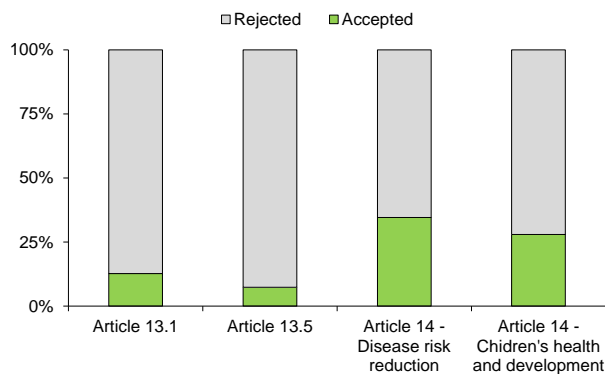
Figure III. Percentage of claims given a favourable opinion by EFSA. The figure was adapted from [14].

1.3. Need for new biomarkers of nutrition-derived health claims

One of the limiting factors in the use of nutrition in the prevention of NCDs is that, contrary to pharmacological treatments, dietary treatment are meant to be used in the first steps of the progression of the diseases or, even better, in a situation of pre-disease where still it is not fully initiated the process of its development. This is due to the fact that current biomarkers of disease are actually endpoints, identifying a state in which the disease is so advanced in its progression that nutrition is not capable of reversing the situation and only drugs from pharmacological treatments can control or palliate the alteration [15]. Therefore, there is a need for new early biomarkers of the onset of diseases. Only with these novel biomarkers that would allow to determine the primarily metabolic disturbances that lead into a plethora of diseases, nutrition will have the opportunity to be really efficient in this intended purpose [16] (**Fig. IV**).
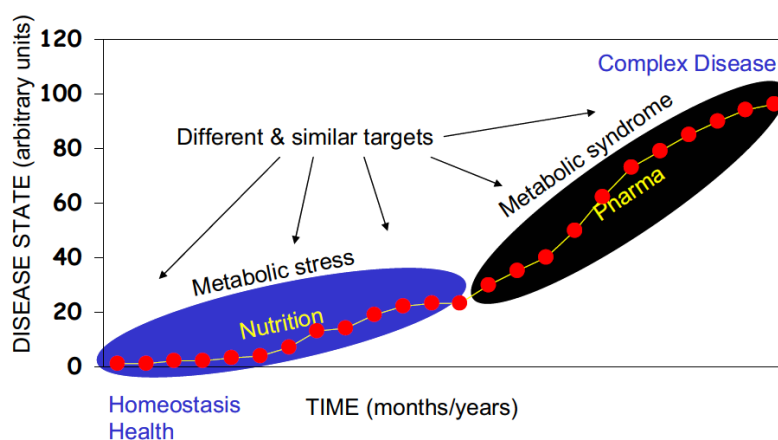
Figure IV. Effect of nutrition and pharma in the treatment and progression of metabolic syndrome. The figure was obtained from [15].

This is very clear for example in the case of Alzheimer disease. The pharmacological treatments addressed to this pathology are very limited. However, there is growing evidence in the literature that shows that Alzheimer can be, at some level, preventable by means of brain-protective changes in the lifestyle habits of the individuals at risk during the preclinical phase, where still no clear signs of the disease are present. But, for this to be possible, it is necessary to identify new biomarkers of Alzheimer disease that allow to identify it prior to irreversible neuronal damage [17].

For this reason, some researchers are currently focusing their experiments in the search of novel biomarkers. Several strategies are being used to identify them for example those that include the use of phenotypic flexibility as mechanism to detect biomarkers at early stages. These strategies are based on the fact that humans, as the rest of animals, are able to respond to different kind of stress and other alterations in order to maintain homeostasis [18]. In fact, it is believed that unbalances in the maintenance of this equilibrium are some of the reasons underlying the onset of NCDs. In this line, several researchers have demonstrated that the use of different challenges such as intense fasting, acute ingestion of some nutrients or cold temperatures intended to generate a situation of extreme stress forces the dilation of the homeostatic potential of the organism and allows to identify new early biomarkers that otherwise would remain unidentified [19] (**Fig. V**).
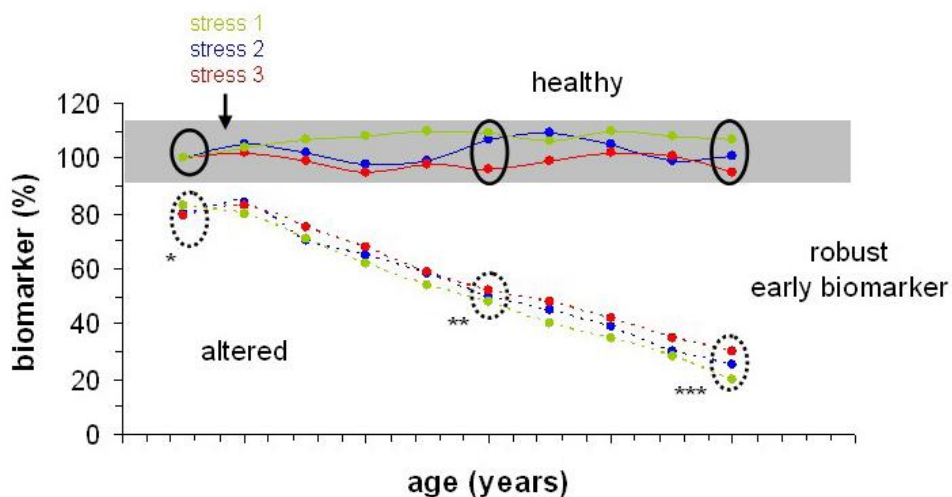
Figure V. Impact of nutritional challenges on ideal biomarkers. The figure was obtained from [20].

A good example of this type of studies is the performed by Krug *et al.* [21]. In this pilot experiment conducted in humans (n = 15), the authors evaluated the impact on the metabolome of a variety of challenges generated in order to identify biomarkers of metabolic disruption. Specifically, in an interval of 4 days, volunteers were submitted to an oral glucose tolerance test, a fat tolerance test, cold stress, fasting, physical activity and a standard liquid diet. Different kinds of samples (including blood, urine, exhaled air, and breath condensate) were evaluated in order to obtain a general vision of the behavior of the organism. Once analyzed all the samples, it was possible to observe a fluctuation of some metabolites in

response to the different stimuli, following what the authors described as an "accordion behavior". The study and comparison of the responses of the volunteers to the challenges allowed the identification of different signatures among them, being able to discriminate subjects according to their physiological traits. Consequently, those molecules responsible for the different reaction of the volunteers to the challenges would be good candidates to be selected as novel biomarkers of homeostasis disruption.

The process of searching new biomarkers through the use of the phenotypic flexibility is not simple. Once identified the molecules by means of the challenges, it is necessary to deep in the metabolic pathways and biological function in which these molecules are involved, prior to confirm is adequacy as putative biomarkers. Furthermore, it is necessary to validate its suitability in different models and experiments, thus complicating the objective. Aiming to help in the identification of novel biomarkers, in 2009 a consortium of researchers from different institutions of Europe initiated the BIOCLAIMS project (BIOmarkers of Robustness of Metabolic Homeostasis for Nutrigenomics-derived Health CLAIMS Made on Food) within the context of the Seventh Framework Programme. The main objective of this project (which lasted up to 2015) was the identification of biomarkers of health robustness, as well as nutrigenomics-based biomarkers. Moreover, the biological acceptability of these biomarkers determined by mechanistic data was also evaluated [20]. As a result, an important step forward in the identification of biomarkers was reached with this project.

1.4. Defining biomarkers

Throughout history humans have search for signals that allow the identification of states of disease in order to be able to act against them in an effective manner. From temperature to blood pressure, initially, these signals were reduced to external-feasible measurements of the physiological state. It was not up to the last century when the term biological marker (usually coined as biomarker) was introduced to refer to molecules, determined by biochemical analysis, which evaluation could be related with a certain situation of alteration in the organism [22].

Although there are several definitions for the term biomarker, the most accepted is the proposed by the National Institutes of Health Biomarkers Definitions Working Group which literally says that "*Biomarker is a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. A biomarker is not an assessment of how*

*an individual feels, functions, or survive*s [23]". Following this definition, an according to its use, biomarkers can be classified in several groups such as diagnostic, prognostic, predictive and monitoring biomarker (**Fig. VI**). For example, diagnostic biomarkers are those that allow the identification of a certain pathology and thus, to differentiate between healthy and diseased individuals. This type of biomarkers can also be very useful in the monitoring of patients. On the other side, prognostic biomarkers are those that, prior to a certain treatment, indicate an estimation of the severity and likely outcome of the disease while a predictive biomarker foresee the response of a patient to a treatment [24].
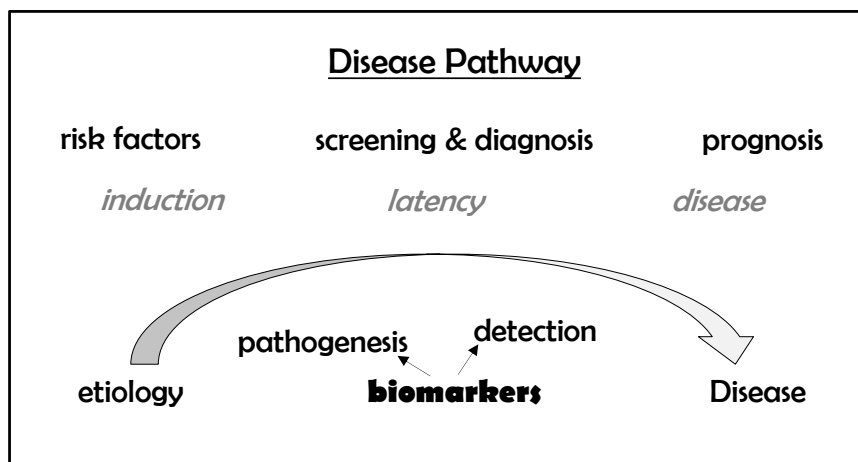
Figure VI. Disease pathway and potential impact of biomarkers.

An ideal biomarker should reunite a series of characteristics in order to be potentially used in routine clinical diagnosis (**Fig. VII**). Hence, a good biomarker has to be specific, reproducible, accurate and sensitive. In addition there are other things that have to be considered when selecting a good biomarker. For example, it is important that the biomarkers be easily interpreted by the clinicians and there shouldn't exist differences across genders and ethnics groups [25]. Furthermore, it is desired that the biomarker could be measured in non-invasive (urine, saliva, feces) or minimum invasive samples (such as plasma or serum) in order to ease the analytical determination of its value in humans and to avoid as much as possible more aggressive procedures such as biopsies from tissues [26]. A clear example of this last point is the actual procedure to diagnose liver disease. Although transaminases and gamma-glutamyl transferase are usually used to diagnose the onset of liver diseases, biopsy is still required as "gold standard" to confirm these pathologies and the degree of the illness [27]. In this sense, it is clear that there is a need for identification of novel biomarkers that would help in the control and management of these diseases [28].

Sample Acquisition
Non-invasive
Urine
Blood
Saliva
Tears

Analytical requirements
Minimal sample preparation
Simple instrumentation
Easily interpretable
Measure proteins or metabolites

Methodology
Sensitive
Accurate
Rapid
Reproducible
Low cost (especially screening)

Sensitivity and specificity
100%
  no false positives
  no false negatives

Figure VII. Characteristics of an ideal biomarker.

Some examples of classical biomarkers are the determination of blood glucose as identifier of type 2 diabetes mellitus or the glomerular filtration rate (based on creatinine determination) as diagnostic biomarker of chronic kidney disease [29]. In the case of cardiovascular disease, two recognized markers of myocardial infarction are creatine kinase and troponin [30]. However, as already explained, there is a need for novel biomarkers. The identification of these new early biomarkers would ease the implementation of strategies to avoid the continuous advance of the NCDs, having a high impact in clinical diagnosis and allowing the optimization of the resources needed for the treatment of the patients [31].

1.5. Role of the omics in the search of novel biomarkers

In the search of new biomarkers useful for nutrition-derived health claims it is important to highlight the role of the group of technologies sheltered under the –omics distinction. This group of techniques is designed to study, under a holistic perspective, a broad range of molecules. From genes to metabolites including proteins, the enormous potential of these technologies lies in the high amount of data that is obtained from its use. Thus, different omics are defined depending on the molecules evaluated (**Fig. VIII**): genomics is focused on the study of the genes, transcriptomics analyzes the transcripts while proteomics and metabolomics study proteins and metabolites, respectively [32]. As new improvements are performed in the pieces of equipment used in these technologies, there is

also a significant enhancement in the general knowledge of the scientific community and this fact, in turns, has a direct effect on the creation of new omics disciplines such as micromics (focused on microRNA) [33] and fluxomics (which studies the metabolic fluxes) [34].

The "Omics" Cascade

What can happen	**GENOME**
What appears to be happening	**TRANSCRIPTOME**
What makes it happen	**PROTEOME**
What has happened and is happening	**METABOLOME**

PHENOTYPE
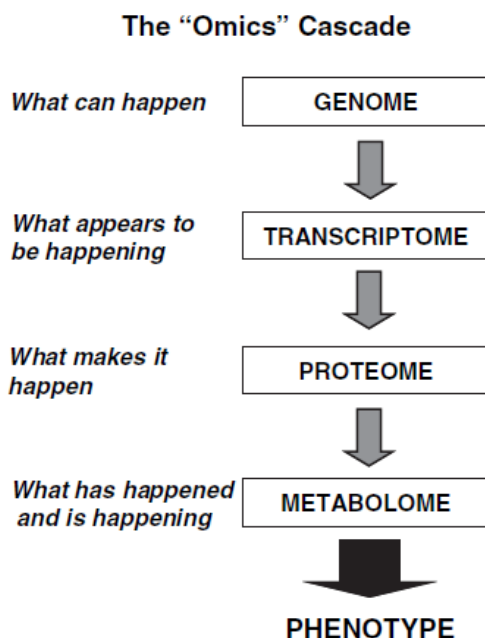
Figure VIII. Omics cascade. The schematic illustration was obtained from [35].

In addition to the development of new equipment, it has also had a great impact in the expanded application of the omics the creation of bioinformatics tools, necessary to ease the process of analyzing the immense amount of data obtained with these techniques. In this sense, bioinformatics engineers from the different companies have dedicated great efforts in order to develop software, applications, libraries and platforms designed to extract the maximum information from the omics studies [36].

Among the different omics, metabolomics, which is focused in the analysis of the complete set of molecules that compose the metabolome (defined as the group of molecules with a molecular weight lower than 2,000 Dalton), is one of the more used in the search of novel biomarkers. This is due to the fact that metabolomics is at the end of the so-called "omics cascade" and as a consequence is very dynamic and susceptible to all the changes that suffers an organism, showing all the molecules that are present in a certain organ or tissue in a determined moment. In other words, is like analyzing a photo in which is shown

the actual state of the organism [37]. On the other hand, results from other omics such as for example proteomics are not always reflecting a realistic situation and have to deal with different problems prior to confirm their suitability as biomarkers (i.e. increased amount of enzyme is not always correlated with increased activity and, in turns, an increased amount of the resulting product) [38]. In this sense, it is clear that a more general approximation, grouping all the omics within the systems biology approach will allow in the process of obtaining more reliable biomarkers to be used in clinical diagnosis [39]. Thus, several authors claim that, combining different biomarkers together will help to obtain improved accuracy in the diagnostics and will allow distinguishing between diseases with similar traits. In this process of defining multi-composed biomarkers it is said that metabolomics will play a crucial role [29].

Referring to metabolomics, the main analytical techniques that are used in this omics are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) coupled to either liquid or gas chromatography (LC or GC, respectively). In a less usual manner, another technique also used in metabolomics is capillary electrophoresis coupled to MS (CE-MS) [40]. The success of these techniques relies in their sensitivity and selectivity. On the one hand, NMR is characterized by its high reproducibility, minimal sample preparation requirements and rapid analysis. In addition, it is a non-destructive technique so the samples can be further analyzed by other complementary methodologies. As main drawbacks of this technique outstands its limited use when metabolites are present in the sample at very low concentrations and the difficulty in the analysis of complex samples in which signals from different metabolites can be overlapped [41]. On the other hand, MS stands out by its great sensitivity and specificity and its adequacy to be used in complex samples. Furthermore, MS can be used in both quantitative and qualitative studies widen the number of experiments in which it can be used [35]. Thus, comparing NMR and MS it seems that the balance leans towards MS due to the high number of molecules that can be detected by means of this last technique [42]. Nevertheless, some authors have claimed about the use of combined methodologies in which both instruments are used in a complementary manner, thus enhancing the obtained results and obtaining a broader image of the whole metabolome [43].

1.6. Metabolomics approaches

Metabolomics analyses can be performed under two different perspectives depending of the intended purpose: targeted and non-targeted metabolomics [44].

Targeted metabolomics is used when only a defined set of molecules is analyzed. This means that the targets of the study are known and, therefore, all the attention can be focused on them. This fact allows a higher sensitivity and specificity of the measurements, improving the signal-to-noise ratio in a significant manner. In general, this modality of metabolomics is conducted by using NMR or LC coupled to a tandem mass spectrometer detector (LC-MS/MS) such as the triple quadrupole detector (QqQ). When using this last equipment, selected metabolites are ionized several times in order to break them in a specific manner, generating fragments of known weight. Hence, it is possible to identify and quantify these metabolites just by monitoring the generated fragments [45].

The other modality of the metabolomics analyses is the non-targeted approach [46]. The use of this kind of analysis implies that not prior information of the molecules that are analyzed is known and, therefore, the objective is to identify as many metabolites as possible. In other words, non-targeted metabolomics consists on obtaining the metabolome fingerprint of a certain sample. For this reason, it is said that this is a not-hypothesis driven approach. Or, what is more important in the case of biomarkers discovery, is a hypothesis generating approach. In this case, analyses are usually performed by LC coupled to a quadrupole time-of-flight detector (LC-Q-TOF) which has an enormous sensibility and accuracy in the molecular weight determination. Thus, in a biomarker-search pipeline, different samples are analyzed and profiled by LC-Q-TOF and then, the molecules that differ among groups in a significant manner are selected as putative biomarkers thus allowing to generate a hypothesis of the metabolic pathways and processes that can be acting different among the samples [47].

Thus, while non-targeted metabolomics is usually used in the first step of novel biomarker discovery, targeted approaches are frequently used in the clinical diagnosis step, when the biomarkers are already established (**Fig. IX**).
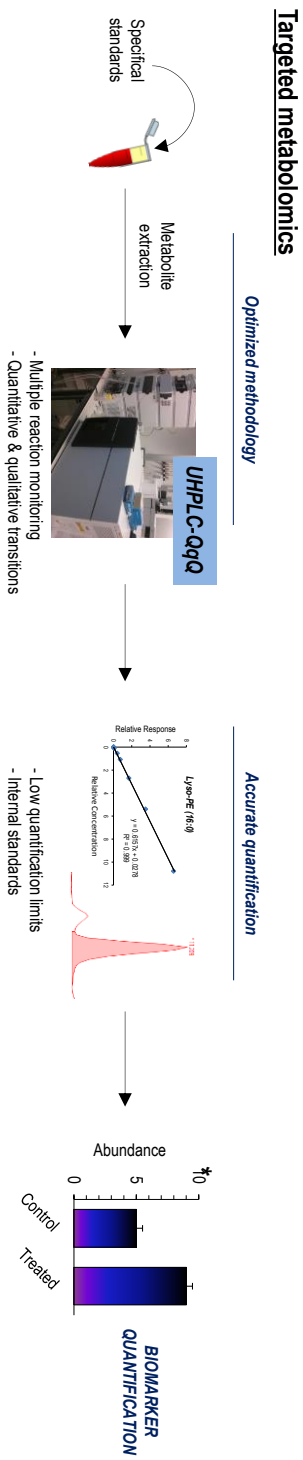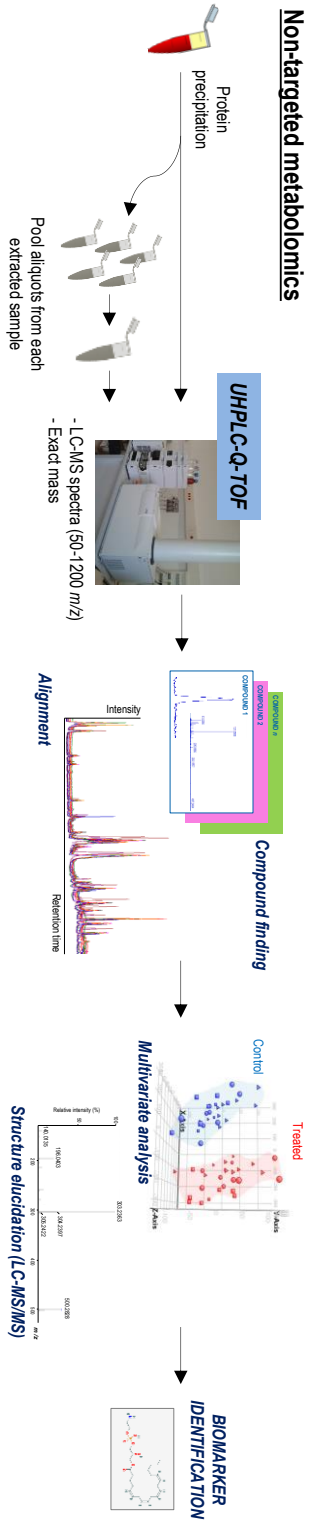
Figure IX. Non-targeted and targeted metabolomics workflow.

Several researchers have used these metabolomics approaches in order to identify novel biomarkers for health related claims. For example, in the manuscript of Laborde *et al.* [48] authors were able to identify biomarkers for the early diagnosis of acute coronary syndrome in plasma. Among others a 5-OH-tryptophan, 2-OH-butyric acid and 3-OH-butyric acid were potentially selected as biomarkers of this disease. In another study [49], following a non-targeted approach authors were able to tentatively identify novel biomarkers of prostate cancer. From a list of metabolites that significantly differed in the disease group the authors selected three of them, namely 2-isopropyl citrate, cytidine and D-asparagine, as the most adequate to be used in this type of cancer diagnosis based on multivariate statistical evaluation of the results. However, further studies were still required to confirm its validity and application.

In this search of novel biomarkers an interesting and recent study was the performed by Fan *et al.* [50]. In this work, novel biomarkers for early stage epithelial ovarian cancer were evaluated. By means of plasma metabolomics, authors identified five potential biomarkers (including lysophospholipids and adrenoyl ethanolamide) due to its positive association with epithelial ovarian cancer.

Therefore, attending at the large number of research articles that are focused on the search of biomarkers by metabolomics approaches, it is clear that this is a promising field in which scientists trust in order to enhance the management and diagnostic of chronic diseases.

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2. HYPOTHESIS & OBJECTIVES

Hypothesis and objectives

There are increasing evidence regarding the importance of nutrition in the maintenance of well-being. Likewise, poor eating habits have a direct impact on the emergence of noncommunicable diseases of high prevalence in developed societies, such as cardiovascular diseases and hepatic disorders. In this line, it has been seen that the beneficial effects of nutrition are only effective in the initial stages of the disorder, by way of correction and/or prevention. Nevertheless, the traditional biomarkers currently used in clinical diagnosis have a delayed response, at which point, the harmful effects of the disease can only be reversed through the use of drugs. That is why the need arises to find new biomarkers that assist on the early diagnosis of the pathology, when the situation can still be controlled through good dietary practices and use of nutritional supplements. It is also important that sample collection for the evaluation of the health markers can be carried out using non-invasive techniques, to enable the subsequent implementation in diagnosis. The emergence of the omics sciences along with the development of high precision and sensitivity equipment and tools for the treatment of large volumes of data are very useful in the search for these novel biomarkers.

Taking into account all the information presented above, the hypothesis of the present thesis is that **different nutritional and behavioral interventions cause modifications in the metabolome which can be elucidated by the application of omics techniques for the identification of new early biomarkers useful in clinical diagnosis.**

For this purpose, specific objectives were proposed:

1. **To identify novel circulating biomarkers of homeostasis disruption using non-targeted metabolomics techniques (CHAPTER 1)**

   1.1. **To evaluate the influence of chronic consumption of a diet that promotes the development of metabolic syndrome, namely cafeteria diet, and different intensities of physical exercise in the circulating metabolome (MANUSCRIPT 1)**

   Cafeteria feeding is an energetically dense diet composed of different highly palatable foodstuffs from which the animal can choose which ones to eat. Thus, the cafeteria diet-fed rodent reflects perfectly the eating habits responsible for many of the nutrition-related diseases in Western societies while it constitutes, after long-term administration, a robust model for the

study of metabolic syndrome developing all the complications present in humans. Consequently, we decided to start determining the metabolic effects of this type of feeding in combination with the regular practice of physical activity, which is known to be able to modulate many of the parameters contributing to the metabolic syndrome.

Therefore, in this first study, the following partial objectives were defined:

1.1.1.   To identify novel compounds in serum whose levels are altered as a consequence of the chronic intake of cafeteria diet.

1.1.2.   To investigate the metabolic changes associated with the practice of daily exercise to broaden the knowledge concerning the mechanisms of action.

1.1.3.   To explore whether significant variations exist between different intensities of physical training.

1.1.4.   To evaluate the differences in the exercise effect depending on the health state of the animals.

1.2. To select non-invasive biomarkers of early progression of lipid unbalances that reveal the etiology of the disorder (MANUSCRIPT 2)

The second investigation carried out using a non-targeted approach was designed aimed to elucidate specifically the metabolic alterations induced by a diet high in fats. On this basis, we decided to employ Golden Syrian hamster among other rodents because it offers unique advantages in the modeling of human diseases, particularly those related to lipid disturbances. An additional goal of this study was to discern whether the selected biomarkers come from the diet or whether these circulating metabolites are veritable biomarkers of dyslipidemia. For this task, we introduced a supplementary group in which the hyperlipidemia was generated by chemical treatment. Furthermore, circulating levels of these metabolites were evaluated over time in order to investigate the response of the biomarkers to both types of intervention.

2. To study in depth the metabolites selected in Objective 1 for the evaluation of their suitability as biomarkers (CHAPTER 2)

2.1. To determine accurately the circulating levels of these putative biomarkers using a targeted quantification method (MANUSCRIPT 3)

To meet the objective of Chapter 2, it was necessary to develop and validate an analytical method that was specific to the metabolites of interest. Once the methodology was optimized (**Annex I**), the research adressed in Manuscript 3 was able to focus on the accurate assessment of the specific changes undergone by the putative circulating biomarkers in the same cohort of animals of Manuscript 1, thus verifying the results obtained with the non-targeted evaluation and the adequacy of the molecules as representatives of the overall metabolome.

2.2. To determine the distribution of the selected biomarkers and evaluate the involvement of organs with a key role in their metabolism (MANUSCRIPT 4)

To complete the purpose of Chapter 2 and perform the quantification in tissues, we decided to design an animal experiment in which we deepened in the biological significance of the selected biomarkers.

3. To evaluate the adequacy of the putative biomarkers in a cohort of humans (MANUSCRIPT 4)

As last step, we intended to clarify the potential of the biomarkers in humans. For this purpose, we planified a preliminary study using a reduced human cohort. The results of this experiment would shed light in the potential use of these biomarkers in clinical diagnosis.

Hipòtesi i objectius

Existeixen proves creixents sobre la importància de la nutrició en el manteniment del benestar. A més a més, els mals hàbits alimentaris tenen un impacte directe en l'aparició de malalties no transmissibles d'alta prevalença en les societats desenvolupades, com per exemple les malalties cardiovasculars i els trastorns hepàtics. En aquesta línia, s'ha vist que els efectes beneficiosos de la nutrició només són efectius en les etapes inicials del trastorn, de forma correctiva i/o preventiva. No obstant, els biomarcadors tradicionals utilitzats actualment en el diagnòstic clínic indiquen una situació avançada de la malaltia, moment en el qual, els efectes nocius de la malaltia només es poden revertir mitjançant l'ús de fàrmacs. Per això, sorgeix la necessitat de trobar nous biomarcadors que ajudin al diagnòstic precoç de la patologia, quan la situació encara es pot controlar mitjançant bones pràctiques dietètiques i l'ús de suplements nutricionals. A banda d'això, també és important que la recollida de mostres per a avaluar els biomarcadors de salut es pugui realitzar mitjançant tècniques no invasives, que facilitin la posterior implementació en diagnòstic de forma efectiva. En aquesta línia, l'aparició de les ciències òmiques, juntament amb el desenvolupament d'equips i eines de gran precisió i sensibilitat per al tractament de grans volums de dades, són molt útils en la cerca d'aquests nous biomarcadors.

Tenint en compte tota la informació presentada anteriorment, la hipòtesi de la present tesi és que les **diferents intervencions nutricionals i de comportament causen modificacions en el metaboloma que es poden avaluar mitjançant l'aplicació de tècniques òmiques per a la identificació de nous biomarcadors primerencs útils en el diagnòstic clínic**.

Amb aquest propòsit, es van plantejar els següents objectius específics:

1. **Identificar nous biomarcadors circulants d'alteració de l'homeòstasi mitjançant tècniques de metabolòmica no dirigida (CAPÍTOL 1)**

   1.1. **Avaluar la influència del consum crònic d'una dieta que promou el desenvolupament de la síndrome metabòlica, la dieta de la cafeteria, així com diferents intensitats d'exercici físic en el metabolisme circulant. (MANUSCRIT 1)**

      La dieta de cafeteria es caracteritza per ser energèticament densa, formada per diferents aliments altament gustosos, dels quals l'animal pot triar els que es mengen. D'aquesta manera, el rosegador alimentat per la

dieta de cafeteria reflecteix perfectament els hàbits alimentaris responsables de moltes de les malalties relacionades amb la nutrició a les societats occidentals, alhora que, administrada a llarg termini, constitueix un model robust per a l'estudi de la síndrome metabòlica, desenvolupant totes les complicacions presents a els éssers humans. En conseqüència, vam decidir començar realitzant un primer estudi en el que es van determinar els efectes metabòlics d'aquest tipus d'alimentació en combinació amb la pràctica habitual d'activitat física, la qual se sap que pot modular molts dels paràmetres que contribueixen a la síndrome metabòlica.

Per tant, en aquest primer estudi, es van definir els següents objectius parcials:

1.1.1.   Identificar nous compostos en el sèrum els nivells dels quals es modifiquen com a conseqüència de la ingesta crònica de la dieta de cafeteria.

1.1.2.   Investigar els canvis metabòlics associats amb la pràctica d'exercici diari per tal d'ampliar el coneixement sobre els seus mecanismes d'acció.

1.1.3.   Explorar si existeixen variacions significatives entre diferents intensitats d'activitat física.

1.1.4.   Avaluar les diferències en l'efecte de l'exercici en funció de l'estat de salut dels animals.

1.2. Seleccionar biomarcadors no invasius de progressió precoç dels desequilibris lipídics que revelin l'etiologia del trastorn (MANUSCRIT 2)

La segona activitat realitzada amb un enfoc no dirigit va ser dissenyada per aclarir específicament les alteracions metabòliques induïdes per una dieta alta en greixos. Sobre aquesta base, vam decidir utilitzar el model de hàmster Golden Syrian entre altres rosegadors, ja que ofereix avantatges úniques en la simulació de malalties humanes, especialment les relacionades amb trastorns lipídics. Un objectiu addicional d'aquest estudi va ser discernir si els biomarcadors seleccionats provenien de la dieta o si per el contrari aquests metabòlits circulants eren realment biomarcadors

de dislipidèmia. Per aquesta tasca, es va introduir un grup suplementari en el qual la hiperlipidèmia es va generar mitjançant un tractament químic. A més, els nivells circulants d'aquests metabòlits es van avaluar a diferent temps per tal d'investigar la resposta d'aquests biomarcadors als dos tipus d'intervenció.

2. **Estudiar en profunditat els metabòlits seleccionats en l'Objectiu 1 per avaluar la seva idoneïtat com a biomarcadors (CAPÍTOL 2)**

2.1. **Determinar amb precisió els nivells circulants d'aquests biomarcadors putatius utilitzant un mètode de quantificació específic (MANUSCRIT 3)**

Per complir l'objectiu del Capítol 2, era necessari desenvolupar i validar un mètode analític específic dels metabòlits d'interès. Un cop optimitzada la metodologia (**Annex I**), la recerca realitzada al Manuscrit 3 va poder centrar-se en l'avaluació precisa dels canvis específics que van patir els possibles biomarcadors circulants en la mateixa cohort d'animals del Manuscrit 1, verificant així els resultats obtinguts amb la metabolòmica no dirigida i l'adequació de les molècules com a representants del metabolome global.

2.2. **Determinar la distribució dels biomarcadors seleccionats i avaluar la implicació d'òrgans amb un paper clau en el seu metabolisme (MANUSCRIT 4)**

Per completar el propòsit del Capítol 2 i realitzar la quantificació en teixits, vam decidir dissenyar un experiment animal en el qual aprofundir en la importància biològica dels biomarcadors seleccionats.

3. **Avaluar l'adequació dels biomarcadors putatius en una cohort d'humans (MANUSCRIT 4)**

Com a últim pas, ens vam proposar aclarir el potencial dels biomarcadors en humans. Amb aquesta finalitat, es va planificar un estudi preliminar mitjançant una cohort d'humans reduïda. Els resultats d'aquest experiment donarien llum al potencial ús d'aquests biomarcadors en el diagnòstic clínic.

3. RESULTS & DISCUSSION

CHAPTER 1

Non-targeted metabolomics studies in animal models of lipid disorder

Manuscript 1

Impact of a cafeteria diet and daily physical training on the rat serum metabolome

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Lluís Arola, Manuel Suárez

PREFACE

As a preliminary approach to the issue of interest, namely the search of novel biomarkers of homeostasis disruption, we decided to apply a holistic approximation by means of the use of non-targeted metabolomics. In the Introduction section we have already explained the great interest that this methodology has gained in the last years due to its potential to generate new hypotheses and enhance the knowledge in the biochemical field. Therefore, in **Chapter 1**, we intended to identify the effect that different nutritional and behavioural interventions had in the circulating metabolome, as an initial step in the process of searching of new biomarkers.

Specifically, in **Manuscript 1**, our main objective was to evaluate the impact of a chronic consumption of cafeteria diet (CAF) and exercise on the metabolome. We selected CAF-fed rat as animal model in our first study because it is considered an exceptional tool to study obesity and metabolic syndrome (MetS), which nowadays is pandemic in Western civilisation. Along with healthy dietary patterns, the practice of physical activity routinely is usually recommended for the prevention and treatment of MetS. Nevertheless, little is known regarding the relevance of physical exercise on the modulation of the metabolome. Therefore, different intensities of exercise carried out in treadmills without inclination were included in the experimental design. Female Sprague Dawley rats were selected because they tolerate higher intensities of forced exercise compared to males and are more active in the voluntary wheel running. The evaluation of these two interventions which deeply modify the lifestyle of the animals, both as individual factors and the interaction between them, allow us to obtain a general overview of the metabolome and thereby identifying the metabolites mostly affected by the treatments. The identification of these biomarkers gives us an idea of the biochemical processes affected by CAF and exercise and to assess whether these altered pathways differ between animals with and without MetS.

Impact of a cafeteria diet and daily physical training on the rat serum metabolome

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Abstract

Regular physical activity and healthy dietary patterns are commonly recommended for the prevention and treatment of metabolic syndrome (MetS), which is diagnosed at an alarmingly increasing rate, especially among adolescents. Nevertheless, little is known regarding the relevance of physical exercise on the modulation of the metabolome in healthy people and those with MetS. We have previously shown that treadmill exercise ameliorated different symptoms of MetS. The aim of this study was to investigate the impact of a MetS-inducing diet and different intensities of aerobic training on the overall serum metabolome of adolescent rats. For 8 weeks, young rats were fed either standard chow (ST) or cafeteria diet (CAF) and were subjected to a daily program of training on a treadmill at different speeds. Non-targeted metabolomics was used to identify changes in circulating metabolites, and a combination of multivariate analysis techniques was implemented to achieve a holistic understanding of the metabolome. Among all the identified circulating metabolites influenced by CAF, lysophosphatidylcholines were the most represented family. Serum sphingolipids, bile acids, acylcarnitines, unsaturated fatty acids and vitamin E and A derivatives also changed significantly in CAF-fed rats. These findings suggest that an enduring systemic inflammatory state is induced by CAF. The impact of physical training on the metabolome was less striking than the impact of diet and mainly altered circulating bile acids and glycerophospholipids. Furthermore, the serum levels of monocyte chemoattractant protein-1 were increased in CAF-fed rats, and C-reactive protein was decreased in trained groups. The leptin/adiponectin ratio, a useful marker of MetS, was increased in CAF groups, but

decreased in proportion to training intensity. Multivariate analysis revealed that ST-fed animals were more susceptible to exercise-induced changes in metabolites than animals with MetS, in which moderate-intensity seems more effective than high-intensity training. Our results indicate that CAF has a strong negative impact on the metabolome of animals that is difficult to reverse by daily exercise.

Keywords: metabolic syndrome; cafeteria diet; physical activity; treadmill; non-targeted metabolomics; circulating metabolome; dyslipidemia

1. Introduction

Metabolic syndrome (MetS) is a combination of metabolic disturbances, including insulin resistance, dyslipidemia, obesity and hypertension, that is becoming increasingly prevalent in our society due to sedentary lifestyles and dietary patterns [1–3]. Considering that this disorder may result in cardiovascular disease [4] and type II diabetes [5], great efforts are being made to prevent its development. It has been shown that physical activity provides beneficial effects, including weight loss, changes in body fat percentage, and improvements in blood pressure, lipoprotein profile, cholesterol levels and insulin sensitivity [6–8]. Particularly in adolescents, the substitution of sedentary habits such as watching television for many hours with increased physical activity is the main strategy for the prevention and treatment of obesity [9].

A good model for studying MetS is the cafeteria diet (CAF)-fed rodent, as it develops all the complications present in the human syndrome [10,11]. The CAF-fed rat model is a robust model for diet-induced human obesity associated with muscle, adipose tissue and serum inflammation that leads to the development of MetS [12]. Cigarroa *et al.* [13] showed that CAF administered to weaning rats for 8 weeks induced a sharp rise in body and relative retroperitoneal white adipose tissue (% RWAT) weight, showing these animals a significant increase of 30% and 120% on these parameters, respectively, when compared with rats fed ST. Furthermore, CAF intake during this period of time also produced hyperleptinemia, hypertriglyceridemia, hyperglycemia and insulin resistance. Although in that study, a treadmill intervention at low (12 m/min) or high (17 m/min) intensity did not produce a body weight-lowering effect in CAF-fed animals, we demonstrated that daily physical training had beneficial effects on RWAT weight and triglycerides, partially counteracting the increase in these parameters induced by CAF. In the present study, we provide an omics approach to investigate, in the same cohort of animals, the impact of the chronic intake of CAF and different intensities of daily exercise on the overall serum metabolome of adolescent rats.

Some researchers have attempted to identify distinctive metabolite profiles associated with obesity in adults [14] and youths [15]. All these studies point to the importance of biomarker discovery for early diagnosis, treatment and assessment of lifestyle-related diseases. Recent studies have applied comprehensive metabolomics to uncover metabolite perturbations in CAF-fed rats [12,16] and in trained rodents [17,18]; however the combined influence of CAF and daily physical activity on the circulating metabolome has not yet been investigated.

The objectives of this study were (1) to identify novel circulating metabolites influenced by chronic CAF intake in rats, (2) to elucidate further changes in the metabolome that may help us understand the mechanisms whereby periodic training exerts its beneficial (or detrimental) effects in young animals, (3) to examine whether differences exist between the effects of low- and high-intensity exercises, and (4) to verify whether these biochemical processes differ between animals with and without MetS.

Our goal was accomplished using liquid chromatography coupled to mass spectrometry (LC-MS/MS), a powerful technique very suitable for metabolomics studies that allows the identification of molecular alterations among experimental groups. The use of comparative untargeted metabolomics allows for the identification of potential biomarkers and characteristic metabolic signatures that may be predictive of the health status of the animals.

2. Material and methods

2.1. Chemicals

Acetonitrile (Merck, Darmstadt, Germany), glacial acetic acid (Panreac, Barcelona, Spain), methanol and formic acid (Scharlab S.L., Barcelona, Spain) were of high-performance liquid chromatography (HPLC) analytical grade. Ultrapure water was obtained using a Milli-Q advantage A10 system (Madrid, Spain). For mass spectrometry, paracetamol and pyrocatechol (Fluka/Sigma-Aldrich, Madrid, Spain) were used as internal standards in positive (+ESI) and negative (-ESI) electrospray ionization mode, respectively. Both were dissolved in methanol at 1 mg/mL and stored at -20°C.

2.2. Animals, diets and training sessions

Female Sprague-Dawley rats weighting 62 ± 2 g were weaned at 21-23 days of age and housed 2 per cage at 22°C with a light/dark period of 12 h. Female animals were selected because they tolerate higher intensities of forced exercise compared to males and are more active in the voluntary wheel running [19,20]. The animals were randomly distributed into 6 groups (n=9-12) according to the diet (ST or CAF) and the intensity of the treadmill intervention received during 8 weeks: control-ST (CON-ST), treadmill-low intensity-ST (TML-ST), treadmill-high intensity-ST (TMH-ST), control-CAF (CON-CAF), treadmill-low intensity-CAF (TML-CAF) or treadmill-high intensity-CAF (TMH-CAF).

The CAF included the following components (quantity per rat/day): ST (6-10 g), bacon (8-12 g), biscuits with pâté (12-15 g) or cream cheese (10-12 g), sweet roll (8-10 g), carrot (6-9 g) and milk with sugar (220 g/L; 50 mL). ST had a calorie breakdown of 24%

protein, 18% fat and 58% carbohydrates, whereas CAF had 10% protein, 41% fat and 49% carbohydrates [21]. The animals consumed tap water and diet *ad libitum* throughout the experiment, and CAF was renewed daily.

Training sessions were performed as previously described [13]. Briefly, the rats were trained on a treadmill 5 days per week for 30 min. Initially, the animals were accustomed to the treadmill (0 m/min), and the speed was progressively increased until it reached 12 m/min in TML and 17 m/min in TMH groups. These values were maintained until the end of the experiment. Neither electrical shock nor physical prodding was used to motivate the animals. The control rats stayed on the treadmill (0 m/min) for an equivalent amount of time as the trained rats.

The animals were fasted for 12 h and then sacrificed by beheading to avoid interferences in the circulating metabolome due to drugs. Total blood was collected and serum was obtained by centrifugation at 2,000 g for 15 min after 1 h at room temperature.

2.3. Ethics statement

All procedures were approved by the Generalitat de Catalunya (DAAM 6836) and they have been performed in accordance with the European Communities Council Directive (86/609/EEC).

2.4. Determination of serum lipids, hormones and cytokines

Enzymatic kits were used to determine triglycerides, total cholesterol (QCA, Barcelona, Spain) and high- and low-density lipoprotein cholesterols (HDLc and LDLc; Spinreact, Girona, Spain). Leptin, adiponectin, C-reactive protein (CRP) (Millipore, Barcelona, Spain) and monocyte chemoattractant protein-1 (MCP-1) (Thermo Fisher Scientific, Pittsburgh, USA) were measured using rat ELISA kits. The irisin levels were determined using a human/rat/mouse ELISA kit (Phoenix Pharmaceuticals Inc., Burlingame, California, USA).

2.5. Sample preparation for metabolomics analysis

Metabolites were extracted from serum using a hydroalcoholic solution. 800 µL of methanol:water (8:1 vol/vol) were added to 100 µL of serum, and 50 µL of pyrocatechol (1 ppm) and 50 µL of paracetamol (1 ppm) were added as internal standards. The mixture was homogenized by vortexing (30 s) and ultrasonication (30 s). Then, samples were incubated on ice for 10 min to precipitate proteins and centrifuged at 19,500 g for 10 min at 4°C. The

supernatant was collected and dried under nitrogen flow to eliminate the solvent. Finally, dried samples were re-dissolved in 200 µL of methanol:water (8:1 vol/vol) prior to injection.

2.6. LC-MS and LC-MS/MS analyses

Non-targeted analysis of the serum extracts was performed using an HPLC 1200 series coupled to an ESI-TOF 6210 (Agilent Technologies, Palo Alto, California, USA). Each sample (7.5 µL) was injected into a Zorbax SB-Aq (3.5 µm particle size, 2.1 mm internal diameter x 150 mm length) chromatographic column equipped with a Zorbax SB-C18 (3.5 µm, 2.1 x 15 mm) pre-column, also from Agilent Technologies. The ionization in the mass spectrometer was performed in +ESI and -ESI to cover all the ranges of metabolites. In +ESI, the mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). In -ESI, solvent A was 0.2% acetic acid, and solvent B was acetonitrile. The chromatographic separation was performed using a continuous gradient elution at flow rate of 0.4 mL/min starting at 10% B and increasing to 100% B over 45 min, after which time it was maintained for 10 min. The ionization source parameters were as follows: nitrogen was used as the nebulizer gas with a pressure of 45 psi; desolvation gas flow rate, 9 L/min at 325°C; source temperature and gas flow rate, 150°C and 12 L/min, respectively; capillary voltage 4 kV; and fragmentor was set to 125 V. LC-MS accurate mass spectra were acquired from 50 to 1200 *m/z* at a scan rate of 1 spectra/s. A reference solution was used for the continuous calibration using the following reference masses: 121.0509 and 922.0098 *m/z* for +ESI and 119.0363 and 980.0164 *m/z* for -ESI.

For targeted analyses, extracts were injected in a UHPLC 1290 series coupled to a Q-TOF 6550, also from Agilent Technologies, operated either in MS or MS/MS modes. The LC-MS/MS analyses were performed using the same analytical conditions described above. The data were collected in the range of *m/z* 100-1000 with a scan rate of 1.5 spectra/s. The MS/MS spectra of metabolites were obtained by different collision energies (10, 20 and 40 eV).

2.7. Untargeted data processing and metabolite identification

All the software programs used to process the data were also from Agilent Technologies. Untargeted data were acquired using MassHunter Data Acquisition, whereas Qualitative Analysis was used to obtain the molecular features of the samples using the 'Molecular Feature Extractor' algorithm, which removes the background ions and groups related ions in a unique feature based on the presence of adducts and dimers, the isotopic distribution and the charge-state envelope. Mass Profiler Professional was used to perform the alignment of the features present in the chromatograms and to carry out multivariate

statistical analysis. Data from each ionization mode, positive and negative, were analysed separately. A list of chemical entities was obtained, and only those features with a minimum of 2 related ions were selected for the subsequent chemometric analysis. Multiple charge states were not considered. The retention time and mass window used for alignment were 0.5% ± 0.15 min and 10.0 ppm ± 2.0 mDa, respectively. Abundance values corresponding to each entity were transformed to a base-2 logarithm and normalized to the internal standard. Uncommon features were discarded, and only those that were found in at least 75% of the samples within the same group were selected.

A multivariate analysis based on a combination of hierarchical clustering analysis (HCA), principal components analysis (PCA), and partial least squares for discriminant analysis (PLS-DA) was performed to evaluate the influence of diet and physical exercise on the metabolome. The HCA was conducted on both features and conditions. The distance metric selected for the analysis was euclidean, and the linkage rule was centroid. Analysis of variance (ANOVA) was used to detect significant differences in the normalized abundances of the filtered features. To handle false discovery rates from multiple comparisons, the cut-off point for significance was calculated according to the Benjamini-Hochberg correction [22] at a level of 5%. All significant masses were extracted manually from the chromatograms to verify that they were in sufficient abundance in the spectra at the specific retention time to be subsequently fragmented by LC-MS/MS. After cleaning the spectrum for each entity, the molecular formula generator algorithm included in the MassHunter Qual software was used to obtain a list of candidate molecular formulas. This algorithm considers more information contained in the spectra than the value of the monoisotopic mass (isotope abundance, spacing between isotope peaks, etc.) [23]. Taking into consideration all the information about the nature of the entities, various databases such as METLIN [24], HMDB [25], LIPID MAPS [26] and KEGG [27] were used for the tentative identification of the molecules. Finally, to confirm the identity of the metabolites of interest, LC-MS/MS was used to obtain the fragmentation patterns of the molecules and these were compared with spectral information for the candidates.

2.8. Univariate statistical analysis

After this previous screening, several metabolites were identified and studied more thoroughly. The differences among groups were assessed using one- and two-way ANOVA. First, two-way ANOVA was carried out to evaluate the effects of diet, exercise and their interactions. The results were reported in tables and bar charts with italic capital letters indicating a significant effect of diet (*D*), exercise (*E*) or their interaction (*DxE*). When one or both main effects were statistically significant, one-way ANOVA was used to determine the

differences between the means. When only the interaction between diet and exercise was statistically significant according to the two-way ANOVA model, one-way ANOVA was carried out to compute pairwise comparisons between diet groups (i.e., the effect of exercise within diet groups). The assumption of normality was determined using the Shapiro-Wilk test, and the homoscedasticity between groups was determined using Levene's test. Tukey *post hoc* contrast was used when variances between groups were similar, and Dunnett's T3 test was used if this assumption was not fulfilled. Data are expressed as the means with their standard errors (SEM), and the results of the *post hoc* contrasts are shown using lower-case letters. Student's t-test was used for single statistical comparisons. A two-tailed value of $p < 0.05$ was considered statistically significant for all tests. Statistical analyses were performed using the Statistical Package for Social Sciences (IBM SPSS Statistics, ver19.0).

3. Results

3.1. Targeted analysis of serum metabolites

3.1.1. Dyslipidemic parameters

We have previously shown in Cigarroa *et al.* [13] that CAF-fed rats have higher amounts of triglycerides in their serum than animals fed ST, and this increase gradually drops with exercise until triglycerides nearly return to ST-fed levels in the TMH-CAF group (**Fig. 1A**). CAF feeding also had significant effects on the remaining dyslipidemic variables ($p < 0.05$, two-way ANOVA). Curiously, CAF-fed animals had lower circulating levels of total cholesterol, HDLc and LDLc than ST-fed groups. (**Fig. 1B-D**). Interestingly, although LDLc levels remained unchanged among ST-fed animals, a significant decrease was detected in the serum of CAF-fed rats as a result of running at low-intensity compared to the sedentary group ($p = 0.04$, Student's t-test) (**Fig. 1D**).

A *Triglycerides*

B *Total cholesterol*

C *HDL cholesterol*

D *LDL cholesterol*

☐ CON-ST ▨ TML-ST ▩ TMH-ST ☐ CON-CAF ▨ TML-CAF ▩ TMH-CAF
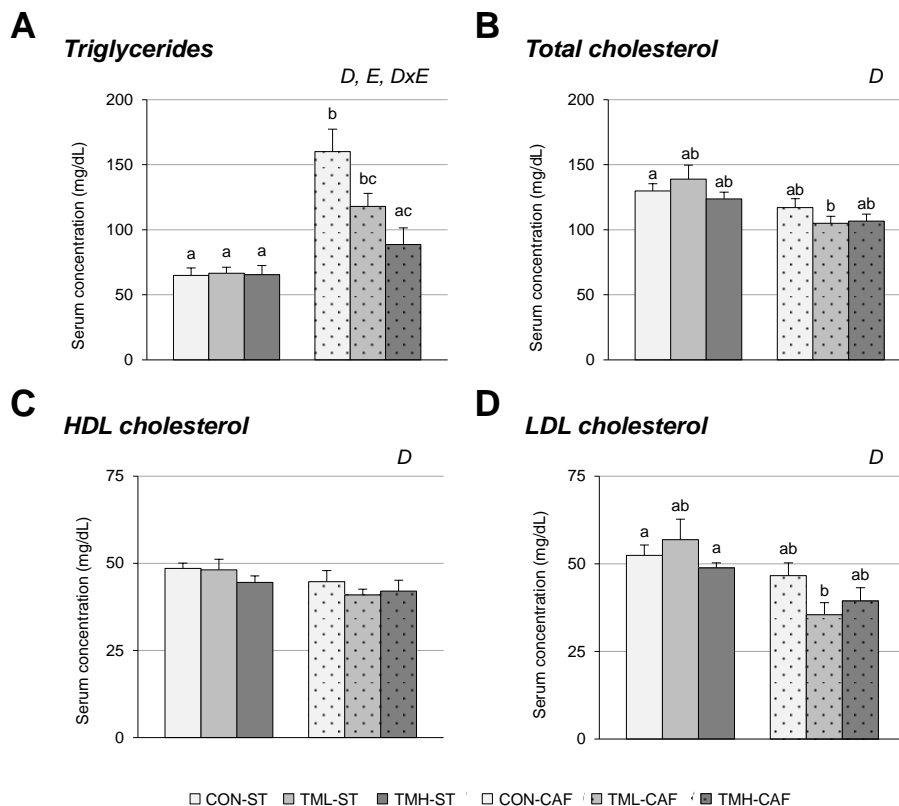
Figure 1. Dyslipidemic parameters. Animals were distributed into 6 groups (n=9-12) based on diet and training condition: control-standard diet (CON-ST), treadmill-low intensity-standard diet (TML-ST), treadmill-high intensity-standard diet (TMH-ST), control-cafeteria diet (CON-CAF), treadmill-low intensity-cafeteria diet (TML-CAF) and treadmill-high intensity-cafeteria diet (TMH-CAF). Diets and training sessions started after the weaning period and were extended for 8 weeks. Lipid serum concentrations were determined at the end of the experiment after 12 h of fasting. The data are given as the mean ± SEM. The statistical comparison among groups was conducted using two- and one-way ANOVA. *D*: the effect of diet; *E*: the effect of exercise; *DxE*: the interaction between the two main factors. [abc]Mean values with different small letters indicate significant differences between groups (one-way ANOVA and Dunnett's T3 *post hoc* contrast, p<0.05).

3.1.2. Leptin/adiponectin ratio (LAR)

We observed a clear effect of both CAF feeding (p<0.01) and physical exercise (p<0.05, two-way ANOVA) on LAR (**Fig. 2A**). This index was higher in CAF-fed rats than in ST-fed animals, and this increase was progressively reversed by the practice of running until it practically returned to ST-fed levels in the animals trained at high-intensity. Moreover, LAR was the only parameter on which physical exercise had significant effects in ST-fed groups. The progressive reduction of the ratio due to training was observed in animals regardless of diet.

3.1.3. Circulating levels of the exercise hormone irisin

Contrary to what was expected, the levels of irisin were largely affected by diet (p<0.01, two-way ANOVA) and not by physical training. As shown in **Fig. 2B**, CAF feeding induced elevated circulating levels of this hormone, especially in the group of high-intensity runners.
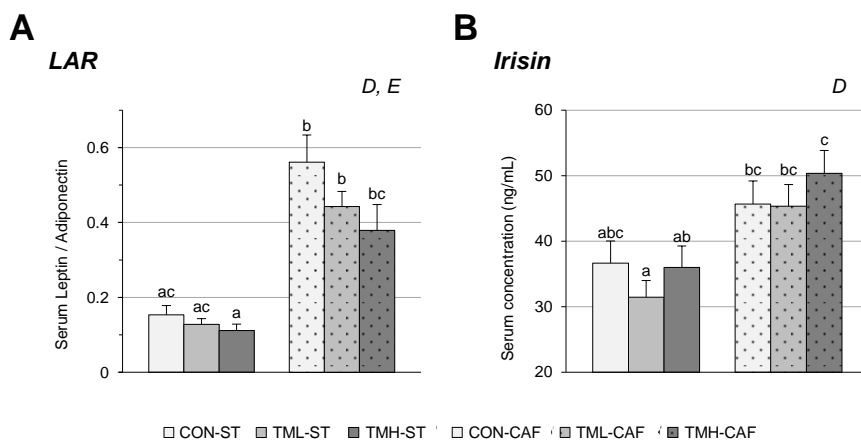
Figure 2. Circulating levels of hormones associated with lifestyle-related diseases. Animals were fed standard chow (ST) or cafeteria diet (CAF) for 2 months and periodically trained on a treadmill at different intensities (CON: 0; TML: 12; TMH: 17 m/min). The serum levels of adiponectin, leptin and irisin were determined at the end of the experiment after 12 h of fasting. The data are given as the mean ± SEM (n=9-12). The statistical comparison among groups was conducted using two- and one-way ANOVA. *D*: the effect of diet; *E*: the effect of exercise. [abc]Mean values with different small letters were significantly different (one-way ANOVA and Tukey/ Dunnett's T3 *post hoc* contrast, p<0.05).

3.1.4. Systemic inflammatory levels

Due to the pro-inflammatory effects of CAF consumption in rodents, the serum concentrations of two inflammatory markers were assessed (**Fig. 3**). The CAF-fed animals had increased levels of the chemotactic cytokine MCP-1 (p=0.04, two-way ANOVA). Although no significant effect of physical training on MCP-1 was established by two-way ANOVA, CAF-fed animals showed a significant decrease due to the treadmill intervention at high-intensity relative to moderate training (p=0.04, Student's t-test) (**Fig. 3A**). Contrasting MCP-1, serum levels of the cytokine CRP were influenced only by physical exercise (p=0.04, two-way ANOVA). In ST-fed rats, the exercise produced a similar decline (approximately 45%) in CRP levels in both trained groups relative to those of the CON-ST animals. Remarkably, CRP values of CAF-fed rats experienced the highest decrease (of 60%) relative to the sedentary group as a consequence of moderate training (p<0.05, Student's t-test), whereas TMH-CAF levels were similar to those for the CON-CAF group (**Fig. 3B**). These findings suggest that the two training intensities did not yield similar effects on inflammation levels in CAF-fed animals.
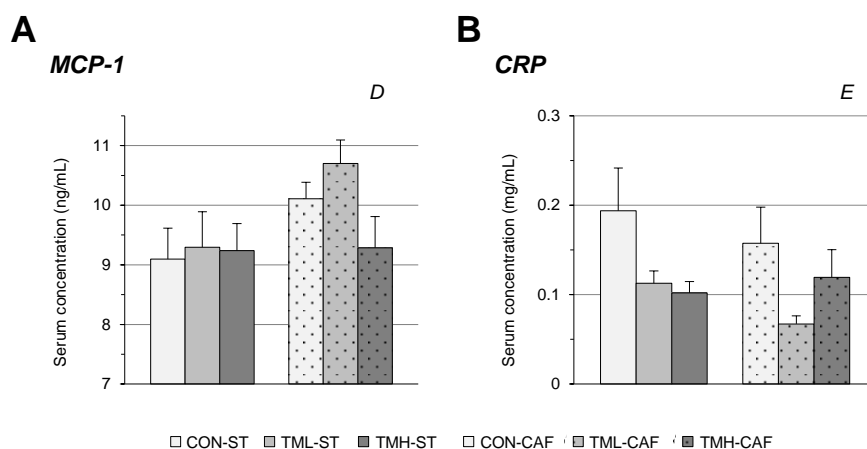
Figure 3. Circulating inflammatory markers. Animals were fed standard chow (ST) or cafeteria diet (CAF) and trained 5 days per week for 30 min on a treadmill at different intensities (CON: 0; TML: 12; TMH: 17 m/min). Diets and training sessions were continued for 8 weeks. The serum levels of the cytokines monocyte chemoattractant protein-1 (MCP-1) and C-reactive protein (CRP) were determined at the end of the experiment. The data are given as the mean ± SEM (n=9-12). *D:* the effect of diet; *E:* the effect of exercise (p<0.05, two-way ANOVA).

3.2. Untargeted evaluation of circulating metabolome

Analysis of the LC-ESI-MS data revealed that 6,594 molecular features were aligned in +ESI. From these, 1,207 features were present in at least 75% of the samples within the same group. These features were selected for subsequent analysis. Similarly, when analysing data from -ESI 5,973 molecular features were aligned. Of these, the 1,479 features present in at least 75% of the samples within the same group were selected.

By means of two-way ANOVA adjusted using the Benjamini-Hochberg correction, we found 87 and 160 metabolites with altered concentration between groups (p(Corr)<0.05) in +ESI and -ESI, respectively. As shown in the Venn diagram (**Fig. 4**), these significant differences were mainly due to diet rather than physical exercise in +ESI. However, in -ESI, there were no significant differences observed due to exercise.

POSITIVE IONIZATION   **NEGATIVE IONIZATION**
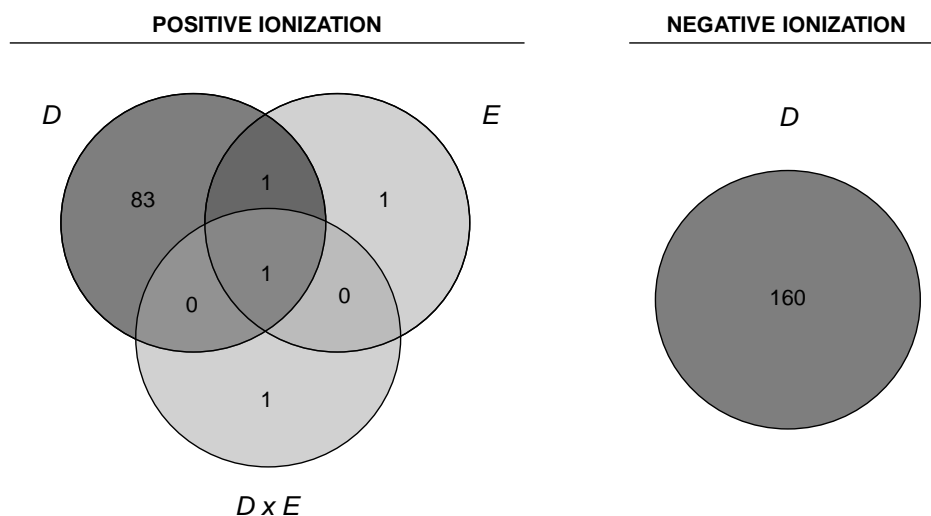
Figure 4. Venn diagrams showing the number of significant entities from each experimental parameter. Data from each ionization mode, positive and negative, were analysed using two-way ANOVA. To handle false discovery rates from multiple comparisons, the cut-off point for significance was calculated according to the Benjamini-Hochberg correction at a level of 5%. *D*: the effect of diet; *E*: the effect of exercise; *DxE*: the interaction between the two main factors. The areas where the circles overlap show the number of significant entities shared by the parameters.

To evaluate the influence of daily exercise and chronic intake of CAF on the metabolome, we used a combined multivariate analysis. The relative average normalized abundances of the differential metabolites were plotted using a heat map (**Fig. 5A and 5D**),

and in both ionization modes, the importance of the two parameters was evident, although the influence of diet was far greater than those exerted by training. HCA from +ESI data also indicated that the effect of exercise was more marked when animals were fed ST rather than CAF. Interestingly, while both exercise intensities caused a similar change in the metabolome of ST-fed groups, when animals were fed CAF, the practice of running at a low speed influenced the metabolome in a diverse manner compared to what happened in the other two groups of CAF-fed rats, approaching the TML-CAF metabolome to ST-fed rats (**Fig. 5A**). PCA of +ESI and -ESI data, explaining a 43% and 47% of the variance, respectively, resulted in a clear clustering of animals depending on diet but not on physical activity (**Fig. 5B and 5E**). PLS-DA was used to assess whether the 6 groups could be clustered separately using a supervised approach (**Fig. 5C and 5F**). The classification accuracies associated to the predictive PLS-DA model were 82% and 85% in +ESI and -ESI, respectively, suggesting an acceptable performance and therefore a good clustering trend.
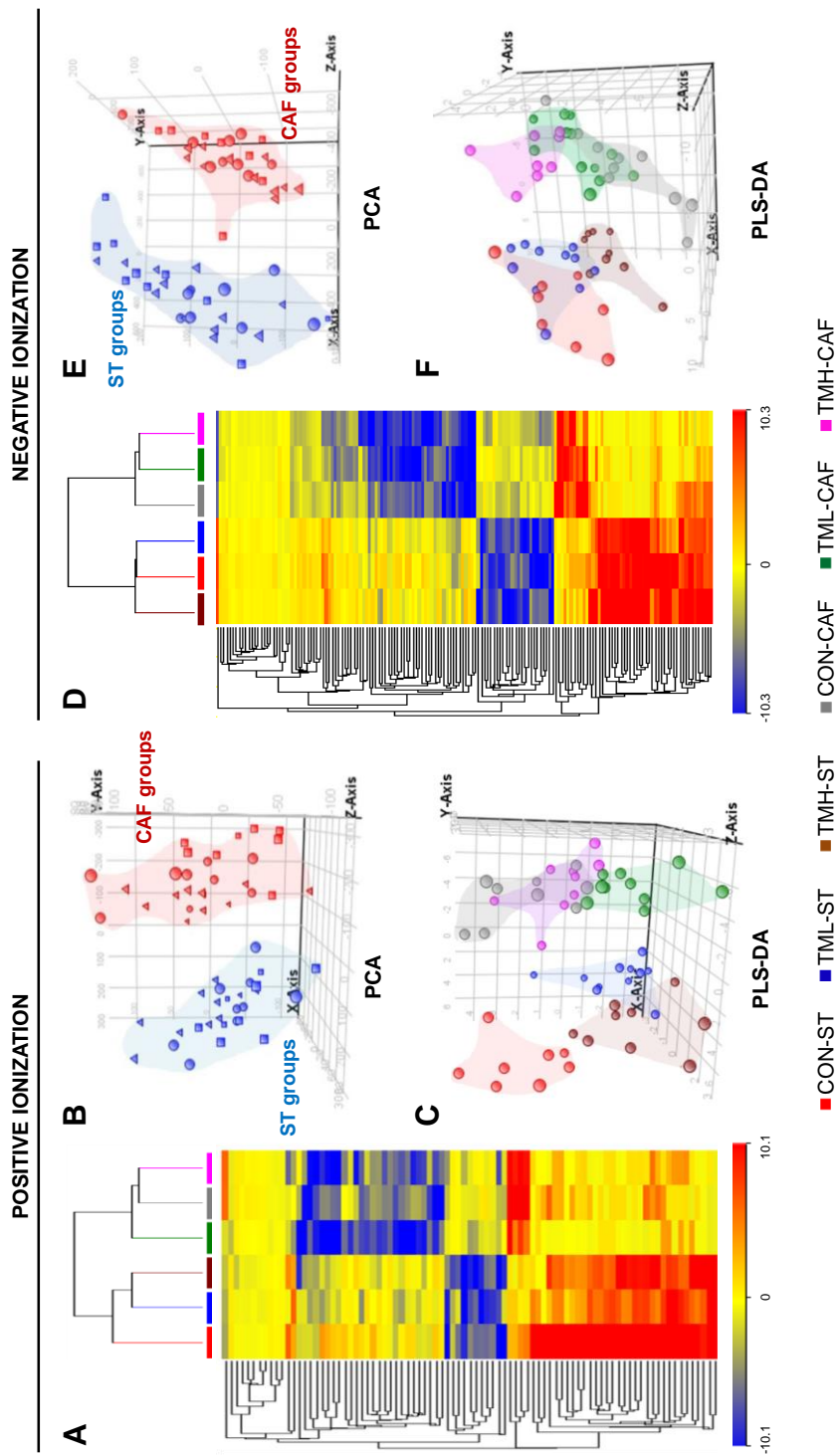
Figure 5. Multivariate analysis demonstrating the effect of diet and physical exercise on the animal metabolome. Serum extracts were analysed using LC-ESI-MS in both positive and negative ionization modes. (**A, D**) Heat map representations of hierarchical clustering of significant entities found in each group of animals. Each row represents an exact mass coloured by its abundance intensity, normalized to an internal standard and baselined to the mean of all samples. The scale from -10 (blue) to +10 (red) represents this normalized abundance in arbitrary units. The PCA (**B, E**) and PLS-DA (**C, F**) graphs show that the effect of cafeteria diet had prevalence over the periodic training on the treadmill at diverse intensities. Abbreviations: ST, standard chow; CAF, cafeteria diet; CON, control animals; TML, treadmill-low intensity runners; TMH, treadmill-high intensity runners.

3.2.1. Metabolites associated with cafeteria diet consumption

Having determined the entities that were significantly modified by diet, a tentative identification of their nature was carried out. This identification was performed by LC-ESI-MS/MS using a comparison of exact mass, retention time, and spectral and fragmentation information with those in metabolite databases (**Table 1**). The results indicate that CAF altered the levels of seven of the eight lipid categories included in LIPID MAPS: glycerophospholipids, mainly lysophosphatidylcholines (Lyso-PC); sphingolipids and glycerolipids, both increased in CAF-fed animals; sterols such as bile acids, prenol lipids including vitamin E derivatives and retinoids and a flavonoid named equol that belongs to the polyketides category, all decreased after chronic intake of CAF; and a fatty acyl category consisting of unsaturated free fatty acids and acylcarnitines, which decreased and increased, respectively, in the serum of CAF-fed animals. Lyso-PC was the most represented family influenced by diet with a heterogeneous regulation in response to CAF feeding.

Table 1. Identified metabolites that differed significantly with the diet

Category	Compound	Entity (M@RT)	Fragmentation pattern [Δ]	CE (V)	Formula	Abundance in CAF groups
Glycerophospholipids	Lyso-PC (16:1)	493.3171@22.3	184.1, 104.1, 86.1	40	$C_{24}H_{48}NO_7P$	Increased
	Lyso-PC (17:1)	507.3325@23.5	184.1, 104.1, 86.1	40	$C_{25}H_{50}NO_7P$	
	Lyso-PC (18:0)	523.2944@24.1	184.1, 104.1, 86.1	40	$C_{26}H_{54}NO_7P$	
	PAF	637.3937@27.5	184.1, 86.1	20	-	
	Lyso-PE (O-18:0)	467.3378@25.4	447.3, 429.3	20	$C_{23}H_{50}NO_6P$	Decreased
	Lyso-PC (20:2)	547.364@25.7	184.1, 104.1, 86.1	40	$C_{28}H_{54}NO_7P$	
	Lyso-PC (20:1)	549.3788@27.2	184.1, 104.1, 86.1	40	$C_{28}H_{56}NO_7P$	
Sphingolipids	Sphinganine	301.2975@21.7	60.0, 284.3, 254.3	20	$C_{18}H_{39}NO_2$	Increased
	Sphinganine-1P	381.2639@21.3	266.3, 284.3	20	$C_{18}H_{40}NO_5P$	
Glycerolipids	MG (16:0)	330.2759@30.1	57.1, 71.1, 85.1	20	$C_{19}H_{38}O_4$	Increased
Sterol lipids	Glycocholic acid	465.3089@17.2	76.0, 209.1, 319.2, 337.2, 412.3	40	$C_{26}H_{43}NO_6$	Decreased
	Hydroxylated bile acid	406.2714@18.2	145.1, 107.1, 197.1, 81.1	40	$C_{24}H_{38}O_5$	
	Hydroxylated bile acid	390.2775@22.2	145.1, 109.1, 57.1, 199.1	40	$C_{24}H_{38}O_4$	

Category	Compound	$M@RT$	Fragments	CE	Formula	Change
PUFAs	Linolenic acid (18:3)	278.2242@30.2	81.1, 95.1, 67.1, 121.1	20	$C_{18}H_{30}O_2$	Decreased
	DHA (22:6)	328.2391@31.7	91.1, 79.1, 67.1, 55.1	40	$C_{22}H_{32}O_2$	
MUFAs	DiHOME (18:1)	314.2465@22.2	67.1, 81.1, 279.2	20	$C_{18}H_{34}O_4$	Decreased
Carnitines	Butyryl carnitine	231.1471@1.99	85.0, 57.0	40	$C_{11}H_{21}NO_4$	Increased
	Myristoyl carnitine	371.3033@22.3	85.0, 60.1	40	$C_{21}H_{41}NO_4$	
Prenol lipids	γ-CEHC	264.1365@16.1	151.1, 123.1, 95.1, 67.1	40	$C_{15}H_{20}O_4$	Decreased
	α-CEHC	278.1514@17.6	165.1, 137.1, 122.1, 67.1	40	$C_{16}H_{22}O_4$	
	Retinoyl glucuronide	476.2396@17.7	255.2, 301.2	20	$C_{26}H_{36}O_8$	
Flavonoids	Equol	242.0953@11.4	123.0, 133.0, 107.0	10	$C_{15}H_{14}O_3$	Decreased
Other benzenoids	Phenyl butyrate	164.0834@17.6	77.0	40	$C_{10}H_{12}O_2$	Decreased
	Hippuric acid	179.059@4.49	77.0, 51.0, 105.0	40	$C_9H_9NO_3$	Decreased

‡Each entity comprises a particular neutral mass (M) and retention time (RT), and both values are separated by the symbol "@"

ΔDistinctive fragments from LC-+ESI-MS/MS analysis at the most appropriate collision energy (CE) for each molecule

Abbreviations: CAF, cafeteria diet; Lyso-PC, lysophosphatidylcholine; PAF, platelet activating factor; Lyso-PE, lysophosphatidylethanolamine; MG, monoacylglycerol; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acid; DiHOME, dihydroxy-octadecenoic acid; γ-CEHC, gamma-tocopheronolactone; α-CEHC, alpha-tocopheronolactone.

3.2.2. Changes in metabolome related to physical activity

Focusing on the metabolites on which physical exercise had a significant effect, four biomarkers (**Fig. 6A, 6B, 6D and 6E**) were identified. Of these, three were directly modified by exercise (retinoyl glucuronide, a lysophosphatidylethanolamine, and a bile acid), as their serum concentrations significantly decreased with the practice of running in both ST and CAF-fed rats. A significant interaction between diet and exercise was found for the levels of stearoylcarnitine, which were significantly lower in TML-CAF rats compared to those of the CON-CAF and the TMH-CAF groups (**Fig. 6D**).

Furthermore, the HCA of CAF-fed groups indicates that there is a differential effect on the metabolome between the two groups of rats submitted to different intensities of exercise. Contrary to what was expected, the more intense training approaches the metabolome of TMH-CAF rats to the CON-CAF group. This may suggest that the level of exercise influences the metabolome in a different manner, and essential information, such as exercise biomarkers, may be masked and lost. Therefore, we continued the restrictive statistical analysis comparing the CON conditions with each training level separately. Six additional exercise biomarkers were discovered, from which five were effectively identified (**Fig. 6C and 6F-I**). Again, all metabolites were lipids, namely a steroid hormone derivative, the dihydroxy-octadecenoic acid known as DiHETE, two phosphatidylglycerols, and a sexual hormone derivative. All of these were down-regulated by exercise: the bile acid, both phosphatidylglycerols and DiHETE were reduced by low-intensity running, and the unidentified sexual hormone metabolite was reduced by high-intensity running.
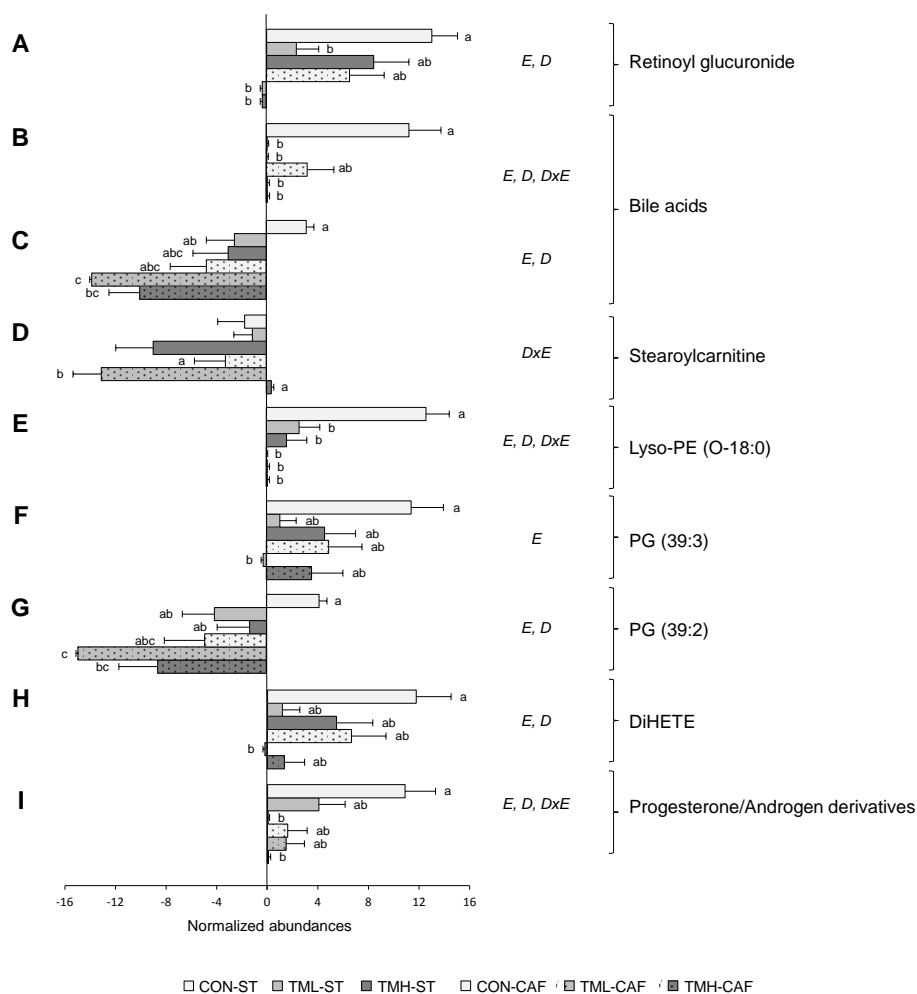
Figure 6. Identified metabolites altered with the periodic practice of running on a treadmill at diverse intensities. The abundance values for each metabolite were transformed to a base-2 logarithm, normalized by the internal standard, and baselined to the mean of all samples. The data are given as the mean ± SEM (n=9-12). To avoid the occurrence of false positives, a primary screen was carried out using two-way ANOVA and adjusting the p-value of the exercise parameter using the Benjamini-Hochberg correction. To more exhaustively evaluate the differences among groups, a posterior analysis without a p-value correction was performed. *E*: the effect of exercise; *D*: the effect of diet; *DxE*: the interaction between the two factors (two-way ANOVA, p<0.05). [abc]Mean normalized values with different small letters were significantly different (one-way ANOVA and Tukey/Dunnett's T3 *post hoc* contrast, p<0.05). Abbreviations: Lyso-PE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; DiHETE, dihydroxy-eicosatetraenoic acid.

4. Discussion

This study is the first to apply a non-targeted metabolomics approach to investigate the impact of the combination of CAF and regular exercise training on the circulating metabolome. All of the animals within each group responded consistently to the treatments, ranging the variance from 1.9 to 4.7 % RSD in relation to body weight gain [13]. This suggests that all the differences observed in the metabolome are due to the treatments evaluated, diet and exercise. Thus, we have shown that CAF feeding administered from a weaning age and continued until late adolescence leads to the accumulation of acylcarnitines in the serum of rats (**Table 1**), suggesting a disruption in fatty acid oxidation. This is in accordance with Sampey *et al.* [12], who showed that this increase is also appreciable in muscle and white adipose tissue of CAF-fed rats, suggesting that the results found in the blood reflects the tissue's acylcarnitine metabolism. Human studies have also revealed the implications of acylcarnitines in youth with obesity and diabetes [15]. Interestingly, herein, the practice of running at low-intensity had an opposite effect if we consider the decreased circulating abundance of stearoylcarnitine in low-intensity runners fed CAF (**Fig. 6D**). Our results suggest that the chronic exposure of muscle to elevated lipids resulted in the over-expression of genes involved in fatty acid β-oxidation leading to the accumulation of acylcarnitines, and exercise might ameliorate mitochondrial activity and counteract this over-expression [28].

We found two sphingolipids, sphinganine and sphinganine-1-phosphate, whose levels were elevated in CAF-fed animals (**Table 1**). Sphingolipids are synthetized in hepatocytes in a process that involves inflammatory cytokines and are then incorporated into lipoproteins. The alteration of the lipoprotein composition may be responsible for the increased atherogenicity of these particles [29,30]. Relatedly, previous studies have shown that sphingolipids contribute not only to atherosclerosis but also to insulin resistance, diabetes and cancer [31–33]. Specifically, plasma sphingolipids have been identified as biomarkers of MetS in non-human primates by Brozinick *et al.* [34]. Furthermore, sphinganine-1-phosphate is also released by erythrocytes, and this release is accompanied by an increase in sphingosine, a strong extracellular messenger involved in important cellular functions such as cell migration, proliferation and death [35].

We also detected changes in markers of vitamin A and E consumption and metabolism as a consequence of both CAF and treadmill interventions. The reduction in the serum concentration of retinoyl glucuronide as a consequence of CAF intake (**Table 1**) can be a signal of vitamin A deficiency. Its role as an exercise biomarker (**Fig. 6A**) may be explained by its capability to serve as reservoir of retinoic acid, which is involved in the

regulation of energy homeostasis and insulin responses [36]. On the other hand, α- and γ-tocopheronolactone (α-CEHC and γ-CEHC, respectively) are synthesized from dietary α- and γ-tocopherol, respectively. Many lines of evidence suggest that α-tocopherol has an inhibitory effect on the conversion of arachidonic acid to pro-inflammatory prostaglandins [37,38] and decreases LDLc oxidation [39], pro-inflammatory cytokines and adhesion of monocytes to endothelium [40,41]. Additionally, γ-CEHC and γ-tocopherol molecules exhibited an inhibitory influence on prostaglandin E_2 synthesis [42,43], a key intermediate in the early inflammatory response with pro-inflammatory activity. The significant decrease observed in tocopherol metabolites as a consequence of CAF intake (**Table 1**) might be related to the systemic pro-inflammatory and pro-oxidant state of these animals [11,12].

Similarly, we observed decreased bile acids levels in the serum of CAF-fed rats (**Table 1**), and this decrease was even greater in trained groups (**Fig. 6B and 6C**), which may suggest a disruption in cholesterol homeostasis. This event may also be linked to inflammation because many studies have shown that inflammatory cytokines may inhibit the expression of CYP7A1 and other rate-limiting enzymes in the synthesis of bile acids [44–46].

Long chain omega 3 polyunsaturated fatty acids (ω-3 PUFA) decrease the production of pro-inflammatory eicosanoids from arachidonic acid and support anti-inflammatory eicosanoids production for the purpose of resolving acute inflammation [47,48]. However, in metabolic diseases such as obesity, type II diabetes or atherosclerosis, this situation is unbalanced in favour of pro-inflammatory eicosanoids leading to an enduring inflammatory state [48]. Our data showed decreased levels of ω-3 PUFA (linolenic and docosahexaenoic acid) as a consequence of chronic intake of CAF (**Table 1**). Therefore, the metabolome of CAF-fed animals reflected nutritional deficiencies that drive disorders in lipid metabolism and inflammation. The serum accumulation of Lyso-PC may be associated with this fact because increased phospholipase A_2 activity promotes the release of PUFA for subsequent synthesis of bioactive eicosanoids [49]. In fact, Lyso-PC have been linked to acute and chronic inflammation [50,51] as well as obesity [52,53]. Physical training had a subtle effect on PUFA, as only the DiHETE serum concentration was altered. Although increased levels of DiHETE are associated with type II diabetes and other inflammatory diseases [54,55], we observed decreased levels as a consequence of physical exercise, especially at low-intensity (**Fig. 6H**).

Many studies carried out with rodents using a targeted strategy indicate that inflammation is a clear metabolic consequence of chronic CAF consumption [11,56–58]. The results obtained in the present study showed that the pro-inflammatory condition induced by the CAF has a significant holistic effect in the circulating metabolome. In accordance with this, the serum levels of the pro-inflammatory cytokine MCP-1 were increased in CAF-fed

rats (**Fig. 3A**). In particular, MCP-1 has been described as the main responsible for the recruitment of monocytes in the inflammatory response, as well as a potential intervention point for the treatment of various diseases associated to MetS and inflammation, including insulin resistance, atherosclerosis and rheumatoid arthritis [59,60]. Some authors have also shown that physical exercise has a significant effect in reducing circulating levels of MCP-1 in rats and subjects with MetS [59,61]. However, our results did not reveal a remarkable influence of the treadmill intervention in serum MCP-1.

Enduring inflammation could lead to a decrease in total and lipoprotein-associated cholesterol [44,62], explaining why we observed the lowest circulating levels of total cholesterol and its fraction associated with lipoproteins in animals fed CAF (**Fig. 1B-D**). We also observed that the changes in dyslipidemic variables associated with the practice of running were found in animals fed the MetS-inducing diet but not in the ST-fed rodents. Serum triglycerides of CAF-fed animals were clearly modulated by treadmill intervention, decreasing its concentration proportionally to training intensity (**Fig. 1A**). A lowering effect of training is also found in the circulating values of LDLc of TML-CAF rats in comparison with sedentary group (**Fig. 1D**). The lack of modifications in dyslipidemic parameters of animals without MetS may indicate a corrective effect of exercise specifically in dyslipidemia.

Nevertheless, a significant effect of physical training was perceptible in the CRP levels of animals fed both ST and CAF (**Fig. 3B**). In fact, recent studies carried out in obese and non-obese subjects have shown a reduction in the circulating levels of the inflammatory marker CRP in response to regular physical activity [63–65]. In the present study, the low concentrations of CRP found in the serum of trained animals suggest that aerobic training can reduce inflammation. Decreased circulating levels of adiponectin were observed in obese children and adolescents [66,67] and are correlated with MetS and inflammation [2,68]. On the other hand, many studies have concluded that elevated concentrations of plasma leptin are strongly associated with MetS [2,67,69,70]. Because the two adipokines have opposite effects, the plasma LAR has been proposed to be a useful diagnostic marker of MetS in humans [69,70]. According to these authors, we found higher values of LAR in CAF groups relative to the ST-fed rats (**Fig. 2A**). Moreover, the physical training reduced the ratio in both ST and CAF groups, and this response was proportional to training intensity.

We also analysed the serum concentration of the hormone irisin, which has been proposed to mediate some of the beneficial effects of exercise in obesity by driving brown-fat development of the subcutaneous adipose tissue [71]. Surprisingly, we did not detect changes in trained animals, but its levels were increased after CAF intake (**Fig. 2B**). The absence of changes in the irisin levels of trained animals might be related to the fact that it is only transiently expressed after training [72]. To our knowledge, it has never been described

that CAF boosts irisin levels; however, recent reports have shown high levels of circulating irisin in obese subjects [73,74]. In fact, at the time of the sacrifice, a higher amount of brown adipose tissue in CAF-fed rats was evident (data not shown). Originally, irisin was identified as a myokine [75], but now it is known that it is also synthetized by white adipose tissue [76]; so it is not surprising that is found in higher concentrations in overweight animals.

Our study used a non-targeted metabolomics approach and suggests that a systemic inflammatory state in adolescent rats is a consequence of the chronic intake of a high-fat and high-carbohydrate diet. The periodic training was only partially able to reverse the detrimental effects induced by the CAF intake, and the moderate exercise seemed more effective than high-intensity training. In fact, multivariate analysis showed that the metabolome of animals fed ST was more affected by the physical training than CAF-fed rats. However, it has to be taken into consideration that the results of the experiment were obtained using female animals. This fact could limit the degree of extrapolation of our results because the metabolome of females is also influenced by the hormonal cycle [77]. Thus it would be necessary to evaluate in subsequent studies if male rats have a similar response regarding overall metabolism when submitted to the same treatments [78].

In future studies, it may also be interesting to analyse, in the same conditions, the hepatic expression of genes related to the metabolism of bile acids, such as CYP7A1 or SREBP2 [79,80], to study the mechanisms through which the chronic inflammation could be hampering the cholesterol homeostasis. Furthermore, it would be appropriate to evaluate the serum concentrations of proteins involved in the extracellular releasing of lysoglycerophospholipids, since they are the family of altered metabolites most representative of our study and are not just linked to inflammatory events but also to other important physiological and pathological processes [81].

From the results of the present study, we conclude that an unhealthy diet has a strong impact on the animal metabolome that is difficult to revert with the practice of running, even when maintained daily.

5. Acknowledgements

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Manuscript 2

Serum lysophospholipid levels are altered in dyslipidemic hamsters

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PREFACE

Continuing with the objective of **Chapter 1** focused on the identification of metabolic changes in response to lifestyle interventions, in **Manuscript 2** we intended to determine which are the principal effectors involved in the regulation of lipid disorders generated as a consequence of nutritional treatment.

As a result of the animal study performed in **Manuscript 1**, we observed that CAF had a strong impact on the serum metabolome masking the response of the organism to those interventions. Therefore, we decided to design another study in which the development of dyslipidemia due to diet occur in a more progressive manner. For this reason, we selected Golden Syrian hamsters fed a high-fat diet (HFD). The election of this hamster as animal model is motivated by the fact that is the rodent that shows highest similarity to humans regarding lipoprotein metabolism. The HFD is a defined stablished chow which allows us to manage the amount of fats provided to the animals to induce the disorder. Moreover, the content of fats in the HFD administered in the following study was lower than others widely used in the bibliography, thus generating slightly metabolic disorders.

To achieve this objective, in this manuscript firstly we performed an animal study in which under a non-targeted analysis we determined the metabolites most affected by the intervention. Secondly, in another study, we examined the levels of these metabolites in a shorter intervention time to verify if they were already altered. In addition, to discard the molecules that are actually diet-consumption biomarkers we included in the experimental design an additional group in which the dyslipidemia was originated by the periodic administration of a chemical treatment (Poloxamer 407, a hyperlipidemic agent). Altogether, it is intended to identify biomarkers of dyslipidemia progression, which in turn disclose its etiology.

Serum lysophospholipids levels are altered in dyslipidemic hamsters

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Abstract

Dyslipidemias are common disorders that predispose individuals to severe diseases. It is known that healthy living habits can prevent dyslipidemias if they are diagnosed properly. Therefore, biomarkers that assist in diagnosis are essential. The aim of this study was to identify biomarkers of dyslipidemia progression, which in turn disclose its etiology. These findings will pave the way for examinations of the regulatory mechanisms involved in dyslipidemias. Hamsters were fed either a low-fat diet (LFD) or a high-fat diet. Some of the LFD-fed animals were further treated with the hyperlipidemic agent Poloxamer 407. Non-targeted metabolomics was used to investigate progressive changes in unknown serum metabolites. The hepatic expression of putative biomarker-related genes was also analyzed. The serum levels of lysophospholipids (Lyso-PLs) and their related enzymes lecithin-cholesterol acyltransferase (LCAT), secreted phospholipase A_2 (sPLA$_2$) and paraoxonase-1 were altered in dyslipidemic hamsters. Lysophosphatidylcholine levels were increased in diet-induced dyslipidemic groups, whereas lysophosphatidylethanolamine levels increased in response to the chemical treatment. The liver was significantly involved in regulating the levels of these molecules, based on the modified expression of endothelial lipase (*Lipg*), sPLA$_2$ (*Pla2g2a*) and acyltransferases (*Lcat* and *Lpcat3*). We concluded that Lyso-PL evaluation could aid in the comprehensive diagnosis and management of lipid disorders.

Keywords: dyslipidemia; biomarkers; metabolomics; lysophospholipids; Poloxamer 407; lecithin-cholesterol acyltransferase; phospholipase; endothelial lipase; lysophosphatidylethanolamine (20:4)

1. Introduction

Dyslipidemias are disturbances in lipid metabolism that have a high prevalence in both developed and developing countries and lead to changes in the levels and/or functions of the plasma lipoproteins [1–3]. These lipid disorders can appear as an elevation in the levels of circulating triglycerides, total cholesterol (TC) and low-density lipoprotein cholesterol (LDLc), as well as a reduction in high-density lipoprotein cholesterol (HDLc) levels [4]. Alterations in lipid metabolism and transport can have severe implications, predisposing the patients to the development of cardiovascular diseases, fatty liver disorders and different types of cancer [5–8].

In terms of the etiology, the development of dyslipidemia in humans depends on both genetic and environmental factors. Thus, lipid management begins with the exclusion of secondary causes of metabolic disorder [9]. A diet rich in saturated fats and cholesterol is a well-established source of acquired dyslipidemias [4–6], but accumulating evidence has shown that certain exogenous compounds, such as alcohol, tobacco or some drugs, also induce lipid disorders when consumed regularly over a long period of time [9–11]. Advances in our understanding of the mechanisms involved in dyslipidemia progression that depend on the type of triggering factor are necessary to improve the diagnosis and treatment of dyslipidemia-related diseases.

The Golden Syrian hamster is the rodent that displays greatest similarity to humans regarding lipoprotein metabolism [12]. In the present work, this hamster model was selected to investigate the biological alterations that occur after the chronic intake of a high-fat diet (HFD) for a maximum of 30 days. Furthermore, an additional group of animals was treated with the hyperlipidemic agent Poloxamer 407 (P407) to generate a different form of dyslipidemia that was not induced by diet. P407 is a hydrophilic, non-ionic, surface-active compound with low toxicity that has been extensively used in pharmaceutical preparations and in common personal care products, such as toothpastes and cosmetics [13]. After parental administration, P407 induces hypertriglyceridemia and hypercholesterolemia in rodents by inhibiting the heparin-releasable fraction of lipoprotein lipase (LPL) and the cholesterol 7α-hydroxylase (C7αH), respectively [14,15]. However, chronic treatment with P407 does not seem to stimulate 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme involved in the synthesis of cholesterol, target of many anti-dyslipidemic drugs [16]. P407-induced hyperlipidemia, which exhibits more pronounced plasma triglyceride levels than cholesterol levels, is dose-dependent and remains at steady levels with repetitive injections of the drug approximately every 3 days [17]. Thus, the P407-treated rodent and the HFD-fed hamster are well-established models of atherogenesis that

properly reproduce the adverse events that precede the development of atherosclerotic lesions in humans [18,19].

In this context, the use of an omics-based strategy allowed us to provide a global characterization of the changes in the circulating metabolome associated with the long-term administration of both pro-dyslipidemic treatments, as well as to investigate the main hepatic mechanisms. Some studies have been performed in HFD-fed hamsters [20–22] and dyslipidemic subjects [23,24] using non-targeted metabolomics techniques, but, to date, research on P407-treated rodents has not utilized this approach. All these studies point to the importance of comprehensive investigations for expanding our understanding of the complex networks involved in the development of lipid disorders and the identification of novel biomarkers for the early diagnosis and management of related diseases.

Therefore, the main objective of the present study was to identify non-invasive biomarkers of dyslipidemia progression that reveal the etiology of the disorder. Therefore, we used ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS) to evaluate the alterations in the serum metabolite levels of adult hamsters in response to different periods of HFD feeding and treatment with P407. Furthermore, after the untargeted identification of the metabolites associated with each type of dyslipidemia, the hepatic mechanisms involved in the metabolism of these molecules were also examined to extend our knowledge of the modes of action of the two treatments and their disparities.

2. Material and methods

2.1. Chemicals

Methanol (Scharlab S.L., Barcelona, Spain) and glacial acetic acid (Panreac, Barcelona, Spain) were of high-performance liquid chromatography (HPLC) analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Phenylalanine ^{13}C (Fluka/Sigma-Aldrich, Madrid, Spain) was used as an internal standard (IS) for the untargeted metabolomics analysis. The standard was dissolved in methanol at 1 mg/mL and stored at - 20°C prior to use. Butylated hydroxytoluene (BHT, Fluka/Sigma-Aldrich, Madrid, Spain) was added to the extraction solution to avoid metabolite oxidation during the sample extraction. P407 (Fluka/Sigma-Aldrich, Madrid, Spain) was administered to the animals to induce dyslipidemia.

2.2. Animal studies

Two different *in vivo* studies were performed to identify serum biomarkers of dyslipidemia. All of procedures were approved by the Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and they have been performed in accordance with the European Communities Council Directive (86/609/EEC).

A first exploratory study was performed using male Golden Syrian hamsters (Charles River Laboratories, Barcelona, Spain) weighing 130 g. Animals were housed singly at 22°C with a light/dark period of 12 h (lights on at 09:00 h) and with free access to food and water. After an adaptation period of 4 days, the hamsters were randomly distributed into two experimental groups (n = 16 per group) and fed either a low-fat diet (LFD; D10051906) or a high-fat diet (HFD; D10051907) (Research Diets Inc., New Brunswick, NJ, USA) *ad libitum*. The composition of the diets is shown in **Table 1**. The LFD provided 10% energy as fat, whereas the HFD provided 21% energy as fat because of the high content of lard. Eight animals of each group were sacrificed on day 15 (15d) and the other 8 on day 30 (30d).

Table 1. Detailed composition of the diets

	LFD	HFD
Macronutrients		
Protein (g/kg)	22	24
Carbohydrate (g/kg)	65	57
Fat		
g/kg	4	10
%	10	21
Energy (kcal/g)	3.9	4.1
Ingredients (g/kg)		
Casein, 80 mesh	220	220
L-Cysteine	3	3
Wheat starch	386.5	281
Maltodextrin 10	100	100
Dextrose	50	50
Sucrose	100	100
Cellulose, BW200	50	50
Coconut oil, 76	7.74	5
Flaxseed oil	5.16	4
Sunflower oil	30.1	10
Lard	0	71
Mineral mix S10022G	35	35
Vitamin mix V10037	10	10
Choline bitartrate	2.5	2.5
Cholesterol	0.03	0.92
Fatty acids		
SFA		
g/kg	10.9	28.6
%	26.5	33.7
MUFA		
g/kg	8.2	28.0
%	19.9	33.1
PUFA		
g/kg	22.1	28.2
%	53.6	33.2

LFD, low-fat diet; HFD, high-fat diet.

A second study was conducted using a different strategy to induce dyslipidemia. For this purpose, male Golden Syrian hamsters (Janvier, Le Genest-St-Isle, France) weighing 110 g were housed individually in cages at 22°C with a light/dark period of 12 h and free access to food and water. After an adaptation period of 2 weeks in which animals were fed an LFD, they were randomly assigned to six groups (n = 9 - 10 per group). Three groups continued the experiment for 4 days (4d) to detect early changes in the metabolome profile between groups, whereas the other three groups were sacrificed at 30d. The experimental design is described below. Two groups were maintained on the LFD and served as control animals (C-4d and C-30d groups), two other groups were fed the lard-based HFD (HFD-4d and HFD-30d groups) and the last two groups were fed the LFD and periodically injected with 50 mg of P407 per kg body weight every 72 h to induce dyslipidemia (P407-4d and P407-30d groups).

Until now, P407 has been extensively used in mice [15,25] and rats [14,26,27] but, to the best of our knowledge, only one study has been conducted in Golden Syrian hamsters that were periodically injected with P407 to induce dose-dependent hyperlipidemia [28]. In the present work, we have utilized a lower dose of P407 than the dose used by Liu *et al.* [28]. Based on preliminary studies, this quantity was selected as the adequate dose to achieve the development of moderate hypertriglyceridemia and hypercholesterolemia in hamsters (**Fig. 1**). The animals that were not treated with P407 received injections of vehicle (0.9% NaCl) with equal frequency. The hamsters were sacrificed 24 h after the last administration of P407 or vehicle. Lean and fat mass measurements (in grams) were performed without anesthesia on the first and the last days of the study using an EchoMRI-700™ (Echo Medical Systems, LLC., TX, USA).

In both experiments, the hamsters were deprived of food for 6 h on the day of sacrifice and euthanized under anesthesia (pentobarbital sodium, 80 mg per kg body weight). Blood was collected by cardiac puncture and serum was obtained by centrifugation (2,000 g for 15 min). The livers were dissected, weighted and immediately frozen in liquid nitrogen. All the samples were stored at - 80°C until further analyses.
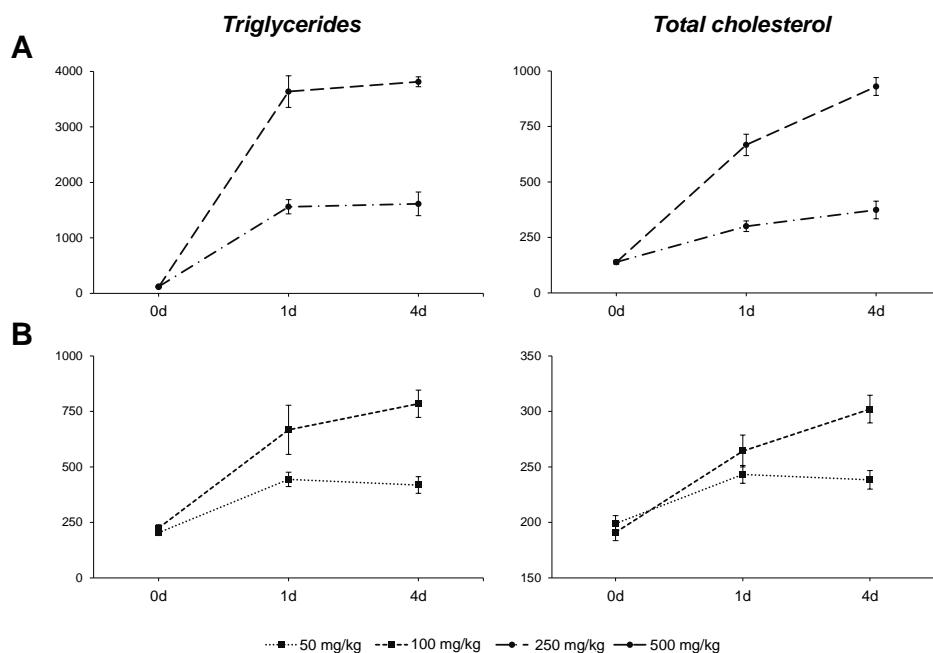
Figure 1. Determination of the adequate dose of P407 required to induce moderate hyperlipidemia in hamsters. In preliminary studies, animals were assigned to two groups (n = 7 per group) and treated with different doses of P407. These hamsters were fed a low-fat diet and received two intraperitoneal injections of the drug 3 days apart (days 0 and 3) to induce hypertriglyceridemia and hypercholesterolemia. Blood was collected under fasting conditions 6 h before the first administration of P407 (0d) and 24 h after each injection, on days 1 (1d) and 4 (4d). (**A**) As a first approximation, we tested two concentrations similar to those commonly used in rodents (250 and 500 mg/kg), and both resulted in exaggerated hyperlipidemia. (**B**) In a second study, two lower doses were evaluated (50 and 100 mg/kg), and we selected 50 mg/kg as the most suitable dose of P407 among the doses assessed that induced stable and moderate dyslipidemia in adult hamsters.

2.3. Serum measurements

Enzymatic colorimetric kits were used for the determination of glucose and triglycerides (QCA, Barcelona, Spain), phospholipids (Spinreact, Girona, Spain), non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany), TC, HDLc and LDLc (Bioassay systems, Hayward, CA, USA). The circulating levels of the enzymes lecithin-cholesterol acyltransferase (LCAT), group IIA secreted phospholipase A_2 (sPLA$_2$-IIA), and paraoxonase-1 (PON1) were measured with hamster ELISA kits (MyBioSource, San Diego, CA, USA).

2.4. Sample preparation for non-targeted metabolomics

Serum extracts were prepared using a procedure similar to the method described by Jové *et al.* [20]. Briefly, 90 µL of cold methanol containing phenylalanine ^{13}C as the IS (10 ppm) and BHT (1 µM) as an antioxidant were added to 30 µL of serum, vortexed for 1 minute and incubated at - 20°C for 1 h to precipitate proteins. Samples were centrifuged at 12,000 g for 5 min at 4°C and the supernatants were collected and dried in a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). Samples were re-suspended in 100 µL of Milli-Q water containing 0.2% acetic acid:methanol 0.2% acetic acid (1:1) prior to injection.

2.5. LC-MS and LC-MS/MS analyses

Both untargeted and targeted analyses were performed on serum extracts using an UHPLC 1290 coupled to a Q-TOF 6550 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The temperature in the autosampler was maintained at 4°C throughout the analysis, and the samples were randomized.

Solvent A consisted of 0.2% acetic acid and solvent B consisted of methanol with 0.2% acetic acid. Two microliters of sample were applied to a Zorbax SB-Aq (1.8 µm particle size, 2.1 mm internal diameter x 50 mm length) analytical column maintained at 60°C and equipped with a Zorbax SB-C8 (3.5 µm, 2.1 x 30 mm) guard column, also from Agilent Technologies. Chromatographic separation was performed by continuous gradient elution at flow rate of 0.6 mL/min starting at 2% B and increasing to 98% B in 13 min, where it was maintained isocratically for 6 min. The chromatographic system was returned to the initial conditions in 1 min, followed by a 5-min equilibration prior to the subsequent injection. Ionization in the mass spectrometer was performed by AJS ESI operated either in positive (+ESI) and negative (-ESI) mode with the following settings: nebulizer gas, nitrogen at a pressure of 45 psi; desolvation gas flow rate, 9 L/min at 325°C; source temperature and gas flow rate, 150°C and 12 L/min, respectively; capillary voltage, 4 kV; fragmentor, 125 V. Accurate LC-MS mass spectra were acquired over the 40 - 1600 *m/z* range at a scan rate of

1.5 spectra/s. A reference solution was used for continuous calibration with the following reference masses: 121.0509 and 922.0098 *m/z* for +ESI and 119.0363 and 980.0164 *m/z* for -ESI. Quality controls (QC) were prepared mixing 10 µL of each serum extract. At the start of the run, eight QC were injected for column conditioning and then every ten samples to assess instrument stability. For targeted analyses, the instrument was operating in MS and MS/MS modes, and the other analytical conditions were the same as described above. The MS/MS spectra of the metabolites were obtained at collision energies of 10 and 20 eV.

2.6. Data processing and metabolite identification

All the software programs were provided by Agilent Technologies (Palo Alto, CA, USA). MassHunter Data Acquisition software was used to generate profile peak data from the spectra and MassHunter Qualitative Analysis software was used to obtain the molecular features of the samples. The peaks were aligned using Mass Profiler Professional (MPP) and mass and retention time (RT) windows of 0.1% ± 0.15 min and 15.0 ppm ± 2.0 mDa, respectively. Each chemical entity obtained from MPP is associated with a particular neutral mass, RT and abundance value. The list of molecular entities was filtered by selecting only those entities that were present in at least 80% of the samples in the same group. The normalized abundances were obtained by performing a base-2 logarithmic transformation of the ratio of each analyte to the IS. Candidate biomarkers were first identified using metabolite databases (METLIN [29], HMDB [30], and LIPID MAPS [31]). Finally, the structural information about the molecules of interest was provided by the fragmentation data obtained at different collision energies.

2.7. Total RNA isolation and gene expression analysis

Livers used for mRNA analysis were homogenized and total RNA was extracted with TRIzol Reagent and purified on RNeasy Mini Kit spin columns (Qiagen, Barcelona, Spain) according to the manufacturer's protocols. RNA yield was quantified on a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain) and tested for purity by measuring A260/280 ratio. Total RNA (0.5 µg; in a final volume of 20 µL) was reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). cDNAs were subjected to quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) amplification using SYBR Green PCR Master Mix (Bio-Rad, Barcelona, Spain). All reactions were performed in triplicate. When possible, primers were designed to span an exon-exon junction at the conserved domain of the gene of interest, avoiding the problem of the possible contamination by genomic DNA. Primer sequences for the target genes are listed in **Table 2** and were obtained from Biomers.net (Ulm, Germany). The relative mRNA expression levels were

calculated using the $2^{-\Delta\Delta Ct}$ method [32] with *β-actin* as the reference gene and were normalized to the C-4d group.

Table 2. Hamster-specific primer sequences used for the gene expression analysis

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Lipc	GTGGTCGGGCTCAGAAAGTG	GTATGCCAGGGAGACCCAAT
Lipg	CCAAGGACCCAGAACACGAA	GCCACTCATCGTCCATCCAT
Pla2g2a	TGGCCCAATCCAGATCCAAG	AGTCGGTCGTAGCAACAGTC
Abhd3	GCTGTGCCTAAATGCTGTGG	TGGCCGCCGTAAGAAGTAAG
Pon1	GGGACTGCTGTTGGCATTCT	AGTCCAGTGCTGAGGAAAGC
Lcat	ACACTGGTGCAGAATCTGGTTA	ACTGTCTCATCCCGCACATAC
Lpcat3	TATGACAGCCGCCCTTTCTG	TCCTTCTGTGACCAGCCAAC
Actb	ACGTCGACATCCGCAAAGACCTC	TGATCTCCTTCTGCATCCGGTCA

Lipc, hepatic lipase; *Lipg,* endothelial lipase; *Pla2g2a*, phospholipase A_2 group IIA; *Abhd3,* abhydrolase domain-containing 3; *Pon1*, Paraoxonase 1; *Lcat,* lecithin-cholesterol acyltransferase; *Lpcat3,* lysophosphatidylcholine acyltransferase 3; *Actb*, beta-actin (housekeeping gene).

2.8. Statistical analysis

The results are presented as the means ± SEM from the indicated number of hamsters. The assumption of normality was determined using the Shapiro-Wilk test, and the homoscedasticity between groups was assessed using Levene's test. Once these conditions were verified, differences among groups were assessed using two-way ANOVA to evaluate the main effects of the time (duration of the experiment) and the pro-dyslipidemic intervention (HFD and P407) and their interaction. For the untargeted analysis, the p-values were calculated using the Benjamini-Hochberg correction. When any of the effects was statistically significant, one-way ANOVA was used to determine the differences among all the means at once. Tukey's *post hoc* test was applied when the variances were similar and the Games-Howell test was applied if this assumption was not fulfilled. Student's t-test was used for single statistical comparisons. A two-tailed value of $p < 0.05$ was considered statistically

significant. All statistical analyses were performed with Statistical Package for Social Sciences (IBM SPSS Statistics, version 19.0).

3. Results

3.1. Identification of a class of lipids involved in dyslipidemia progression

Based on the results from the initial study, the development of dyslipidemia was successfully induced by the chronic administration of the HFD to the animals for 15d and 30d (**Fig. 2**). Hamsters fed the HFD showed higher amounts of TC, LDLc and triglycerides in serum, higher values for the atherogenic index (TC/HDLc) and greater relative liver weights than animals fed the LFD ($p < 0.005$, two-way ANOVA), without alterations in the body weight. However, time did not significantly affect any of these parameters, and only the circulating triglyceride levels were influenced by the interaction between diet and time ($p = 0.008$, two-way ANOVA).

Based on these findings, the state of dyslipidemia in hamsters fed the HFD for 15d was too similar to the hamsters fed for 30d. Nevertheless, the untargeted comparative analysis of the animal serum revealed that two lipid subclasses were differentially modified by HFD feeding according to the length of the treatment (**Table 3**). On the one hand, the circulating levels of five monoacylglycerophosphocholines, which are usually named lysophosphatidylcholines (Lyso-PCs), were increased in response to HFD intake ($p < 0.050$, two-way ANOVA), and the increase in all of the identified Lyso-PCs was restricted at 15d. On the other hand, although the diet had an overall effect on lysophosphatidylinositol (18:1) (Lyso-PI (18:1)) ($p = 0.006$, two-way ANOVA), the serum levels of all of the Lyso-PIs were at least residually increased in the HFD-fed animals compared to LFD-fed animals at 30d ($p < 0.050$, Student's t-test), which was a later response than Lyso-PCs to HFD feeding. Other metabolites, including monoacylglycerols, amino acids and fatty acids such as arachidonic acid, were altered in the HFD-fed groups, but the glycerol-based lysophospholipids (Lyso-PLs) were the most representative lipid class. Concretely, this means that Lyso-PL category was the family of metabolites that exhibited the most numerous and deepest changes in the overall metabolome in this study. Furthermore, Lyso-PLs have been previously described as indicators of the health status in both animal and human studies [33-35]. Therefore, taking in consideration all this information and evidence, this first study highlighted the importance of this family of compounds and their potential as suitable candidate biomarkers of dyslipidemia progression.
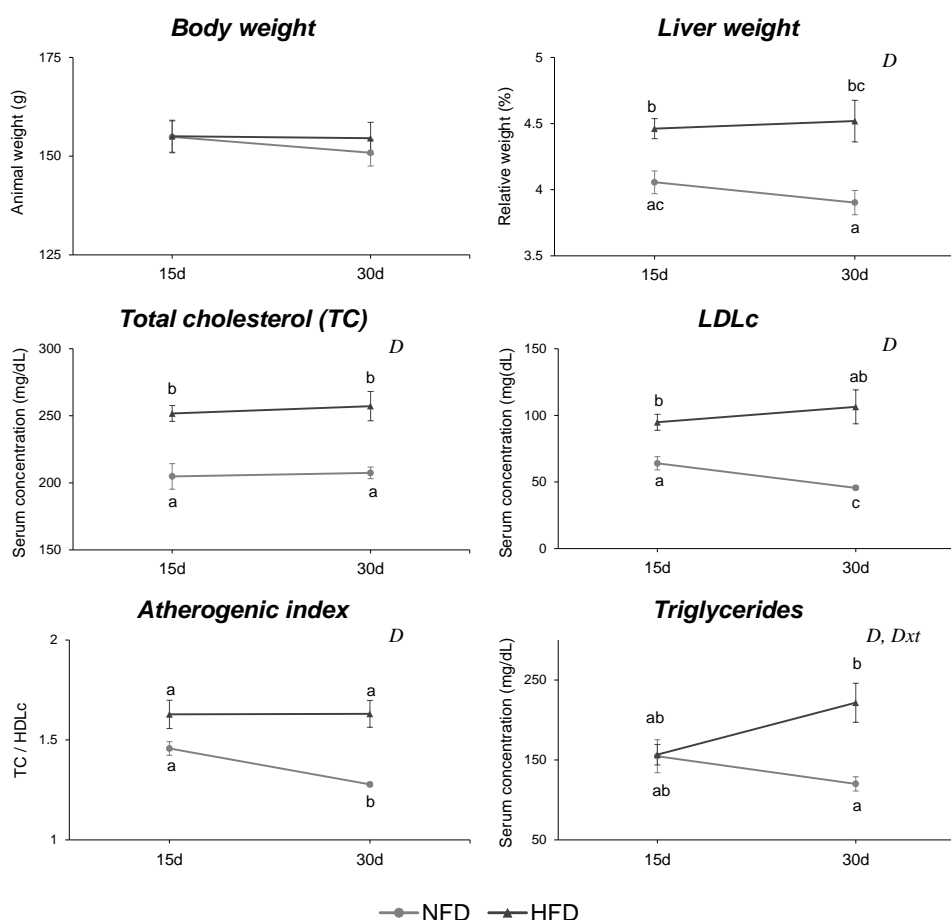
Figure 2. Biological characteristics of the hamsters from the initial study. Adult hamsters were distributed into two groups and fed either a low-fat diet (LFD) or a high-fat diet (HFD). Half of the animals in each group were sacrificed on day 15 (15d) and the other half were sacrificed on day 30 (30d). Serum parameters were determined after a 6 h fast. The data are presented as means ± SEM (n = 8 per group). The statistical comparisons among groups were conducted using two- and one-way ANOVAs. *D*: the effect of the diet; *Dxt*: the interaction between diet and time (two-way ANOVA, $p < 0.05$). [abc]Mean values with different lowercase letters were significantly different (one-way ANOVA and Tukey's or Games-Howell *post hoc* tests, $p < 0.05$). TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol.

Table 3. Identification of the serum lysoglycerophospholipids (Lyso-PLs) that were significantly altered by the high-fat diet (HFD) compared to the low-fat diet (LFD) after 15 and 30 days of feeding in the initial study

Ionization mode	Ion *m/z* (MS)	Distinctive fragments (MS/MS)	Tentative identification †	Lipid subclass	Increased with HFD feeding
+ESI	[M+H]†: 524.3743	184.1, 104.1, 60.1	Lyso-PC (18:0)		
	[M+H]†: 520.3440	184.1, 104.1, 60.1	Lyso-PC (18:2)		
	[M+H]†: 518.3251	184.1, 104.1, 60.1	Lyso-PC (18:3)	Monoacylglycerophospho cholines	15d
	[M+H]†: 550.3871	184.1, 104.1, 60.1	Lyso-PC (20:1)		
	[M+H]†: 548.3719	184.1, 104.1, 60.1	Lyso-PC (20:2)		
-ESI	[M-H]: 599.3218	419.2, 283.3, 79.0	Lyso-PI (18:0)		
	[M-H]: 597.3060	417.2, 281.8, 79.0	Lyso-PI (18:1)	Monoacylglycerophospho inositols	30d
	[M-H]: 621.3060	441.0, 305.1, 79.0	Lyso-PI (20:3)		
	[M-H]: 619.2906	439.0, 303.2, 79.0	Lyso-PI (20:4)		

† According to the LIPID MAPS [31] glycerophospholipid nomenclature, the structure of the acyl chain is indicated within parentheses in the 'Headgroup (*sn1* or *sn2*)' format. The headgroup is assumed to be attached to the *sn3* position of glycerol.

Abbreviations: Lyso-PC, lysophosphatidylcholine; Lyso-PI, lysophosphatidylinositol

3.2. Evaluation of the involvement of Lyso-PLs in the etiology and early progression of dyslipidemias

We performed a second animal study in which we induced an earlier stage of dyslipidemia by only feeding the animals the HFD for 4d to analyze the ability of this class of compounds to serve as biomarkers of dyslipidemia-related diseases. Furthermore, an additional strategy was used to induce hyperlipidemia that was not influenced by the diet in this second study. Therefore, two groups of animals were treated with P407 for 4d or 30d.

3.2.1. Health status of animals with two diverse forms of dyslipidemia

The biometric and serum analyses of the six groups of hamsters are reported in **Table 4**. At the start of the experiment, all the groups exhibited similar body weights and fat percentages. Total body weight was not affected by the treatments throughout the study, whereas the overall and relative liver weights of the HFD- and P407-treated groups increased, mainly in the animals submitted to the 30d treatments, in which significant differences were observed compared with the C-30d group. Nonetheless, only significant effects of the HFD on adiposity were observed, but not P407 administration. HFD-fed animals displayed numerically greater adiposity than the C groups at both times (**Table 4**). Although the *post hoc* analysis only revealed a significant difference in response to HFD intake at 4d, a residual increase was also noted in the HFD-30d animals compared to the corresponding C group (p = 0.014, Student's t-test). Regarding the biochemical parameters, the pro-dyslipidemic intervention had an overall significant effect by increasing the circulating levels of TC and its main fractions. Although both treatments influenced the TC levels, the increase in the levels of the fraction corresponding to the evaluated lipoproteins is mainly due to HFD intake. According to the *post hoc* analysis, the TC and LDLc levels in the HFD-fed animals were altered throughout the study, but the response of HDLc was the slowest and increased after 30d of feeding. Conversely, the TC levels in the P407-treated animals increased rapidly but then stabilized and approached the levels of the C-30d group. The atherogenic index, which is calculated as the ratio of TC and HDLc, was also modulated by the pro-dyslipidemic intervention. More specifically, P407-treated animals had increased values of the index compared to each C group beginning at 4d, whereas significant alterations in response to HFD feeding were only observed at 30d. On the other hand, sharp increases in the serum triglyceride levels of approximately 200% and 150% were produced by P407 after 4d and 30d of treatment, respectively, whereas no significant changes were detected between the HFD-fed animals and C groups. The intervention also induced a significant increase in the serum phospholipid and glucose levels that was more pronounced for phospholipids than for glucose. The increase in the phospholipid levels was generally induced by both pro-

dyslipidemic treatments, although the *post hoc* analysis only revealed a significant difference in the P407-treated animals at 4d. However, the overall effects on glucose were due to the diet, which showed an increasing trend, particularly at 30d (p = 0.016, Student's t-test), but P407 did not induce alterations in the glucose levels. Finally, the serum NEFA levels were the only component that was not affected by either treatment throughout the study.

In addition, some of the enzymes involved in the extracellular release of Lyso-PLs were evaluated in these hamsters. As shown in **Fig. 3**, we investigated the serum levels of the enzymes LCAT, $sPLA_2$-IIA and PON1. Overall, both treatments induced a decrease in the serum LCAT levels (p < 0.001, two-way ANOVA), which was much more evident in the groups treated with P407 for 4d and 30d compared with their respective control counterparts (32% and 14% decreases at 4d and 30d, respectively) (**Fig. 3a**). As shown in **Fig. 3b**, the serum $sPLA_2$-IIA concentrations exhibited similar responses to both HFD intake and the P407 treatment (p = 0.003, two-way ANOVA) as the LCAT levels, with a more marked decline in the P407-treated animals than in HFD-fed groups compared with the controls (24% and 27% decreases at 4d and 30d, respectively; p = 0.032 and p = 0.049, Student's t-test). Although the two-way ANOVA revealed overall effects of the pro-dyslipidemic intervention (p = 0.005) and time (p = 0.036) on the circulating levels of PON1, the pair-wise comparisons did not reveal significant differences at any time when the HFD-fed and the P407-treated animals were compared with the C groups (**Fig. 3c**). However, the P407-treated animals displayed numerically greater serum PON1 levels than C groups, mainly at 4d (p = 0.031; Student's t-test).

Based on these results, the P407 treatment induced greater changes in the levels of enzymes involved in the release of Lyso-PLs into the bloodstream than HFD intake.
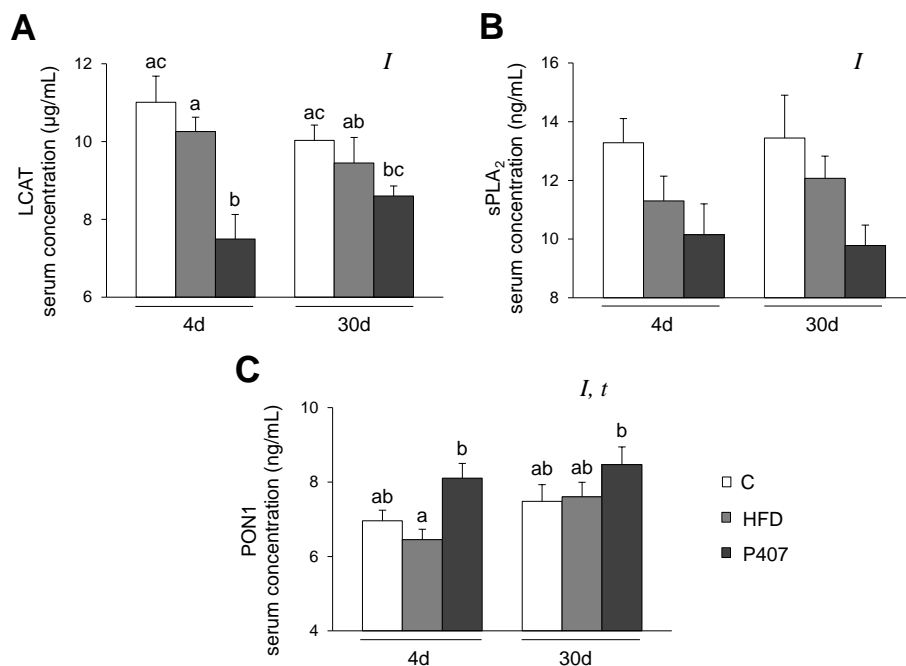
Figure 3. Circulating levels of different enzymes involved in Lyso-PL synthesis in hamsters with different degrees of dyslipidemia. The animals were distributed into three groups: C, group fed a low-fat diet (LFD); HFD, group fed a high-fat diet; and P407, group fed an LFD and periodically injected with the pro-dyslipidemic agent P407. Half of the animals in each group were sacrificed on day 4 (4d) and the other half were sacrificed on day 30 (30d). The dose of P407 was 50 mg/kg every 3 days. Non-P407 hamsters received injections of vehicle (0.9% NaCl) with equal periodicity. All animals were sacrificed 24 h after the last administration of P407 or vehicle and after a 6 h fast. The concentrations of serum parameters were determined at the end of the experiment. The data are presented as means ± SEM (n = 9 - 10). The statistical comparisons among groups were conducted using two- and one-way ANOVAs. *I:* the effect of the pro-dyslipidemic intervention (HFD and P407); *t:* the effect of time (two-way ANOVA, $p < 0.05$). [abc]Mean values with different lowercase letters were significantly different (one-way ANOVA and Tukey's or Games-Howell *post hoc* tests, $p < 0.05$).

Table 4. Biometric and serum biochemical parameters measured in each group of hamsters from the second experiment

	C-4d	HFD-4d	P407-4d	C-30d	HFD-30d	P407-30d	Effect
Biometric parameters							
Initial body weight (g)	113.83±2.27	111.66±2.84	112.59±3.36	113.94±1.52	115.45±1.83	112.53±1.83	
Initial fat (%)	10.34±0.74	10.39±0.72	10.48±1.15	11.70±0.41	10.47±0.61	11.02±0.50	
Final body weight (g)	115.56±1.97[a]	114.87±2.80[a]	114.94±3.48[a]	124.20±0.96[ab]	129.60±2.38[b]	125.80±1.93[b]	t
Fat increase (%)	-0.37±0.17[a]	0.70±0.24[b]	-0.33±0.17[a]	1.47±0.43[c]	3.72±0.67[c]	2.13±0.35[c]	I, t
Liver weight (%)	3.47±0.12[a]	3.65±0.04[a]	3.79±0.11[ab]	3.45±0.10[a]	4.07±0.09[b]	4.04±0.08[b]	I, t
Biochemical parameters							
TC (mg/dL)	162.36±6.10[a]	231.31±12.16[bc]	231.41±14.09[bc]	170.16±8.00[a]	251.30±8.91[b]	200.90±8.28[ac]	I, Ixt
HDLc (mg/dL)	73.22±2.64[a]	83.42±3.60[ab]	77.08±3.31[a]	73.70±5.59[a]	94.09±4.39[b]	73.80±3.12[a]	I
LDLc (mg/dL)	53.94±1.43[bc]	69.82±4.25[a]	65.65±3.84[ac]	47.76±1.85[b]	67.92±3.01[a]	46.92±4.12[b]	I, t, Ixt
Atherogenic index (TC/HDLc)	2.34±0.11[ac]	2.77±0.09[ab]	3.02±0.17[b]	2.25±0.10[c]	2.69±0.08[ab]	2.73±0.07[ab]	I
Triglycerides (mg/dL)	149.60±15.78[a]	187.16±15.57[ac]	457.21±58.76[bd]	219.82±11.93[ce]	269.28±16.38[de]	533.02±50.43[b]	I, t
Phospholipids (mg/dL)	277.96±11.82[a]	319.03±11.53[ab]	356.52±15.70[b]	288.21±12.88[ac]	338.91±12.50[ab]	347.34±20.14[bc]	I
Glucose (mg/dL)	231.88±14.99	253.67±9.90	209.26±12.59	200.97±16.97	254.67±10.88	220.71±16.20	I
NEFAs (mM)	0.46±0.09	0.36±0.05	0.50±0.06	0.55±0.10	0.79±0.16	0.77±0.14	t

Table 4. The hamsters were distributed into six groups, depending on the treatment and the duration of the experiment: control group-4 days (C-4d), diet-induced dyslipidemia-4 days (HFD-4d), pro-dyslipidemic agent-4 days (P407-4d), control group-30 days (C-30d), diet-induced dyslipidemia-30 days (HFD-30d), and pro-dyslipidemic agent-30 days (P407-30d). The diet-induced dyslipidemia was generated by a high-fat diet (HFD) providing 21% energy as fat and 0.92% as cholesterol. The pro-dyslipidemic agent was Poloxamer 407 (P407) and the dose was 50 mg/kg of body weight. P407 animals were fed the control diet and received intraperitoneal injections of the drug every 3 days. Non-P407 hamsters received injections of vehicle (0.9% NaCl) with equal periodicity. The animals were sacrificed 24 h after the last administration of P407 or vehicle and after a 6 h fast. The concentrations of serum parameters were determined at the end of the experiment. Relative fat and liver weights were determined using the formula 100*(tissue weight/body weight) and expressed as a percentage of the total body weight. Fat increase was calculated as the difference in the relative fat mass at each time point from the initial value for each animal. The data are presented as means ± SEM (n = 9 - 10). The statistical comparisons among groups were conducted using two- and one-way ANOVAs. A two-tailed p-value < 0.05 was considered statistically significant. For the two-way ANOVA: *I*: the effect of the pro-dyslipidemic intervention (HFD and P407); *t*: the effect of time; *Ixt*: the interaction between the two factors. When one-way ANOVA was also significant, the *post hoc* analysis was used. [abcd]Mean values within a row with different lowercase letters were significantly different (Tukey's or Games-Howell tests). TC, total cholesterol; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; NEFAs, non-esterified free fatty acids.

3.2.2. Relative expression of hepatic genes implicated in the extracellular release of Lyso-PLs

Impaired hepatocyte functions are responsible for alterations in endogenous plasma lipid and lipoprotein levels and the subsequent development of dyslipidemias [36]. For this reason, we evaluated the relative expression levels of some hepatic genes related to the regulation of extracellular Lyso-PL levels (**Fig. 4**).

The pro-dyslipidemic intervention had significant effects on the relative expression levels of the genes encoding the lipase *Lipg,* the phospholipase *Pla2g2a* ($p < 0.001$) and the acyltransferases *Lcat* ($p = 0.010$) and *Lpcat3* ($p = 0.033$) (two-way ANOVA). Specifically, the pair-wise comparisons revealed that the mRNA levels of *Pla2g2a* were significantly higher in P407-4d animals than the levels in the C-4d group, and very similar trends were observed for *Lipc*, *Lipg* and *Lpcat3*, showing that the P407-treated animals expressed higher levels of these genes than the C group at 4d ($p < 0.050$, Student's t-test). Furthermore, after 30d of pharmacological treatment, the P407-30d hamsters displayed a residual increase in *Lcat* expression and decrease in *Abhd3* mRNA levels compared with the C-30d group ($p < 0.050$, Student's t-test). Moreover, HFD feeding induced alterations in the expression of genes related to hepatic Lyso-PL metabolism, but to a lesser extent than the P407 treatment and, generally, with opposite trends. Thus, compared to the corresponding C group at each time, the expression of *Lcat* and *Lipc* was decreased and increased, respectively, in the HFD-4d group ($p = 0.005$ and $p = 0.049$, Student's t-test), whereas *Pla2g2a* expression decreased in the HFD-30d hamsters ($p = 0.002$, Student's t-test). In addition, the levels of the *Lipg* mRNA were decreased in the HFD-fed animals compared with the control hamsters at both times ($p = 0.024$ and $p = 0.001$, Student's t-test). No significant differences in the expression of any evaluated gene were observed between the C groups.
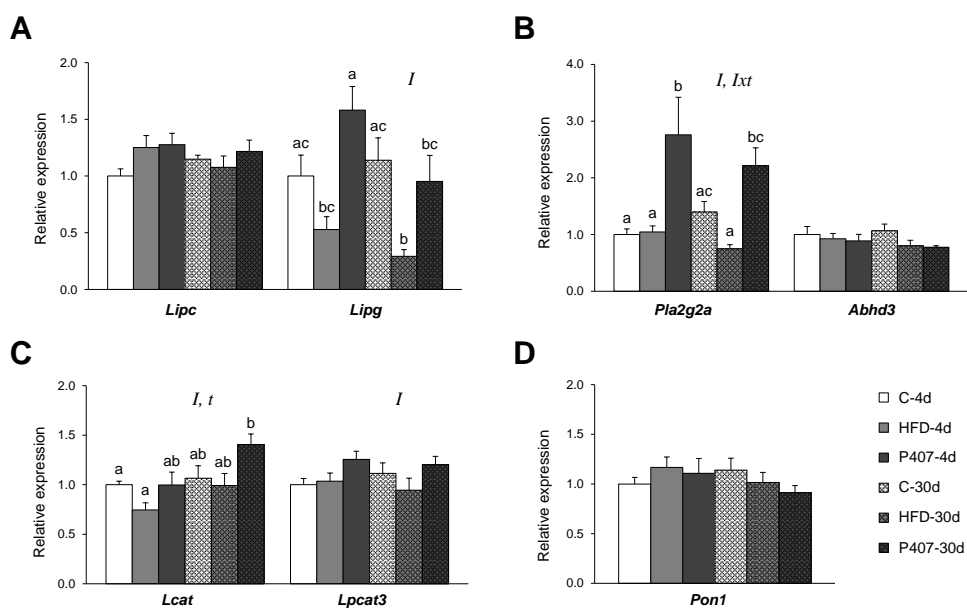
Figure 4. Relative expression levels of hepatic genes implicated in the regulation of the circulating Lyso-PL levels. (**A**) **Lipases:** *Lipc,* hepatic lipase; *Lipg,* endothelial lipase. (**B**) **Acyltransferases:** *Lcat,* lecithin-cholesterol acyltransferase; *Lpcat3,* lysophosphatidylcholine acyltransferase 3. (**C**) **Phospholipases:** *Pla2g2a*, phospholipase A_2 group IIA; *Abhd3,* abhydrolase domain-containing 3. (**D**) *Pon1*, Paraoxonase 1. The hamsters were assigned to six groups, depending on the pro-dyslipidemic treatment and duration of the experiment: control group-4 days (C-4d), high-fat diet-4 days (HFD-4d), pro-dyslipidemic agent-4 days (P407-4d), control group-30 days (C-30d), high-fat diet-30 days (HFD-30d), and pro-dyslipidemic agent-30 days (P407-30d). Total RNA was isolated from the liver and subjected to qRT-PCR analysis. The relative expression levels were determined using *β-actin* as the reference gene and were normalized to the C-4d group. The data are presented as means ± SEM (n = 9 - 10). The statistical comparisons among groups were conducted using two- and one-way ANOVAs. *I:* the effect of the intervention (HFD and P407); *t:* the effect of time; *Ixt*: the interaction between the two factors (two-way ANOVA, $p < 0.05$). [abc]Mean values with different lowercase letters were significantly different (one-way ANOVA and Games-Howell *post hoc* test, $p < 0.05$).

3.2.3. Non-targeted metabolomics of Lyso-PLs and metabolite identification

After confirming that the two pro-dyslipidemic treatments studied here utilize different mechanisms in the liver, we analyzed the circulating metabolome using a non-targeted approach to determine the whole range of altered Lyso-PLs and confirm their suitability as biomarkers of progression and dyslipidemia typology.

After aligning and filtering the molecular entities identified in the six groups of hamsters, the results of the LC-MS/MS analysis showed two characteristic fragmentation patterns that exhibited changes in the dyslipidemic groups (**Table 5**). Based on the exhaustive study of the fragmentation data, we concluded that both patterns represented Lyso-PL species containing the acyl chain in *sn1*-position, which generally is the most common circulating isomer for these metabolites (see **Manuscript 5** in **Annex I).** Thus, when collision energies of 10 V were applied in the positive ionization mode, several daughter ions were generated: a) the loss of the phosphorylethanolamine head group ($[M + H]^+$ - 141), b) the loss of water ($[M + H]^+$ - 18), c) the ion corresponding to the ethanolamine ($[M + H]^+ = 62$) and its fragment, d) the ethylamine ($[M]^+ = 44$), e) the phosphocholine ($[M - H + 2H]^+ = 184$), and f) choline ions ($[M + H]^+ = 104$). The daughter ions described in *a-d* are from lysophosphatidylethanolamine (Lyso-PE) species, whereas the daughter ions described in *e-f* are fragments from the Lyso-PCs identified in the previous study. Published spectra for these lipid subclasses are available in the METLIN database [29] and Liebisch *et al.* [37]. The RTs of Lyso-PLs should also be examined. Lyso-PLs with identical acyl chain lengths but different double bond numbers had consistent behaviors. A greater number of double bonds reduced the retention of the particles on the chromatographic column, corresponding to lower RTs.

According to the two-way ANOVA, six Lyso-PEs and two Lyso-PCs exhibited significant changes in the serum of hamsters subjected to HFD feeding and/or P407 treatment for 4d or 30d (**Table 6**). However, the *post hoc* analysis performed on the six groups revealed a different trend that depended on the Lyso-PL class: Lyso-PC levels - particularly the Lyso-PC (20:2) levels - were increased in the diet-induced dyslipidemic groups, whereas Lyso-PE abundances mainly increased in the P407-treated groups. Moreover, Lyso-PEs allow us to distinguish the groups with P407-induced dyslipidemia. At 4d, the serum Lyso-PE (18:2) and (20:4) levels were already increased, but the (18:3) and (22:6) levels changed over a longer period. Interestingly, as shown in **Table 6** and in **Fig. 5**, Lyso-PE (20:4) was the unique metabolite that showed a significant increase in response to both the HFD and P407 treatments at 4d and at 30d; in addition, its levels remained constant between both C groups. Therefore, at first glance, Lyso-PE (20:4) may be a putative novel biomarker of dyslipidemia.

Table 5. LC-+ESI-MS/MS identification of the altered Lyso-PLs in the serum of hamsters from the second study

Neutral mass	RT (min)	Collision energy		Formula (score) *	Lipid subclass	Tentative identification †
		10 V	20 V			
475.2661	10.85	335.3, 44.1, 458.3	44.1, 335.3	$C_{23}H_{42}NO_7P$ (86.26)	Monoacylglycerophospho ethanolamines	Lyso-PE (18:3)
451.2679	10.92	311.3, 44.1, 434.3	44.1, 311.3	$C_{21}H_{42}NO_7P$ (93.69)		Lyso-PE (16:1)
477.2854	11.20	337.3, 44.1, 62.1, 460.3	44.1, 337.3	$C_{23}H_{44}NO_7P$ (81.06)		Lyso-PE (18:2)
501.2853	11.21	361.3, 44.1, 62.1, 484.3	44.1, 361.3	$C_{25}H_{44}NO_7P$ (91.87)		Lyso-PE (20:4)
525.2847	11.23	385.3, 44.1, 62.1, 508.3	44.1, 385.3	$C_{27}H_{44}NO_7P$ (94.58)		Lyso-PE (22:6)
453.2852	11.41	313.3, 44.1, 62.1, 436.3	44.1, 313.3	$C_{21}H_{44}NO_7P$ (63.07)		Lyso-PE (16:0)
547.3634	11.93	184.1, 104.1	184.1, 104.1, 60.1	$C_{28}H_{54}NO_7P$ (98.74)	Monoacylglycerophospho cholines	Lyso-PC (20:2)
549.3767	12.27	184.1, 104.1	184.1, 104.1, 60.1	$C_{28}H_{56}NO_7P$ (64.24)		Lyso-PC(20:1)

* The molecular formula score was calculated using the 'Molecular Formula Generator' algorithm included in the MassHunter Qual software.

† According to the LIPID MAPS [31] glycerophospholipid nomenclature, the structure of the acyl chain is indicated within parentheses in the 'Headgroup (*sn1* or *sn2*)' format. The headgroup is assumed to be attached at the *sn3* position of glycerol.

Abbreviations: RT, retention time; Lyso-PE, lysophosphatidylethanolamine; Lyso-PC, lysophosphatidylcholine

Table 6. Normalized serum Lyso-PL levels that were significantly altered in the dyslipidemic groups

Compound name		C-4d	HFD-4d	P407-4d	C-30d	HFD-30d	P407-30d	2-way ANOVA (I)	
								p	p (Corr)
Lyso-PE	(20:4)	-0.33 ± 0.05[a]	0.04 ± 0.06[bc]	0.12 ± 0.11[bc]	-0.18 ± 0.08[ac]	0.18 ± 0.08[b]	0.32 ± 0.10[b]	<0.001	<0.001
	(18:2)	-0.17 ± 0.08[a]	-0.06 ± 0.03[ac]	0.28 ± 0.12[bc]	-0.19 ± 0.10[a]	-0.02 ± 0.09[ac]	0.43 ± 0.14[b]	<0.001	<0.001
	(18:3)	-0.10 ± 0.18[a]	-0.22 ± 0.09[a]	0.36 ± 0.17[ab]	-0.23 ± 0.19[a]	-0.30 ± 0.14[a]	0.72 ± 0.19[b]	<0.001	<0.001
	(22:6)	-0.43 ± 0.10[a]	-0.08 ± 0.10[ab]	0.05 ± 0.17[ab]	0.10 ± 0.11[b]	-0.01 ± 0.08[ab]	0.81 ± 0.11[c]	<0.001	<0.001
	(16:1)	-0.26 ± 0.26[a]	-0.25 ± 0.13[a]	0.13 ± 0.24[ab]	0.23 ± 0.17[ab]	-0.20 ± 0.14[a]	0.95 ± 0.21[b]	0.001	0.02
	(16:0)	-0.14 ± 0.11[a]	-0.13 ± 0.09[a]	0.00 ± 0.21[ab]	0.06 ± 0.17[ab]	-0.34 ± 0.20[a]	0.67 ± 0.18[b]	0.005	0.09
Lyso-PC	(20:2)	-0.60 ± 0.17[a]	0.24 ± 0.10[bc]	-0.58 ± 0.22[a]	-0.09 ± 0.19[ac]	0.75 0.19[b]	-0.16 ± 0.21[ac]	<0.001	<0.001
	(20:1)	-0.08 ± 0.16	0.35 ± 0.10	-0.37 ± 0.22	-0.30 ± 0.12	0.27 ± 0.24	-0.29 ± 0.21	0.003	0.08

The levels of each metabolite were transformed to a base-2 logarithm, normalized to the internal standard and to the mean levels of the C-4d samples. The data are presented as the means of the normalized abundances ± SEM (n = 9 - 10). The two-way ANOVA cut-off point was calculated using the Benjamini-Hochberg correction to avoid false positives. The p-values for the main factor 'intervention' without (*p*) and with correction (*p(Corr)*) are shown. *I*: the effect of the pro-dyslipidemic intervention (HFD and P407 administration). When one-way ANOVA was also significant (p < 0.05), the *post hoc* analysis was used. [abc]Mean values within a row with different lowercase letters were significantly different (Tukey's test, p < 0.05).

□ C-4d vs P407-4d   □ C-4d vs HFD-4d   □ C-30d vs P407-30d   □ C-30d vs HFD-30d
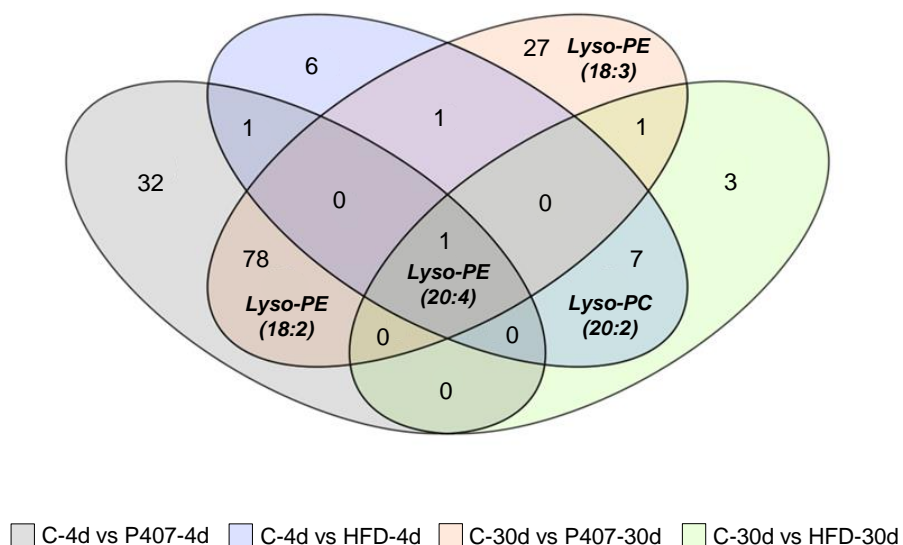
Figure 5. Venn diagram displaying the implications of the identified circulating lyso forms in the four comparisons of interest. The statistical comparisons between the six groups (n = 9 - 10) were assessed using two- and one-way ANOVAs. The cut-off point for significance was $p < 0.05$ and for the two-way ANOVA, the p-value of the 'intervention' effect was calculated using the Benjamini-Hochberg correction. The figures indicate the number of significant metabolites for each comparison of interest, which were determined using Tukey's *post hoc* test. Previously, the metabolites that exhibited significant changes among control groups (C-4d vs C-30d) were discarded. The areas where the ellipses overlap show the significant metabolites, particularly the Lyso-PLs, shared by the specific comparisons. Lyso-PE (20:4) was the only metabolite whose abundance was altered by both diet and the pro-dyslipidemic agent in the short- and long-term assessments.

4. Discussion

Chronic administration of the HFD progressively induced dyslipidemia in adult hamsters after 4, 15 and 30 days of feeding. The biological parameters that first responded to the dietary treatment were the circulating TC and LDLc levels and adiposity index because these parameters increased significantly after 4d of the intervention; at 15d, the consequences of the metabolic disorder started to be observed in the liver weights of the dyslipidemic animals. Finally, the atherogenic index increased after 30d of treatment compared to the controls. These findings are compatible with other studies conducted in HFD-fed hamsters [20,38–40]. Consistent with the main objective of the current study, we introduced an additional group of hamsters in which dyslipidemia was induced through periodic injections of P407 instead of by the diet. Despite the low dose used, P407 induced a type of dyslipidemia characterized by a rapid and dramatic increase in the serum triglyceride levels throughout the study, in addition to early hypercholesterolemia (**Table 4**). The dose-dependent response to P407 was considerably greater for triglycerides than for TC [17]. Nevertheless, the P407-induced hyperlipidemia addressed in the present study was much milder than the form that has been reported in rodents [25,27,28,41] and did not exceed the cholesterol levels of the HFD-fed rodents [42,43]. Furthermore, the adiposity index and serum levels of HDLc and LDLc were not affected by the chemical treatment, whereas the atherogenic index was altered more quickly than in animals administered the HFD, as an increase was already observed at 4d. Thus, hamsters that have been periodically treated with P407 are also a good model for studying atherogenic dyslipidemia [18,25,28]. Interestingly, the liver weights of the animals increased after 30d in the groups treated with both diet and chemical treatments.

Lyso-PLs were the main metabolites with altered circulating levels that were identified in the dyslipidemia-induced hamsters. These biologically active molecules are involved in a broad range of physiological and pathological processes, such as inflammation, apoptosis, reproduction, immunity, carcinogenesis, angiogenesis and regulation of metabolic diseases [44]. Some of these molecules have been proposed as biomarkers of diabetes [45], the progression of atherosclerosis [46,47], obesity [33,48,49] and different types of cancer [35,50,51] in humans. In addition, *in vitro* studies using HepG2 cells have shown differences in the patterns of secreted Lyso-PLs under palmitate-induced, noncytotoxic, steatotic conditions [33]. This result is consistent with the serum levels and hepatic expressions of the Lyso-PL-related enzymes in our study, many of which were modulated by the pro-dyslipidemic intervention (**Fig. 3 and 4**).

Circulating LCAT is exclusively synthetized by the liver and is an important enzyme involved in the reverse transport of cholesterol and maturation of HDL particles [52]. LCAT fulfils its function by transferring fatty acids from glycerophospholipids to cholesterol, contributing to the removal of cholesterol from the circulation and the release of Lyso-PLs [52]. In this study, P407 administration produced 30% and 15% decreases in the circulating LCAT levels after 4d and 30d of treatment, respectively (**Fig. 3a**); however, hepatic LCAT expression was only increased at the longer period (**Fig. 4c**). The increase in the plasma Lyso-PL levels seems to induce the drastic decrease in the LCAT levels after 4d of treatment, and the liver attempts to compensate for this shortage by increasing its expression. Moreover, increases in the biological activity of LCAT have been reported in P407-treated rodents [17,27] as a compensatory mechanism for the high plasma cholesterol loading in these animals. Modifications in exogenous LCAT activity and hepatic mRNA abundance have also been described in hamsters fed different dietary fats [40], but our results did not show a significant effect on LCAT expression in the diet-induced dyslipidemic animals.

Lipases also contribute to cholesterol homeostasis due to their ability to hydrolyze triglycerides, cholesteryl esters and phospholipids within circulating lipoproteins, mediating important processes involved in the remodeling of these heterogeneous particles [52]. Endothelial lipase (EL) predominantly exerts phospholipase activity and prefers phosphatidylethanolamine as a substrate [44,53,54]. The hydrolysis of this favored substrate leads to the synthesis of Lyso-PEs, which constitute the most representative lipid subclass of our second study. Through its phospholipase activity on HDLc, EL enhances cholesterol removal from peripheral tissues and promotes hepatic accumulation [54]. In this study, both pro-dyslipidemic treatments ($p < 0.001$, two-way ANOVA) modified the hepatic expression of EL (*Lipg* locus) (**Fig. 4a**). After 4d, EL expression increased in the P407-treated group and decreased in the HFD-fed hamsters compared to the C-4d group. However, EL expression was also equally influenced by the time and the interaction ($p = 0.062$, two-way ANOVA), and the levels in both treated groups were decreased by half at day 30 compared to the earlier time points. The inactivation of lipases by P407 has been shown in different rodent models [27,55] and is related to liver injury [18]. Animal and human studies have revealed that lipases are negative regulators of HDLc levels [56,57]. Moreover, HFD-fed animals showed the lowest EL expression levels in the liver and the highest plasma HDLc levels (**Table 4**).

sPLA$_2$-IIA is a well-known pro-inflammatory enzyme that is involved in arachidonic acid release and eicosanoid production in the secretory compartment [58]. Interestingly, similar to EL, sPLA$_2$-IIA has strong activity towards anionic phospholipids, such as phosphatidylethanolamines [58]. Some studies have described a sPLA$_2$-mediated progression of liver impairments in rodents when the inducer was a chemical agent [59,60].

Compared to the corresponding controls, we observed a substantial 200% increase in hepatic sPLA$_2$-IIA expression (*Pla2g2a* locus) in the P407-4d animals and 60% increase (p = 0.043, Student's t-test) in the P407-30d group (**Fig. 4b**), but not in the animals fed the HFD. This behavior is related to the substantial increase (p = 0.024, Student's t-test) in EL expression in the P407-4d hamsters compared to the HFD-fed group (**Fig. 4a**) because EL has been positively associated with inflammation in rodents and humans [54,61]. The decreased circulating sPLA$_2$-IIA levels in the P407 groups might be explained by the observation that upon secretion by hepatocytes, the enzyme is rarely into the circulation due to the high rate of synthesis of Lyso-PLs. Based on these findings, animals that have been periodically treated with P407 develop dyslipidemia accompanied by hepatic inflammation, in contrast to the HFD-fed animals.

PON1 is an antioxidant enzyme of primarily hepatic origin that degrades oxidized cholesteryl esters and phospholipids within the lipoproteins [62,63] and has been proposed as a suitable non-invasive biomarker for human liver diseases [64]. Liver injury is usually associated with decreased serum activity and hepatic expression but increased circulating PON1 levels [65,66]. In this study, the pair-wise comparisons did not reveal significant differences between the dyslipidemic and control hamsters, but the highest circulating levels were observed in the animals treated with the drug (**Fig. 3c**) and the largest decrease in hepatic expression of 20% was observed in the P407-30d group (**Fig. 4d**). The relation between P407 and PON1 has been described in injected mice, but our results in hamsters were not as clear [67].

LPCAT3 is responsible for Lyso-PL reacylation and participates in the remodeling of phospholipids and membrane fluidity [68]. The composition of the cell membranes influences the lipid contents in the blood [69]. Mice lacking *Lpcat3* in the liver had lower circulating levels of lipids containing arachidonic acid and inefficiently mobilized triglycerides into very low-density lipoproteins (VLDL), resulting in lipid accumulation in hepatocytes [70]. The P407-treated groups, which were the animals with the highest serum Lyso-PE (20:4) levels, display an early tendency to overexpress *Lpcat3* (**Fig. 4c**), suggesting that hepatic VLDL production may be increased in these animals. In fact, although other authors have reported a shift in the distribution of cholesterol to the VLDL fraction as consequence of P407 administration [42], we observed an increase in the TC levels in P407-4d hamsters, which did not correspond to either HDLc or LDLc (**Table 4**).

Thus, we have verified that dyslipidemia was induced by different hepatic mechanisms, depending on the etiology of the disorder [16,26,42,71]. The dietary intervention seems to induce dyslipidemia by inactivating the hepatic genes involved in

lipolysis, which induces the liver to sequester lipids [26,43,71–74]; however, oxidative stress and inflammation processes predominate in the animals treated with the chemical agent [17,59,61,67]. Moreover, the development of hepatic steatosis without major inflammation has been described in rodents fed an HFD [71,75], whereas a histological examination of livers from P407-treated animals shows defenestration of the sinusoidal endothelium and the accumulation of large foamy Kupffer cells [10,41]. These hepatic alterations are accompanied by the absence of steatosis and are also observed in cirrhosis [76] and aging [77]. In this context, the Lyso-PLs levels may be used as a biomarker to distinguish typologies of hepatic disorders.

The non-targeted metabolomics evaluation presented in the first study suggested that this class of circulating lipids changes in accordance with the progression of the lipid disorder. The second study seems to confirm their suitability for the evaluation of the dyslipidemia state, showing a joint association of the Lyso-PE levels with the progression of the disorder induced by the pharmacological treatment; however, the Lyso-PCs appear as specific biomarkers for the dyslipidemia from dietary origin. Among all of the Lyso-PLs, **Fig. 5** shows the best putative candidate circulating biomarkers and discards those metabolites that were modified over time in the untreated animals. For their possible application in diagnosis, Lyso-PC (20:2), Lyso-PE (18:2) and Lyso-PE (20:4) were the Lyso-PLs that exhibited the earliest alterations in response to the dyslipidemia. Furthermore, Lyso-PE (20:4) was the unique metabolite that was modulated by both treatments throughout the study.

Due to the key role of lipids in the development and stability of atherosclerotic plaques, dyslipidemia is considerate a primary risk factor for atherosclerosis, which can remain asymptomatic for years. As described above, both types of treatments induced significant increases in the atherogenic index of the hamsters. Interestingly, other authors reported elevated amounts of several Lyso-PCs in atherosclerotic human aorta [78] and within symptomatic versus asymptomatic carotid plaques [46]. On the other hand, increases in the plasma Lyso-PE (20:4) and (22:6) levels were associated with the occurrence of stable lesions but not unstable plaques [47].

The elucidation of Lyso-PLs as early biomarkers of pathology is also consistent with recent studies indicating that chronic intake of an HFD dysregulates the plasma and hepatic levels of Lyso-PCs and Lyso-PEs in obese [79] and non-obese [80] mice. Consistent with our results, these authors observed altered circulating levels of Lyso-PCs (18:0), (18:2), (18:3) and (20:1) and Lyso-PEs (18:2), (20:4) and (22:6) in HFD-fed rodents. Actually, regarding hepatic disorders, some studies have examined hepatocarcinogenesis progression [35] and selected certain members of Lyso-PEs as early biomarkers of pathology and altered patterns

of Lyso-PCs as plasma indicators to distinguish the most advanced stage, namely the carcinoma phase. Moreover, increases in the serum Lyso-PEs (18:2) and (20:4) levels associated with early liver injury are reversed by fenofibrate therapy, an anti-dyslipidemic agent [73,81]. Therefore, modifications in circulating Lyso-PE and Lyso-PC levels may be related to hepatic disorders and Lyso-PL patterns change with the progression of dyslipidemia-related diseases. Among all the species of Lyso-PLs, Lyso-PE (20:4) seems to possess specific potential as biomarker of the risk of developing lipid pathology.

We concluded that Lyso-PL evaluation could assist with the exhaustive diagnosis and management of lipid disorders since they provide information about the etiology of the dyslipidemia and, furthermore, allow researchers to determine the extent of dyslipidemia. In any case, the omics-based approach used in this study only provided a first approximation of this interesting issue. Despite the abundant evidence in the literature on this topic, we believe that the future studies should be focused on the exhaustive evaluation of the whole family of Lyso-PLs in these animal models. Subsequently, extensive validation in subjects with dyslipidemia-related diseases is required to verify the suitability of the non-invasive biomarkers for use in the population.

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6. References

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CHAPTER 2

Targeted metabolomics evaluation of lysophospholipids

**Manuscript 3**

Chronic effect of a cafeteria diet and intensity of physical training on the circulating lysophospholipidome of young rats

(Manuscript under preparation)

PREFACE

In the non-targeted metabolomics study of **Manuscript 1**, we have shown that a cafeteria diet (CAF) administered from a weaning age and continued until adolescence leads to drastic modifications in the circulating metabolome related with lipid metabolism and inflammation and promotes the development of MetS in rats. We could also observe that the practice of daily exercise had a minor impact in the metabolome of animals, exerting a therapeutic effect on MetS by means of the reduction of the visceral adipose tissue (RWAT weigth %), leptin/adiponectin ratio in serum and triglycerides levels. Furthermore, the training intensity was directly proportional to the beneficial effects described for animals fed the MetS-inducing diet.

Lysoglycerophospholipids (Lyso-PLs) was the family of altered metabolites most representative of that study. Therefore, our objective in the present investigation was the detailed evaluation of Lyso-PLs levels in the serum of rats fed standard chow (ST) or CAF and submitted to different intensities of physical exercise carried out in a treadmill. Thus, we intend to assess the potential of Lyso-PLs as circulating biomarkers of lipid disorder in rats. For this challenge, we have used targeted metabolomics analysis based on liquid chromatography coupled to tandem mass spectrometry. The analytical method applied in the Lyso-PL profiling and quantification was that developed and validated in the **Annex I**.

Chronic effect of a cafeteria diet and intensity of physical training on the circulating lysophospholipidome of young rats

Abstract

The daily practice of physical exercise and a balanced nutrition are the main advices to prevent the onset of metabolic syndrome (MetS) from youth. Because of MetS is a multifactorial disorder that is associated to development of serious diseases, the advancement of comprehensive biomarkers could aid in an accurate diagnosis of patients. In preliminary studies, we observed that alterations in circulating lysophospholipids (Lyso-PLs) were shared between animals with diet-induced MetS and those performing resistance exercise assiduosly. Therefore, our objective in the present investigation was the determination of Lyso-PL levels in the serum of young rats fed standard (ST) or cafeteria diet (CAF) and subjected to different training intensities to evaluate the potential of Lyso-PLs as biomarkers of detrimental lifestyle. For this purpose, we have used targeted metabolomics intended to the analysis of lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) in rat serum. Furthermore, Lyso-PL profile was examined using multivariate statistics to achieve an integral understanding. The lifestyle intervention induced modifications in most of the Lyso-PL levels. Multivariate statistical analysis showed that the indicative trace left by Lyso-PLs in serum is representative of the overall metabolome of animals. The chronic intake of CAF had a prevalent influence on the lysophospholipidome modulating the serological levels of both Lyso-PL categories. Among the twenty-two Lyso-PLs directly altered by CAF, Lyso-PCs (14:0), (17:1) and (20:2) and Lyso-PEs (18:2) and (18:3) were enough to achieve an optimal prediction. The effect of physical training was particularly focused on lowering Lyso-PE levels with disparities among training intensities for each diet. Whereas ST-fed groups generally presented a gradual response regarding exercise intensity, the moderate training was most effective on CAF-fed animals. Lyso-PC (16:0) and Lyso-PEs (16:0), (18:0), (20:4) and (22:6) were the most important metabolites discriminating sedentary groups. In conclusion, the examination of the circulating lysophospholipidome could be a good strategy to be used in the diagnosis of dyslipidemia-related diseases.

Keywords: metabolic syndrome; cafeteria diet; physical activity; lysophospholipidome

1. Introduction

Metabolic syndrome (MetS) represents a serious public health problem due to the uneven worldwide prevalence can reach around 80% in some regions [1]. The incidence depends of multiple factors including age, gender, ethnicity and social status of cultural and economic type. Among others, the composition of diet, overnutrition and level of physical activity can constitute important risk factors for MetS and its components [1,2]. However, a detrimental lifestyle can be easily corrected if diagnosed properly, contributing to the prevention and treatment of metabolic disorders, specially in young people [3,4].

In preliminary studies, we have observed that a highly palatable cafeteria diet (CAF) provided from a weaning age and continued until late adolescence induce a profound impact on the serum metabolome impairing the lipid metabolism and inflammatory response and leading to development of MetS in rats [5]. In particular, CAF feeding increased the body weigth gain and RWAT depot mass and induced the advance of hypertriglyceridemia, hyperleptinemia, hyperglycemia and insulin resistance in young rats [6]. We could also observe that the practice of daily exercise had a minor impact on the animal metabolome by reducing the serum levels of different lipid species. Regular training exerted a therapeutic effect on MetS by means of the reduction of the RWAT weigth, leptin/adiponectin ratio in serum and triglycerides levels [5,6]. Other salutary properties described for endurance exercise training are improvements in blood pressure and heart rate, weight loss and a systemic lipid-lowering effect associated with reduction in the fat content of adipose tissue and liver [7,8]. Therefore, a resistance training is aimed at preventing of diseases associated with lipid oversupply.

Extracellular lysophospholipids (Lyso-PLs), concretely those with a structure based on glycerol, was the family of altered metabolites most representative of that study [5]. They are deacylated forms of phospholipids that can be synthesized *de novo* from glycerol-3-phosphate and fatty acyl-CoA or through the enzymatic hydrolysis of glycerophospholipids under phospholipase activity [9]. For a long time, lipids have been solely considered as a source of energy reserve and structural elements in biological membranes. However, nowadays Lyso-PLs are included within bioactive lipids with signaling and regulatory capacities and their relevance in the field of biomedicine is growing [10]. With the progress in the last years of the omics technologies, imbalances in Lyso-PL levels have been closely related to atherosclerosis [11,12], nonalcoholic fatty liver diseases [13–15] and childhood and adult obesity [16–19]. In this context, Pietiläinen *et al.* demonstrated in young adult twins that circulating lysophosphatidylcholine (Lyso-PC) content was associated with acquired obesity independently of genetic factors [20]. We further observed in non-obese dyslipidemic animals

that the alterations in lysophosphatidylethanolamines (Lyso-PEs) were due to the endogenous metabolism rather than diet (see **Manuscript 2**). It is also known that the dietary habits conducive to treating MetS have compensatory effects in the plasma levels of both classes of Lyso-PLs [21–23]; however, there is very little research about endurance exercise and the influence of different training intensities on the Lyso-PL-related metabolome associated to MetS.

Therefore, the main objective of the present investigation was the detailed evaluation of Lyso-PLs levels in the serum of young rats fed standard chow (ST) or CAF and submitted to different intensities of aerobic training performed voluntarily in a treadmill without inclination. Thus, we intend to assess the potential of Lyso-PLs as circulating biomarkers of lipid disorder in youth. For this challenge, we have used a targeted metabolomics analysis intended for thirty-one Lyso-PLs in rat serum [24]. Metabolites were firstly separated by ultra-high-performance liquid chromatography and, after that, their molecular structure and sample concentration were determined using tandem mass spectrometry based on soft ionization.

2. Material and methods

2.1. Ethics statement

The animal protocol was approved by the Generalitat de Catalunya. All of procedures have been performed following the "Principles of laboratory animal care" and according to the European Communities Council Directive regarding the protection of experimental animals (86/609/EEC).

2.2. Animals

For the present study, it was used the same cohort of animals described for the non-targeted metabolomics evaluation carried out in a previous phase of the investigation (**see Material and Methods in Manuscript 1**) [5]. Briefly, the animals were female Sprague Dawley rats weaned at 21 - 23 days of age and divided into 6 groups (n = 9 – 12 per group) with comparable body weights. For 8 weeks, the animals were fed *ad libitum* either ST (Harlan, Barcelona, Spain) or CAF. Both dietary groups were further submitted to a periodic training on a treadmill at different intensities (CON: 0; TML: 12; TMH: 17 m/min). The training sessions were organized 5 days per week and extended for 30 min.

The animals were fasted overnight (12 h) and alternately sacrificed by beheading. Total blood was collected and serum was obtained by centrifugation (2,000 g, 15 min, 4°C). Samples were conserved at - 80°C until metabolite extraction and LC-MS/MS analysis.

2.3. Chemicals

The mobile phases used for the <u>chromatographic separation</u> of Lyso-PLs were prepared with methanol (MeOH) (Scharlab, Barcelona, Spain), acetonitrile (Millipore, Darmstadt, Germany), isopropanol and 7.5 M ammonium acetate solution (Sigma-Aldrich, St. Louis, MO, USA). All of them were of the highest grade commercially available. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain).

The standards using in the <u>LC-MS/MS analysis</u> of Lyso-PLs were 1-tridecanoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (13:0); 1-palmitoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (16:0); 1-stearoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (18:0); 1-arachidoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (20:0); 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine, Lyso-PE (16:0); 1-stearoyl-*sn*-glycero-3-phosphoethanolamine, Lyso-PE (18:0); and 1-oleoyl-*sn*-glycero-3-phosphoethanolamine, Lyso-PE (18:1). All of them were over 99% purity and were purchased from Avanti Polar Lipids (Birmingham, AL, USA). A solution containing chloroform (Sigma-Aldrich, St. Louis, MO, USA) was used for the dilution of standards.

Butylated hydroxytoluene (BHT, Sigma-Aldrich, St. Louis, MO, USA) was added to MeOH to avoid metabolite oxidation during <u>sample extraction</u>.

2.4. Preparation of standard curves

Lyso-PLs were individually dissolved in MeOH / chloroform / water (65:35:8 v / v / v) at 2 mg/mL and preserved in dark-glass vials at - 20°C. The day of the LC-MS/MS analysis, mixed standard solutions with concentrations of 1, 10 and 100 mg/L were prepared using MeOH. Similarly to the calibrators, Lyso-PC (13:0) was handled separately to be used as internal standard (IS).

The calibration curves were prepared by the addition of increasing amounts of the standard mixtures to constant final volumes of water / isopropanol / acetonitrile (4:3:3 v / v / v) in the presence of the IS. Concentrations of the calibrators were ranging from 0 to 5 mg/L, whereas the IS was added at a final concentration of 500 or 360 µg/L depending on the later procedure of extraction (**see section 2.5 above**). The preparations were extracted and subsequently analysed by using the same procedure as the serum samples. The calibration curves were finally generated for each standard by plotting the peak abundance ratios (analyte / IS) versus the concentration ratios (analyte / IS) and fitting to a linear regression (**Fig. 1**).
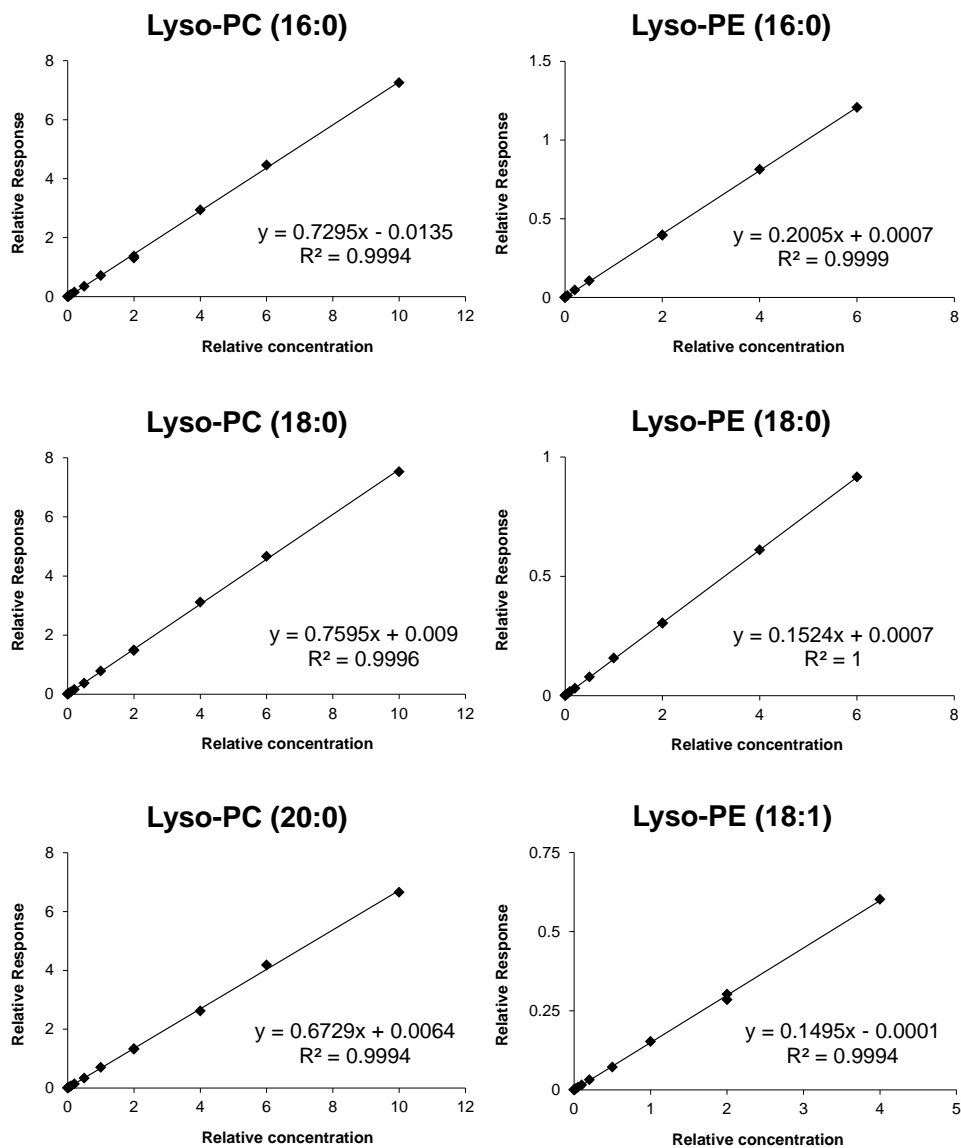
Figure 1. Standard curves used for the analysis of endogenous Lyso-PLs.

2.5. Sample processing

Lyso-PLs are in a wide range of concentrations into the circulation [24]. Due to that, two different procedures for serum processing were required in the present study. Both of them are based on liquid-liquid extractions. The first one, it was aimed at the analysis of the most abundant compounds, mainly Lyso-PCs, and needs only 5 µL of sample; so we have called it the **"Low sample volume" (LSV)** extraction. The second procedure instead, assisted in the determination of serum Lyso-PEs and starts with a greater volume (50 µL) of sample; so it was called the **"High sample volume" (HSV)** method. **Fig. 2** shows an outline of the steps to follow in each procedure. For more information regarding sample processing, refer to **Material and Methods in Annex I.**
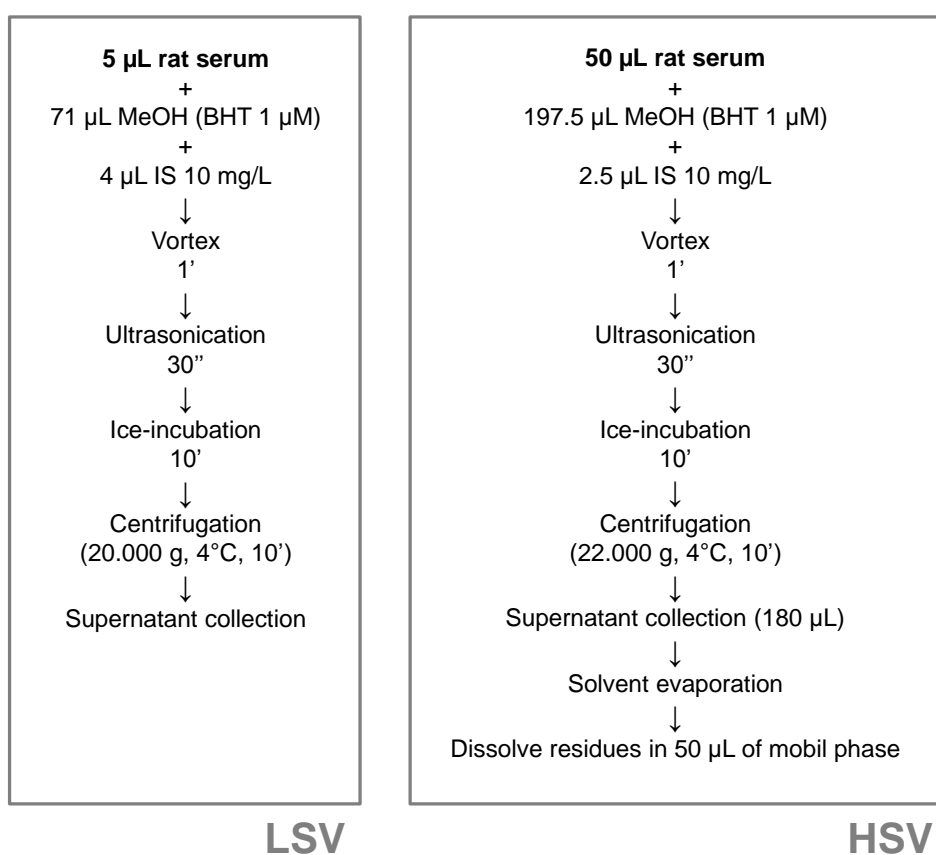

Figure 2. Complementary procedures of sample processing for the exhaustive metabolomics analysis of Lyso-PLs.

2.6. Targeted metabolomics analysis of Lyso-PLs

The quantitative evaluation of Lyso-PLs in biological samples was carried out by means of UHPLC-+ESI-MS/MS analysis following the methodology developed and validated in **Annex I** [24].

The chromatographic separation was performed in a UHPLC 1290 (Agilent Technologies, Palo Alto, CA, USA) composed of a degasser, a binary pump, and thermostatted autosampler and column compartments kept at 4°C and 50°C, respectively, during the analysis. The mobile phase consisted of water / isopropanol / acetonitrile / 500 mM ammonium acetate (89:5:5:1 v / v / v / v) as solvent A and isopropanol / acetonitrile / 500 mM ammonium acetate (50:49:1 v / v / v) as solvent B. Each sample (2 µL) was loaded into a 0.3 mL/min flow of 30% B passing through a reversed-phase column (Acquity UPLC BEH C8) with 1.7 µm of particle size and 2.1 mm internal diameter x 150 mm length (Waters Corporation, Milford, MA, USA). After that, the analytes eluted along a continuous gradient to 75% B over 20 min. The complete elution gradient and specific retention times of Lyso-PLs were those reported in **Annex I**.

The tandem mass spectrometer analysis was achieved through positive electrospray ionization (+ESI) performed within a QqQ 6490, also from Agilent Technologies. The ionization source parameters were as follows: nebulizer gas (nitrogen) pressure, 25 psi; gas flow, 12 L/min at 240°C; sheath gas flow, 12 L/min at 350°C; capillary and nozzle voltages, 4.5 kV and 500 V, respectively; and fragmentor and cell accelerator voltages, 380 and 5 V, respectively. The selected scan mode was dynamic multiple reaction monitoring (MRM). More details regarding MS/MS analysis of Lyso-PLs, such as monitored transitions and optimal collision energies, can be found in **Table 1 of Annex I**.

2.7. Statistical analyses

2.7.1. Univariate analysis

The results are presented as the means ± standard errors (SEM) from the indicated number of rats. Shapiro-Wilk test was used for the assessment of the normality of the data. After that, differences among the six groups of rats were determined using two and one-way ANOVAs. First, analysis based on two-way ANOVA was used to evaluate the main effects of the diet and the physical exercise and their interaction. When any of the effects was statistically significant, one-way ANOVA was used to determine the differences among means. The homoscedasticity between groups was assessed using Levene's test. Tukey's *post hoc* contrast was applied when the variances were similar, whereas Dunnett's T3 *post*

hoc contrast was applied if this assumption was not fulfilled. A two-tailed value of $p < 0.05$ was considered statistically significant. The univariate statistical analysis was performed with Statistical Package for Social Sciences (IBM SPSS Statistics, version 19.0).

2.7.2. Multivariate analysis

A multivariate statistical evaluation based on a combination of principal components analysis (PCA), partial least squares discriminant analysis (PLS-DA) and hierarchical clustering analysis was performed to determine the influence of diet and physical exercise on the Lyso-PL-related metabolome. Receiver operating characteristic (ROC) curves, permutation tests and random forest classification were also conducted to validate the multivariate biomarker. All analyses were performed after range scaling with the use of the software MetaboAnalyst (version 3.0) available online [25].

3. Results

3.1. Determination of the systemic levels of Lyso-PLs in the experimental animals

Targeted metabolomics analysis of the thirty-one Lyso-PLs identified in rat serum was conducted in the experimental groups. The circulating levels expressed on micro molarity (μM) are reported in **Table 1**. In general, Lyso-PEs with maximum levels of scarcely a dozen μM were in lower serum concentration than Lyso-PCs, among which those with saturated acyl chains of 16 and 18 carbons reached several hundred μM. Other abundant Lyso-PLs in rat serum were Lyso-PCs (20:4) and (18:2). Similar pattern of concentrations was followed within Lyso-PEs.

Table 1. Lysophospholipid levels in serum of rats fed a standard chow (ST) or a cafeteria diet (CAF) and periodically trained in a treadmill at different intensities (CON: 0; TML: 12; TMH: 17 m/min)

Metabolite (µM)	Animal group						2-way ANOVA
	CON-ST	TML-ST	TMH-ST	CON-CAF	TML-CAF	TMH-CAF	
Lyso-PCs							
(14:0)	2.062 ± 0.059 [a]	1.988 ± 0.064 [a]	1.969 ± 0.113 [a]	2.894 ± 0.143 [b]	2.632 ± 0.108 [b]	2.674 ± 0.129 [b]	D
(15:0)	1.530 ± 0.075 [a]	1.323 ± 0.057 [ab]	1.298 ± 0.064 [ab]	1.220 ± 0.044 [b]	1.113 ± 0.038 [b]	1.168 ± 0.051 [b]	D, E
(16:0)	154.774 ± 0.740 [a]	138.183 ± 7.787 [ab]	134.710 ± 4.210 [b]	145.448 ± 3.447 [ab]	126.934 ± 4.947 [b]	131.106 ± 7.666 [ab]	E
(16:1)	2.298 ± 0.135 [a]	2.405 ± 0.176 [ac]	2.191 ± 0.122 [a]	4.378 ± 0.326 [b]	3.405 ± 0.127 [b]	3.744 ± 0.342 [bc]	D, DxE
(17:0)	3.374 ± 0.209 [a]	3.521 ± 0.278 [ab]	2.962 ± 0.207 [ac]	2.517 ± 0.122 [bc]	2.289 ± 0.116 [c]	2.195 ± 0.177 [c]	D
(17:1)	0.113 ± 0.006 [a]	0.106 ± 0.008 [a]	0.107 ± 0.007 [a]	0.227 ± 0.017 [b]	0.188 ± 0.012 [b]	0.207 ± 0.020 [b]	D
(18:0)	223.830 ± 8.906	254.579 ± 16.940	220.397 ± 10.399	235.556 ± 12.324	211.205 ± 9.884	223.689 ± 10.308	
(18:1)	10.704 ± 0.282 [a]	12.041 ± 1.212 [a]	10.943 ± 0.652 [a]	22.178 ± 1.427 [b]	17.845 ± 1.032 [b]	21.538 ± 1.940 [b]	D, DxE
(18:2)	31.608 ± 1.393 [a]	31.560 ± 2.673 [ab]	28.4467 ± 1.703 [ab]	23.611 ± 1.627 [bc]	21.097 ± 1.051 [c]	24.427 ± 2.059 [ac]	D
(18:3)	0.471 ± 0.039	0.579 ± 0.074	0.512 ± 0.075	0.382 ± 0.049	0.318 ± 0.039	0.317 ± 0.048	D
(20:0)	0.504 ± 0.031 [a]	0.530 ± 0.045 [ab]	0.434 ± 0.013 [ab]	0.308 ± 0.022 [bc]	0.271 ± 0.021 [c]	0.276 ± 0.028 [c]	D
(20:1)	1.425 ± 0.079 [a]	1.441 ± 0.106 [a]	1.122 ± 0.089 [ab]	0.888 ± 0.048 [bc]	0.763 ± 0.056 [c]	1.109 ± 0.157 [ac]	D, DxE
(20:2)	0.552 ± 0.059 [a]	0.622 ± 0.062 [a]	0.506 ± 0.052 [a]	0.219 ± 0.028 [b]	0.203 ± 0.018 [b]	0.227 ± 0.025 [b]	D
(20:3)	0.757 ± 0.062 [ac]	0.803 ± 0.115 [ac]	0.722 ± 0.107 [a]	1.724 ± 0.136 [b]	1.647 ± 0.122 [b]	1.814 ± 0.273 [bc]	D
(20:4)	53.596 ± 2.983	55.154 ± 5.681	46.297 ± 3.136	61.430 ± 2.813	51.069 ± 3.096	55.131 ± 4.538	
(20:5)	0.117 ± 0.027 [a]	0.137 ± 0.029 [ab]	0.120 ± 0.033 [ab]	0.266 ± 0.035 [b]	0.189 ± 0.025 [ab]	0.250 ± 0.074 [ab]	D
(22:5)	0.595 ± 0.128 [ab]	0.470 ± 0.064 [a]	0.470 ± 0.047 [a]	0.807 ± 0.059 [b]	0.738 ± 0.110 [ab]	0.757 ± 0.141 [ab]	D
(22:6)	6.452 ± 0.675	6.622 ± 0.695	4.863 ± 0.244	6.357 ± 0.388	4.950 ± 0.415	5.253 ± 0.585	

Lyso-PEs							
(16:0)	4.730 ± 0.291 [a]	4.484 ± 0.319 [ab]	3.510 ± 0.165 [b]	4.923 ± 0.215 [a]	3.749 ± 0.183 [b]	4.241 ± 0.355 [ab]	E, DxE
(16:1)	0.027 ± 0.002 [a]	0.030 ± 0.003 [ac]	0.028 ± 0.002 [a]	0.058 ± 0.005 [b]	0.039 ± 0.002 [bc]	0.041 ± 0.002 [bc]	D, E, DxE
(18:0)	9.292 ± 0.427 [ab]	9.590 ± 0.819 [ab]	7.355 ± 0.290 [a]	11.173 ± 0.641 [b]	8.270 ± 0.265 [a]	9.059 ± 0.779 [ab]	E, DxE
(18:1)	1.109 ± 0.055 [ab]	1.024 ± 0.041 [a]	1.038 ± 0.059 [b]	1.364 ± 0.095 [b]	1.077 ± 0.046 [a]	1.280 ± 0.067 [ab]	D, E
(18:2)	1.061 ± 0.086 [a]	1.138 ± 0.080 [a]	1.014 ± 0.040 [a]	0.743 ± 0.092 [ab]	0.574 ± 0.024 [b]	0.646 ± 0.048 [b]	D
(18:3)	0.021 ± 0.002 [a]	0.023 ± 0.002 [a]	0.020 ± 0.001 [a]	0.011 ± 0.001 [b]	0.008 ± 0.000 [b]	0.008 ± 0.000 [b]	D
(20:1)	0.050 ± 0.003	0.047 ± 0.004	0.039 ± 0.002	0.056 ± 0.008	0.043 ± 0.004	0.056 ± 0.004	
(20:2)	0.031 ± 0.002 [a]	0.032 ± 0.003 [a]	0.028 ± 0.001 [a]	0.018 ± 0.002 [b]	0.014 ± 0.001 [b]	0.017 ± 0.001 [b]	D
(20:3)	0.027 ± 0.002 [ac]	0.026 ± 0.001 [a]	0.027 ± 0.003 [abc]	0.043 ± 0.004 [bc]	0.036 ± 0.002 [bc]	0.040 ± 0.003 [b]	D
(20:4)	1.123 ± 0.046 [ab]	1.115 ± 0.094 [a]	1.021 ± 0.077 [a]	1.466 ± 0.099 [b]	1.015 ± 0.048 [a]	1.173 ± 0.090 [ab]	D, E, DxE
(22:4)	0.047 ± 0.002	0.045 ± 0.003	0.044 ± 0.002	0.045 ± 0.003	0.039 ± 0.002	0.043 ± 0.002	
(22:5)	0.046 ± 0.008 [ab]	0.037 ± 0.003 [a]	0.039 ± 0.004 [a]	0.061 ± 0.005 [b]	0.048 ± 0.006 [ab]	0.050 ± 0.007 [ab]	D
(22:6)	0.337 ± 0.030 [ab]	0.336 ± 0.036 [ab]	0.237 ± 0.011 [a]	0.379 ± 0.025 [b]	0.250 ± 0.018 [b]	0.280 ± 0.037 [ab]	E, DxE

Diets and training sessions started in female rats after the weaning period and were extended for 8 weeks. The training program was conducted 5 days per week for 30 min. The circulating levels of lysophospholipids belonging to lysophosphatidylcholine (Lyso-PC) and lysophosphatidylethanolamine (Lyso-PE) lipid subclasses were determined at the end of the study after a 12 h fast. The data are presented as means ± SEM (n = 9 – 12). The statistical comparison among the six groups of animals was conducted using two- and one-way ANOVAs. *D*: the effect of diet; *E*: the effect of exercise; *DxE*: the interaction between the two main factors (two-way ANOVA, p < 0.05). [abc]: Mean values with different lowercase letters were significantly different (one-way ANOVA and Tukey or Dunnett's T3 *post hoc* contrasts, p < 0.05).

Based on the comparison of the six groups by two-way ANOVA, the lifestyle intervention induced significant modifications in the levels of the majority of Lyso-PLs (**Fig. 3**), specifically in twenty-six of them. As **Table 1** shown, these differences were present in both Lyso-PCs and Lyso-PEs and more than 70% were due to the type of feeding. Thus, while the circulating levels of Lyso-PCs (14:0), (16:1), (17:1), (18:1), (20:3), (20:5), (22:5) and Lyso-PEs (16:1), (18:1), (20:3), (20:4), (22:5) were increased; the levels of Lyso-PCs (15:0), (17:0), (18:2), (18:3), (20:0), (20:1), (20:2) and Lyso-PEs (18:2), (18:3), (20:2) decreased in response to CAF intake. As a result, when animals were fed CAF, the levels of Lyso-PLs with different head groups but identical acyl chains typically followed comparable trends.
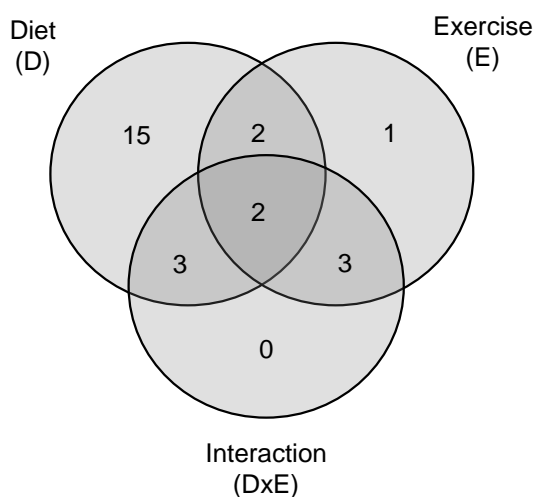
Figure 3. Venn diagram showing the number of serum Lyso-PLs from each experimental factor. Data were analysed using two-way ANOVA. *D*: the effect of diet; *E*: the effect of exercise; *DxE*: the interaction between the two main factors (p < 0.05). The areas where the circles overlap show the number of significant lyso forms shared by the factors.

On the other hand, the effect of physical training was prevalent in Lyso-PEs, influencing the levels of almost half of the molecular species, while only 10% of the Lyso-PCs were altered (p < 0.050, two-way ANOVA). Alongside diet, the levels of Lyso-PEs (18:1) and (20:4) were affected by exercise. The use of the treadmill counteracted the high levels induced by CAF intake mainly in the group that was running at low speed (TML-CAF), in which significant differences in the levels of both metabolites were observed compared to the CON-CAF group (**Table 1**). Circulating Lyso-PE (16:1), also influenced by diet and exercise, behaved similarly to the others but the drop in levels was observed in both trained groups of CAF-fed animals as residual responses (p < 0.020, Student's t-test). No significant changes in these Lyso-PEs were found among ST-fed groups. In fact, Lyso-PEs (16:1) and (20:4) were also significantly affected by interaction of the two experimental factors. Lyso-PC (16:0) instead, was exclusively influenced by exercise which led to a general decrease in the circulating levels of animals fed both diets. According to the *post hoc* analysis, other

decreases in Lyso-PL levels as a consequence of exercise were detected in Lyso-PEs (16:0), (18:0) and (22:6) and, curiously, once more were found between CON-CAF and TML-CAF groups.

Based on these results, the chronic intake of a MetS-inducing diet elicited drastic changes in Lyso-PL levels that involved the two lipid subclasses, Lyso-PCs and Lyso-PEs, and that in many cases were dependent of the fatty acid structure. The effect of physical training was minor and particularly focused on Lyso-PEs, being able to steady the levels of some of them in CAF-fed rats, especially when they had performed the exercise at a moderate intensity.

3.2. Important Lyso-PLs associated with the chronic intake of cafeteria diet

A multivariate statistical analysis was performed in order to uncover the Lyso-PLs that best reflect the CAF-phenotype in rats (**Fig. 4**).
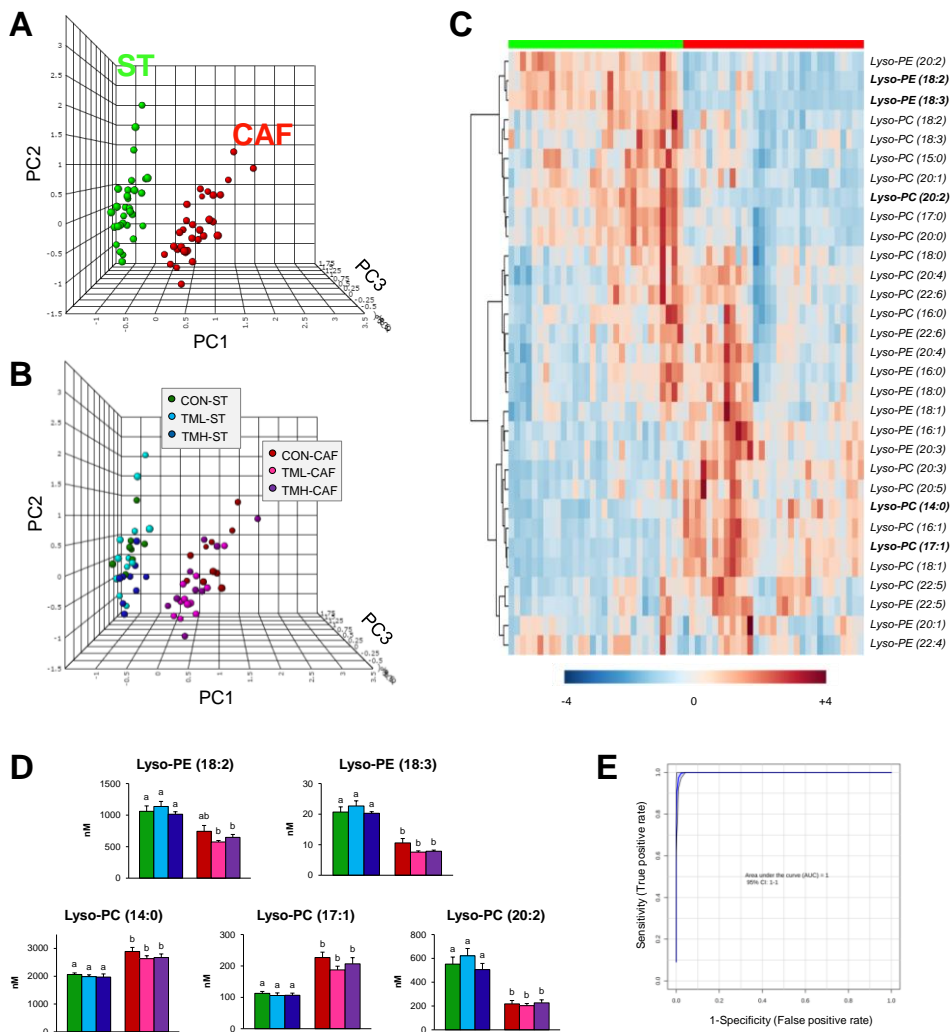
Figure 4. Identification of CAF-related biomarkers. (**A**) Three-dimensional scores plot of principal component (PC) analysis of the thirty-one Lyso-PLs quantified in rat serum. Animals fed standard chow (ST) are represented as green spots and animals fed cafeteria diet (CAF) as red spots. (**B**) Scores plot shown on panel A with spots coloured as ST or CAF-fed animals trained on a treadmill at different intensities (CON: 0; TML: 12; TMH: 17 m/min) for 2 months. (**C**) Heatmap plot of hierarchical clustering analysis of the Lyso-PL levels in each dietary group. Each row represents a molecular specie coloured by its range-scaled abundance intensity. The scale from -4 (blue) to +4 (red) represents this normalized abundance in arbitrary units. (**D**) Circulating levels expressed on nano molarity (nM) of the best cafeteria diet-related biomarkers in the six groups of animals ($n = 9 – 12$). Mean values with different lowercase letters were significantly different (one-way ANOVA and Tukey or Dunnett's T3 *post hoc* contrasts, $p < 0.05$). (**E**) Receiver operating characteristic (ROC) curve analysis using the five Lyso-PLs with the highest variable importance in the discrimination of both dietary groups. The area under the curve (AUC) and the corresponding confidence interval (CI) were optimal.

In a first step, PCA models including all the identified Lyso-PLs were performed to compare the importance of each experimental factor, diet and physical activity, on their levels. As the first scores plot shown, it was possible to distinguish a wide separation between animals according to the diet administered and without resorting to a supervised method of discriminant analysis (**Fig. 4A**). Thus, ~76% of the variance was explained when the scores of the first three principal components were represented. However, the effect of the physical exercise was much lower and when the individual scores were colored according to both diet and training intensity, the discrimination among the three exercise levels was difficult (**Fig. 4B**). The heatmap from hierarchical clustering analysis showed two distinct patterns in Lyso-PL levels according to the diet administered (**Fig. 4C**).

The five Lyso-PLs with greater weigth in the differentiation of the dietary groups were Lyso-PE (18:3) > Lyso-PC (20:2) > Lyso-PC (14:0) > Lyso-PC (17:1) > Lyso-PE (18:2) (**Fig. 4D**). The serum levels of both Lyso-PEs were decreased in CAF-fed groups, whereas Lyso-PC levels followed different trend among them three. The notable importance of these variables were established according to the projection scores (**Table 2**) in the first component of the supervised PLS-DA model (**Fig. 5**). This PLS-DA reported a prediction accuracy of 100% and an excellent quality assesment statistic (Q^2) of 0.94, clearly over the threshold of 0.4 stipulated for biological models [26]. The further validation of the CAF-related biomarker was carried out by plotting ROC curves using different number of variables in consideration. The results showed that the five Lyso-PLs referred to above were sufficient to achieve an optimal prediction, based on the peak values of the area under the curve and its corresponding confidence interval (**Fig. 4E**). This summarised model presented a null error rate classifying the animals according to the diet.

These findings translate Lyso-PLs into excellent predictors of the reiterated consumption of CAF in rat serum even when animals were subjected to low or high intensities of endurance training.

Table 2. Variable importance in projection (VIP) from the PLS-DA of serum Lyso-PLs in rats fed ST and CAF

Metabolite VIPs	Components		
	1	2	3
Lyso-PE (18:3)	1.96	1.96	1.94
Lyso-PC (20:2)	1.66	1.65	1.63
Lyso-PC (14:0)	1.58	1.58	1.56
Lyso-PC (17:1)	1.54	1.54	1.52
Lyso-PE (18:2)	1.42	1.42	1.40
Lyso-PE (20:2)	1.42	1.41	1.39
Lyso-PC (18:1)	1.40	1.40	1.38
Lyso-PC (16:1)	1.38	1.39	1.37
Lyso-PC (20:3)	1.26	1.25	1.24
Lyso-PC (20:0)	1.18	1.18	1.17
Lyso-PC (20:1)	1.07	1.07	1.06
Lyso-PE (20:3)	1.04	1.03	1.02
Lyso-PE (16:1)	1.00	1.00	0.98
Lyso-PC (17:0)	0.94	0.94	0.95
Lyso-PC (18:3)	0.87	0.87	0.86
Lyso-PC (18:2)	0.84	0.84	0.84
Lyso-PC (22:5)	0.81	0.81	0.81
Lyso-PC (15:0)	0.81	0.81	0.80
Lyso-PE (22:5)	0.72	0.72	0.73
Lyso-PC (20:5)	0.65	0.67	0.67
Lyso-PE (18:1)	0.51	0.51	0.52
Lyso-PE (20:4)	0.35	0.36	0.40

VIP > 1

Lyso-PE (22:4)	0.33	0.33	0.37
Lyso-PC (16:0)	0.30	0.35	0.34
Lyso-PC (18:0)	0.26	0.26	0.37
Lyso-PE (20:1)	0.25	0.28	0.40
Lyso-PC (22:6)	0.23	0.26	0.27
Lyso-PE (18:0)	0.23	0.23	0.41
Lyso-PC (20:4)	0.22	0.22	0.40
Lyso-PE (22:6)	0.02	0.18	0.18
Lyso-PE (16:0)	0.01	0.09	0.23

The list of Lyso-PLs belonging to lysophosphatidylcholine (Lyso-PC) and lysophosphatidylethanolamine (Lyso-PE) lipid subclasses was sorted in decreasing order of VIP score of the first component of the PLS-DA model shown in **Fig. 5**. Metabolites above the dashed line had VIP scores over 1.
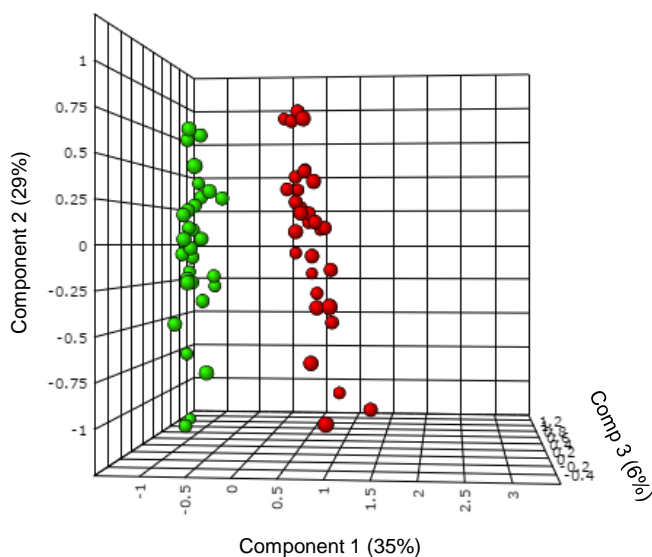
Figure 5. PLS-DA of the thirty-one Lyso-PLs determined in serum of rats fed ST and CAF. Three-dimensional scores plot explaining ~70% of co-variance. Animals fed ST are represented as grey spots and animals fed CAF as red spots. The PLS-DA model showed excellent data fit ($R^2 = 0.95$).

3.3. Lyso-PLs as poor indicators of the practice of daily exercise

Once the prevalent effect of diet was determined, we proceeded to examine the influence of physical exercise on the Lyso-PL-related metabolome (**Fig. 6**). On this occasion, it was necessary the use of a supervised form of discriminant analysis for the separation of the six animal classes in a scores plot explaining ~70% of co-variance (**Fig. 6A**). The extent of fit of the PLS-DA model to the metabolomics data, represented by R^2, was 0.84. However, the internal cross-validation indicated that the prediction accuracy was only 40%. Even though the scores diagram showed a discrimination of the animals mainly in dietary classes along the first component, it was also noticeable that trained groups form distinct clusters regarding sedentary animals along the second component of PLS-DA. In relation to this, the resultant loadings plot indicated that Lyso-PEs (16:0), (18:0), (20:4) and (22:6) and Lyso-PC (16:0) were the physical activity-related metabolites with greater importance in the classification of the sedentary groups (**Fig. 6B**). Therefore, Lyso-PEs were better biomarkers of aerobic training than Lyso-PCs. In fact, the whole Lyso-PE family showed a global involvement in resistance exercise-related metabolism ($p = 0.001$, two-way ANOVA) with a general behaviour similar to that described above for individual Lyso-PEs (**Fig. 6C**). This circulating trend of the Lyso-PE levels was different in relation to diet ($p = 0.015$, two-way ANOVA).

Based on the individual scores plot of ST (**Fig. 6D**) and CAF-fed rats (**Fig. 6E**), the effect of exercise on the Lyso-PL-related metabolome, although very slight, seemed to be gradual exclusively on the ST-fed groups. Thus, the separation from the sedentary animals was larger for the most intensely trained group (TMH-ST) than for the middle condition, which showed a higher degree of data dispersion (**Fig. 6D**). However, even having eliminated the predominant influence of diet, the permutation tests of the two prediction models were not significant and the error rates classifying trained animals within dietary groups were over 50%.

Therefore, although Lyso-PLs, particularly Lyso-PEs, show to be involved in the biological effects of exercise of trained rats, the influence that diet has on the circulating Lyso-PL-related metabolome is such strong that daily resistance training carried out at the analysed intensities was lightly captured on the serum Lyso-PL levels. In addition, differences between the exercise intensities were also observed and they are dependent on the type of feeding.
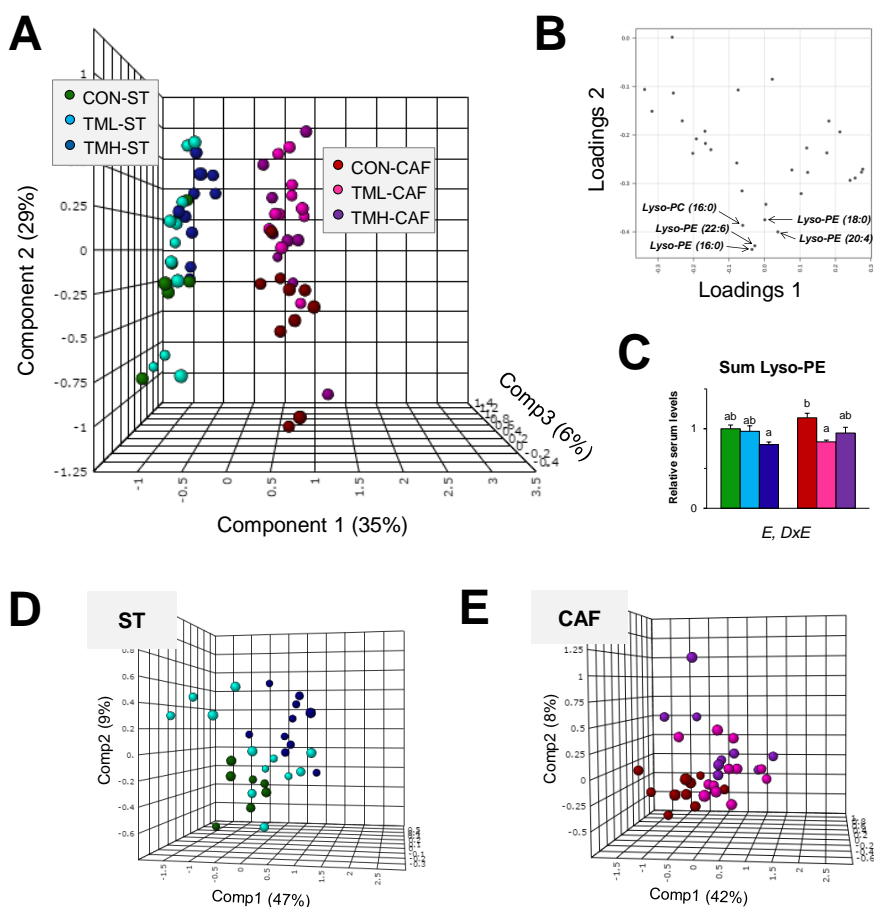
Figure 6. Identification of biomarkers of the daily practice of physical exercise. (**A**) Three-dimensional scores plot of partial least squares discriminant analysis of the thirty-one Lyso-PLs detected in rat serum. Each spot represents an animal coloured according to both diet (ST or CAF) and intensity of training performed in a treadmill (CON: 0; TML: 12; TMH: 17 m/min) for 2 months. (**B**) Two-dimensional loadings plot showing the main underlying Lyso-PLs responsible for the discrimination between trained groups. (**C**) Total sum of the serum Lyso-PE levels (thirteen molecular species). The data are presented as means ± SEM (n = 9 – 12). The statistical comparison among the animal groups was conducted using two- and one-way ANOVAs. *E*: the effect of exercise; *DxE*: the interaction between diet and exercise (two-way ANOVA, p < 0.05). [abc]: Mean values with different lowercase letters were significantly different (one-way ANOVA and Tukey *post hoc* contrast, p < 0.05). Separate scores plot for ST (**D**) and CAF (**E**) groups showing the slight influence of exercise on the Lyso-PL metabolome.

4. Discussion

In the present investigation, we have shown that the administration of CAF to weaning rats for 8 weeks induced several alterations in the lysophospholipidome. These results were consistent with our previous research since the serum levels of Lyso-PCs (16:1) and (17:1) were increased and those of Lyso-PCs (20:1) and (20:2) decreased in CAF-fed rats [5]. However, this study provide further information regarding the relevance of the chronic intake of a MetS-inducing diet and circulating levels of Lyso-PLs in rats. Thus, a considerable ~80% of the Lyso-PCs in serum were implicated, but also more than 60% of the Lyso-PEs, other class of Lyso-PL identified in the first study (**Table 1**). These results are in accordance with other recent studies performed in CAF-fed rats with hypertriglyceridemia [27], different rodent species usually fed diets high in fat [16,28] (**see also Manuscript 2**) and subjects with obesity [17,19,28]. All of them reported several dysregulations in Lyso-PL levels, more pronounced for plasma than tissues in the case of animals.

To simplify the predictive model, we decided to select those compounds that allowed us to distinguish between dietary groups without losing reliability. In this way, the array was limited to five metabolites that as a whole were enough to achieve an ideal prediction regardless of whether the animals performed physical activity (**Fig. 4D-E**). Consistent with our observations, while decreased plasma levels of Lyso-PE (18:2) were detected in rodents with diet-induced obesity [16], a similar trend was identified in the serum of obese children when the linoleic acid (C18:2) was associated to choline as polar group [17]. Other important Lyso-PL whose levels decreased with the chronic ingestion of a MetS-inducing diet was Lyso-PC (20:2). Interestingly, the circulating levels of this metabolite decreased in rats and patients with obesity [27,28] suggesting no difference between these species. Serum increments of Lyso-PC (14:0) have also been described in overweight subjects in comparison with lean subjects [16] and, in another study, this was the best metabolite discriminating among dyslipidemic and healthy men [29].

On the other hand, modifications in the Lyso-PL levels show to be also associated with the practice of exercise in trained rats. However, dietary habits promote a sharp contrast on the circulating Lyso-PL-related metabolome and the consequences of the resistance training are slightly reflected on a global level. These results were consistent with other investigations conducted in trained rodents in which differential patterns in metabolic spectra were identified but these are unable to fully reverse the detrimental effects of a diet rich in fats [5,30,31]. In the present study, Lyso-PE levels are the Lyso-PL class primarily affected by the exercise, specially in animals with MetS in which the moderate intensity was the most influential (**Table 1 and Fig. 6B-C**). The Lyso-PL-lowering effect observed in serum could be

caused by the increased accumulation of these lipid metabolites in tissues. Thus, increments of several Lyso-PLs have been reported in muscle and particularly in liver of trained rodents regarding sedentary animals [32,33]. Intracellular autophagy could be contributing to the alteration of the circulating Lyso-PL profile after physical training and thereby explain the association between these molecules and metabolic diseases. Recently, it has been demonstrated that Lyso-PC (16:0), one of the few Lyso-PCs affected by exercise in this study, upregulates the expression of PPARα target genes in hepatocytes [34]. The levels of this particular specie was decreased in the serum of trained rats regardless of diet and it has also been described as a key compound in the recognition of mice that perform aerobic exercise frequently [35]. Interestingly, other authors have shown that some diets with hypocholesterolemic properties are also able to restrain the circulating level of Lyso-PC (16:0), as well as those of Lyso-PEs (16:0), (18:0) and (20:4) [21,23].

To sum up, the examination of the lysophospholipidome reflects the general state of the metabolome due to MetS-inducing diet intake and the practice of exercise obtained by a non-targeted approach (see **Manuscript 1**), thus highlighting the importance of this family of compounds in lipid disorders. Based on these results, we conclude that the analysis of the plasma lysophospholipidome could be a good strategy to be used in the diagnosis of dyslipidemia-related diseases.

5. Acknowledgements

6. References

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__Manuscript 4__

Altered levels of circulating lysophospholipids are indicative of early steatosis in hamsters

(Manuscript under preparation)

PREFACE

The investigation presented in **Manuscript 2** allowed us to elucidate the involvement of lysophospholipids as indicators of dyslipidemia progression while rejecting the possibility that these circulating molecules were only markers of consumption of a diet rich in fats. These observations accentuate the interest of this family of compounds as putative biomarkers of disease.

With the aim of deepening in the mechanisms by which lysophospholipids are metabolized, we proposed to perform another animal experiment in which the fate of the metabolites could be determined. For this purpose, Golden Syrian hamsters were fed high-fat diet to generate hyperlipidemia and targeted metabolomics was used to quantify the overall lysophospholipidome in liver, aorta, plasma and urine. What underlies the analysis of these tissues is the fact that these organs play a key role in lipid metabolism and development of severe diseases related to unhealthy diets. On the other hand, plasma was selected because it has been reported that, compare with serum, it is a more adequate medium in which to perform lysophospholipid evaluations while urine provides information regarding excretion.

Once confirmed the high potential of these family of compounds in animals, we intended to validate its suitability in a cohort of humans.

Altered levels of circulating lysophospholipids are indicative of early steatosis in hamsters

Abstract

Lysoglycerophospholipids (Lyso-PLs) are bioactive molecules of ubiquitous distribution that, in the last few years, have been associated with the etiology of serious lipid disorders. Accumulations of certain lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) were identified in pathological tissues (e.g. fatty liver and atherosclerotic aorta), where their patterns change as dyslipidemia progresses. However, in hamsters, which are suitable models for dyslipidemic studies, a thorough investigation of the lysophospholipidome has not been performed. Thus, the objective of this study was to determine the Lyso-PC and Lyso-PE levels present in aortic and hepatic tissues of hyperlipidemic hamsters for compare them with control animals. Additionally, the plasma and urine content was evaluated, providing information about excretion. To this end, hamsters were fed either a low-fat diet or a moderate high-fat diet (HFD). After 30 days, samples were obtained for determination of the individual Lyso-PL levels by mass spectrometry-based metabolomics. The lysophospholipidome was further evaluated using multivariate statistics to achieve an integral understanding. Other biochemical and biometric variables were also examined to investigate the biological significance of Lyso-PLs in the onset of dyslipidemias. The adequacy of Lyso-PLs as risk biomarkers was also evaluated in humans. The HFD induces isolated hypercholesterolemia and promotes the development of mild steatosis in hamster liver without alteration of the circulating transaminases. This early pathological event was associated with a generalized drop in the plasma Lyso-PL levels, an intrahepatic accumulation of long chain polyunsaturated acyl Lyso-PLs, as well as an increased excretion of Lyso-PEs through urine. Likewise, decreased levels of circulating Lyso-PLs were identified in humans whose transaminase levels were moderately altered. We concluded that Lyso-PL assessment could aid in the subclinical non-invasive diagnosis of fatty liver diseases, although further research must be conducted on humans.

Keywords: lysophosphatidylcholine; lysophosphatidylethanolamine; steatosis; dyslipidemia; inflammation; hamster; biomarker; metabolomics.

1. Introduction

Lysoglycerophospholipids (Lyso-PLs) are important lipid mediators involved in cellular metabolism belonging to the category of glycerophospholipids. Their molecular structure is constituted by a phosphate and a characteristic head group attached to the *sn3*-position of the glycerol backbone, and a single fatty acyl chain. Although the linkage of the acyl substituent can occur in any of the other two positions, when this is located at *sn2*-position tends to migrate giving rise to the predominant isomeric form [1]. Thus, the nature of the polar group determines the Lyso-PL class, whereas the several individual species present in mammals differ in length and saturation degree of the acyl chain. Besides the role as structural components, Lyso-PLs can act as bioactive molecules by intervening in essential processes of cell recognition and transduction of intracellular signals, as well as by interacting extracellularly with G-protein-coupled receptors (GPCRs) [2,3]. Thus, Lyso-PLs can be found in the extracellular enviroment representing 5-20% of the plasma phospholipids, as well as at sub-cellular level, where they are involved in normal physiological activities and pathological processes [4].

In recent years, the emergence of omics sciences, particularly metabolomics, has aroused a great interest towards these molecules, by providing numerous evidence in terms of Lyso-PLs as important etiological factors of different metabolic diseases [5–10]. In this way, it has been identified accumulation of individual Lyso-PLs in human atherosclerotic tissues and, interestingly, the circulating patterns of lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) change as disease progresses [11–13]. In fact, several proatherogenic properties have been attributed to Lyso-PCs, which are known to be important phospholipid components of the atherogenic lipoproteins [2,5]. Thus, Lyso-PLs have been shown to stimulate the endothelial expression of cell adhesion molecules, a decisive mechanism in endothelial cell activation and progress of atherosclerotic lesions [12,14]. Moreover, greater amounts of Lyso-PCs are associated with worsening of the inflammation levels in human atherosclerotic plaques [11]. Although Lyso-PLs can be generated in blood by enzymatic hydrolysis of circulating phospholipids, direct hepatic secretion is an important source of Lyso-PLs, particularly of those containing polyunsaturated fatty acids (PUFAs) (e.g. arachidonic and docosahexaenoic acids) [4]. Interestingly, it has been demonstrated that the hepatic lysophosphatidylcholine acyltransferase is essential for the triglyceride transport due to its ability to incorporate PUFAs into phospholipids, modulating the maturation of VLDL particles [15]. Other studies also indicated that hepatic Lyso-PLs may inhibit the β-oxidation reducing energy expenditure and thus interfering in the adaptive response to the intake of high-calorie diets [16,17]. In relation to these findings,

altered profiles of Lyso-PCs and Lyso-PEs have been identified in both plasma and liver of rodents chronically fed a high-fat diet (HFD) [18–20] (see also **Manuscript 2**). Furthermore, these levels are normalized when the HFD is supplemented with vegetal extracts that possess anti-dyslipidemic properties [20].

Despite the evidence indicating that modifications in the lysophospholipidome are involved in the onset of lipid disorders, to our knowledge, an exhaustive quantification of the *in vivo* levels has not yet been performed on hyperlipidemic hamsters, although it is known that they are the rodents possessing a greater similarity with humans in terms of lipid metabolism [21]. Therefore, the main objective of the present study was to investigate the individual Lyso-PC and Lyso-PE levels present in aortic and hepatic tissues of HFD-fed hamsters since they are key organs in lipid metabolism and development of diet-induced disorders. In addition, the amount of these lipid metabolites was also evaluated in biofluids, namely plasma and urine, providing information regarding excretion. To this end, the biological samples were analyzed by mass spectrometry (MS)-based metabolomics intended for the simultaneous identification of a total of thirty-one Lyso-PLs. The levels were compared with control animals both individually and with a holistic approach through the utilization of multivariate statistical techniques. Biochemical and biometric analyses were also conducted in order to determine the biological significance of these molecules in the onset of dyslipidemias. Finally, the circulating levels of Lyso-PLs in hamsters were compared with those of humans in order to investigate wheter the results in animals would be extrapolated to humans.

2. Material and methods

2.1. Chemicals

Methanol (Scharlab, Barcelona, Spain), acetonitrile (Millipore, Darmstadt, Germany), isopropanol (Panreac Applichem, Barcelona, Spain), chloroform and 7.5 M ammonium acetate solution (Sigma-Aldrich, St. Louis, MO, USA) were of the highest grade commercially available. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Butylated hydroxytoluene (BHT; Sigma-Aldrich, St. Louis, MO, USA) was used as antioxidant agent.

Lyso-PL standards were all of > 99% purity and were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Lyso-PC standards included 1-tridecanoyl-sn-glycero-3-phosphocoline, Lyso-PC (13:0); 1-palmitoyl-sn-glycero-3-phosphocoline, Lyso-PC (16:0); 1-stearoyl-sn-glycero-3-phosphocoline, Lyso-PC (18:0); and 1-arachidoyl-sn-glycero-3-phosphocoline, Lyso-PC (20:0). Lyso-PE calibrators were 1-palmitoyl-sn-glycero-3-phosphoethanolamine, Lyso-PE (16:0); 1-heptadecenoyl-sn-glycero-3-phosphoethanolamine, Lyso-PE (17:1); 1-stearoyl-sn-glycero-3-phosphoethanolamine, Lyso-PE (18:0); and 1-oleoyl-sn-glycero-3-phosphoethanolamine, Lyso-PE (18:1). Standard stock solutions of each compound were prepared in methanol/chloroform/water (65:35:8 v/v/v) and stored at -20°C prior to use. A standard stock mixture of Lyso-PLs was prepared weekly at a concentration of 100 mg/L. Lyso-PC (13:0) and Lyso-PE (17:1) were separately handled similarly to the standards for use as internal standard of Lyso-PCs and Lyso-PEs, respectively.

2.2. Animals and diets

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).

Male Golden Syrian hamsters weighting 143 ± 2 g were obtained from Charles River Laboratories (Barcelona, Spain) and housed singly at 22°C with a light/dark period of 12 h and free access to water and food. After 2 weeks of acclimatization in which the animals were fed a low-fat diet (LFD), they were randomly distributed into two groups with comparable body weights (n = 8 per group). The hamsters were fed ad libitum either the LFD or a HFD and feed, conserved at 4°C, were renewed daily. As can be observed in **Table 1 of Manuscript 2** (page 98), the LFD (3.9 kcal/g) and HFD (4.1 kcal/g) (Research Diets, New Brunswick, NJ, USA) provide 10% and 21% energy as fat, respectively. The increment is mainly given due to the high lard content of the HFD, which is equivalent to 10.5% calories

provided. Throughout the study, body weight was monitored twice a week and food intake weekly.

On day 30, body mass composition was directly measured using an EchoMRI-700 (Echo Medical Systems, Houston, TX, USA) [22]. Afterwards, the animals were fasted for 6 h and anesthetized with i.p. pentobarbital sodium (80 mg per kg body weight). Blood was withdrawn by cardiac puncture and collected in BD Vacutainer® tubes containing K_2EDTA as anticoagulant (Franklin Lakes, NJ, USA). Blood was conserved at 4°C for 1 h and plasma was separated by centrifugation at 2,000 g for 15 min. Urine was obtained by means of a puncture in the bladder of hamsters. The aorta was excised from the animals and submerged in cold PBS buffer during the cleaning process, which entirely removed residual blood and adipose tissue. The liver was also perfused, patted dry and then weighted for the determination of the relative weight, which was calculated following the formula (tissue weight / body weight * 100) and expressed as percentage of the total body weight. The white adipose tissue including mesenteric (MWAT), epididymal (EWAT) and retroperitoneal (RWAT) depots were also excised for determination of the relative weight. All the samples were snap frozen in liquid nitrogen and then stored at - 80°C until further analyses.

2.3. Liver histology

Frozen liver tissue samples were thawed and fixed by immersion in 4% paraformaldehyde for 24 h, dehydrated successively in graded concentrations of ethanol (70%, 96 % and 100 %; plus xylol), and embedded in paraffin at 52°C (Citadel 2000) for histological examination [23]. The paraffin blocks were sectioned at 2 μm thickness with a microtome (Microm HM 355S), and mounted in slides for automated staining with hematoxylin-eosin (Varistain Gemini ES). All of the equipment was from Thermo Fisher Scientific (Waltham, MA, USA). Morphometric evaluation was conducted by expert pathologists to assess the degree of hepatic steatosis in the biopsies [24]. The steatosis grade was determined estimating the percentage of hepatocytes containing lipid droplets: absent – score 0, <5%; mild – score 1, 5-33%; moderate – score 2, >33%-66%; severe – score 3, >66%. The diagnosis further included determination of the cytoplasmic configuration (microvesicular and macrovesicular steatotic patterns), tissue distribution and inflammation extent.

2.4. Lipid and protein determinations

Enzymatic colorimetric kits were used for the evaluation of total cholesterol, high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol (LDLc), triglycerides, aspartate (AST) and alanine transaminases (ALT) (QCA, Barcelona, Spain).

Circulating levels of the secreted enzyme phospholipase A_2 ($sPLA_2$) were measured using a quantitative sandwich ELISA kit (MyBioSource, San Diego, CA, USA). The limit of detection was 3.12 ng/mL and average CV was less than 15%. The total protein content of tissue samples was determined using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific).

2.5. Metabolomics analysis of Lyso-PLs

Lyso-PLs from biological samples were extracted using previously reported methodology [25] based on protein precipitation with cold methanol assisted by ultrasounds (see **Supplementary Material** for further information). The quantitative evaluation of metabolites was performed using a UHPLC 1290 coupled to a QqQ 6490 mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Palo Alto, CA, USA). The samples were randomized prior to metabolite extraction, and again prior to mass spectrometer analysis. The temperature in the autosampler was held at 4°C.

The mobile phase consisted of water / isopropanol / acetonitrile / 500 mM ammonium acetate (89:5:5:1 v/v/v/v) as solvent A, and isopropanol / acetonitrile / 500 mM ammonium acetate (50:49:1 v/v/v) as solvent B. A sample injection volume of 7.5 µL was loaded onto a 0.3 mL/min flow of 30% B passing through a reverse-phase column (Acquity UPLC BEH C8; 1.7 µm, 2.1 x 150 mm) (Waters Corporation, Milford, MA, USA) held at 50°C. The chromatographic separation was conducted using linear gradient elution to 75% B over 20 min, followed by a further increase to 100% B over 1 min, a 4-min solvent B hold and 2 min for return to the initial conditions. Thereafter, a post-time of 2 min was applied. The mass spectrometer was operated in positive ion mode (+ESI) with the following settings: nebulizer gas (nitrogen) pressure, 25 psi; gas flow rate, 12 L/min at 240°C; sheath gas flow rate, 12 L/min at 350°C; capillary and nozzle voltages, 4,500 and 500 V, respectively; cell accelerator voltage was set to 5 V and fragmentor to 380 V. The scan mode was dynamic multiple reaction monitoring using a retention time (RT) window of 1 min. The RTs of Lyso-PLs, monitored transitions and optimal collision energies were described in a previous study [25]. [M+H]+ ions were used as precursor ions. For each analyte, the most abundant transition was chosen for the quantification, whereas two transitions were used for qualitative purposes. The UHPLC and QqQ systems were operated using MassHunter Data Acquisition, whereas peak detection and integration were performed using MassHunter Quantitative software, both versions 7.0 from Agilent Technologies.

The quantitative analysis of endogenous Lyso-PLs was performed as described previously [25,26]. Briefly, calibration curves were obtained by mixing different concentrations (0.05 – 2000 µg/L) of the standard Lyso-PLs with constant levels (100 µg/L) of the internal

standards Lyso-PC (13:0) and Lyso-PE (17:1) and performing UHPLC-+ESI-MS/MS analysis. The peak abundance ratios (analyte / IS) were plotted versus the concentration ratios (analyte / IS) and fitted to a linear regression. Concentration levels of the Lyso-PLs identified in samples were calculated by using the closest related standard curve and expressed in molarity.

2.6. Evaluation of the adequacy of Lyso-PLs in humans

A total of 48 adult subjects were recruited by the Lipids and Atherosclerosis Research Unit of the *Universitat Rovira i Virgili* to participate in the BIOCLAIMS study. The protocol was approved by the Ethical Committee of Clinical Research of the *Hospital Universitari Sant Joan* (Reus, Spain). Blood samples were taken in over-night fasting conditions to investigate the clinical features of the participants (**Table 1**). Thus, two groups were created based on the transaminase levels, not exceeding 40 U/L for neither of them. Additional exclusion criteria were: hypolipemic treatment, any chronic metabolic disease, triglycerides levels higher than 150 mg/dL, body mass index (BMI) above 30 kg/m2 and/or smokers. Moreover, both subject groups were balanced by gender (12 males/12 females). Lyso-PLs were evaluated using the methodology described above.

Table 1. Clinical characteristics in humans of control group (C) and with altered transaminases (AT)

	Subject group	
	C (n = 24)	AT (n = 24)
Age	41.3 ± 2.4	42.7 ± 2.6
Weight	66.2 ± 1.9	68.2 ± 2.1
BMI	23.1 ± 0.5	24.2 ± 0.6
Triglycerides (mmol/L)	0.8 ± 0.1	0.9 ± 0.1
Total cholesterol (mmol/L)	4.8 ± 0.2	5.3 ± 0.2 *
LDLc (mmol/L)	2.6 ± 0.2	3.2 ± 0.1 *
HDLc (mmol/L)	1.8 ± 0.1	1.7 ± 0.1
ALT (U/L)	13.0 ± 0.4	22.5 ± 1.2 **
AST (U/L)	18.4 ± 0.6	23.4 ± 1.1 **

For each subject group, data are given as mean ± SEM (n = 24). Two-tailed p-values were calculated using Student's t-test: * p<0.05; ** p<0.001. BMI, body mass index; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; ALT, alanine transaminase; AST, aspartate transaminase.

2.7. Statistical analysis

The results are reported as the means ± SEM from the indicated number of hamsters (n = 8) and humans (n = 24) in each group. The differences among groups were assessed using independent Student's t-test with Statistical Package for Social Sciences (IBM SPSS Statistics, version 19.0). A two-tailed value of $p < 0.05$ was considered statistically significant. Lyso-PL content in the different tissues was also comprehensively examined using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Random permutation testing of the class labels was used to assess whether the supervised PLS-DA models exhibited overfitting. The predictive models were further validated by conducting receiver operating characteristic (ROC) curves. The optimal combination of underlying Lyso-PLs in each biological compartment was that associated with the highest predictive accuracy (maximum sensitivity and specificity). Correlations between Lyso-PLs of different tissues were evaluated using Pearson's correlation coefficient (*r*). All these analyses were performed after range scaling and normalization to the LFD-fed group with the use of the software MetaboAnalyst (version 3.0) available online (http://www.metaboanalyst.ca/) [27].

3. Results

3.1. A thirty-day treatment with HFD induces isolated hypercholesterolemia

After 30 days of dietary intervention, no differences were observed in daily caloric intake among the two groups (data not shown). Nevertheless, due to the diet composition the hamsters fed HFD ingested significantly more cholesterol (0.24 ± 0.01 vs 6.46 ± 0.19 mg/day) and fat (31.43 ± 1.31 vs 70.26 ± 2.08 mg/day) than control animals ($p < 0.001$). The body weight was also not affected by the treatment throughout the study, being the weight gain about 7% initial weight for both groups (see **Supplementary Material**). Furthermore, the body mass composition and relative weights of the white adipose tissue depots (MWAT, EWAT and RWAT) were also not increased by the HFD intake. As a result, the chronic treatment with HFD had no effect on the biometric parameters of the animals.

The plasma determinations of both animal groups are reported in **Table 2**, including the dyslipidemic parameters and traditional indicators of liver impairment. Hamsters fed the HFD exhibited higher amount of total cholesterol than LFD-fed animals, without alterations in triglycerides. This resulted in a slight increase (of 13%) of the atherogenic index in these animals, although no significant differences were found in plasma HDLc. Nevertheless, we observed an increasing trend of LDLc levels of 30% in the plasma of HFD-fed hamsters (p =

0.088). The transaminase levels ALT and AST were not affected by the HFD intake, suggesting that liver integrity was not altered by the treatment. Neither did we observe significant differences in plasma glucose levels in relation to the type of diet.

Table 2. Circulating parameters in hamsters fed low- (LFD) or high-fat diet (HFD) for 30 days

	Dietary group		*p-value*
	LFD (n = 8)	**HFD (n = 8)**	
Triglycerides (mmol/L)	1.82 ± 0.19	2.11 ± 0.23	*0.370*
Total cholesterol (TC) (mmol/L)	5.43 ± 0.30	6.60 ± 0.25	*0.011*
LDLc (mmol/L)	1.64 ± 0.12	2.13 ± 0.25	*0.088*
HDLc (mmol/L)	2.03 ± 0.17	2.12 ± 0.09	*0.662*
Atherogenic index (TC/HDLc)	2.71 ± 0.10	3.07 ± 0.13	*0.046*
Glucose (mmol/L)	11.57 ± 1.52	10.42 ± 2.05	*0.660*
ALT (U/L)	49.81 ± 9.65	46.89 ± 5.43	*0.422*
AST (U/L)	29.39 ± 5.79	19.48 ± 3.50	*0.168*
sPLA$_2$ (ng/mL)	35.93 ± 1.62	35.99 ± 1.83	*0.980*

For each animal group, the data are given as the mean ± SEM (n = 8). Two-tailed *p-values* were calculated using independent Student's t-test. Abbreviations: TC, total cholesterol; LDLc, low-density lipoprotein cholesterol; HDLc, high-density lipoprotein cholesterol; ALT, alanine transaminase; AST, aspartate transaminase; sPLA$_2$, secreted phospholipase A$_2$.

3.2. Alteration of Lyso-PL metabolism occurs in the liver of HFD-fed hamsters

Due to the documented association between alterations in Lyso-PLs and development of dyslipidemia (see **Manuscript 2**), the Lyso-PL content was evaluated in plasma, urine, aorta and liver of HFD-fed hamsters and compared with those fed the LFD (control group) (**Table 3**). As a result, we observed that both classes of Lyso-PLs examined, Lyso-PCs and Lyso-PEs, were drastically modified excluding in the aortic tissue, where only some tendencies (p < 0.1) were observed to rise. What was really interesting was the accumulation of lyso forms, notably Lyso-PEs, in the liver of animals fed the HFD, which was accompanied by a generalized drop in the circulating levels of Lyso-PLs. In addition, despite the fact that almost 50% of the Lyso-PL levels were disturbed in plasma, sPLA$_2$ concentration remained unchanged after the treatment with HFD. The unique Lyso-PEs whose hepatic levels were reduced after the chronic intake of HFD were Lyso-PEs (16:0) and (18:0), both excreted 165% and 250% more when comparing their urine levels with the control animals,

respectively. In fact, by distinguishing between both lipid subclasses, we observed that the Lyso-PC / Lyso-PE ratio (**Fig. 1**) was drastically reduced only in the urine because of a significant increase in total Lyso-PE excretion above 200% compared to the control group (p = 0.037).

Table 3. Lyso-PL content in plasma, urine, aorta and liver of hamsters fed low- (LFD) or high-fat diet (HFD)

Metabolite	Plasma (nM)		Urine (pM)		Aorta (µmol/g)		Liver (µmol/g)	
	LFD	HFD	LFD	HFD	LFD	HFD	LFD	HFD
Lyso-PCs								
(14:0)	35.9 ± 1.4	30.7 ± 1.1 *	277.2 ± 0.6	280.6 ± 1.3 #	101.3 ± 2.8	100.7 ± 5.8	79.8 ± 9.9	48.1 ± 1.9 *
(15:0)	38.2 ± 3.3	29.3 ± 1.4 *	268.0 ± 0.9	269.3 ± 1.0	93.1 ± 6.6	86.5 ± 5.3	78.4 ± 7.0	44.4 ± 2.4 **
(16:0)	5,263 ± 303	5,006 ± 172	728.4 ± 129.6	806.3 ± 97.8	6,613 ± 541	7,285 ± 400	9,458 ± 371	8,533 ± 275 #
(16:1)	92.2 ± 5.5	62.3 ± 2.8 ***	264.6 ± 2.9	267.8 ± 1.8	98.3 ± 6.8	88.2 ± 5.8	218.1 ± 24.1	118.7 ± 7.6 **
(17:0)	48.8 ± 5.6	39.1 ± 2.1	221.0 ± 3.1	225.3 ± 3.9	199.6 ± 25.1	182.6 ± 9.9	227.8 ± 26.3	158.3 ± 7.3 *
(17:1)	19.2 ± 1.6	13.6 ± 0.4 *	124.1 ± 50.7	206.7 ± 0.1	43.7 ± 2.9	43.0 ± 3.8	18.5 ± 2.5	9.3 ± 0.9 **
(18:0)	1,327 ± 117	1,498 ± 58	461.0 ± 65.6	613.2 ± 69.1	3,630 ± 349	4,511 ± 296 #	3,723 ± 202	3,511 ± 105
(18:1)	1,470 ± 120	1,365 ± 46	254.9 ± 18.7	290.8 ± 15.7	525.2 ± 45.7	616.1 ± 55.5	821.5 ± 57.4	794.3 ± 50.2
(18:2)	1,532 ± 107	1,616 ± 66	235.0 ± 15.1	260.5 ± 16.0	217.8 ± 20.6	258.2 ± 27.8	364.1 ± 18.7	533.5 ± 47.6 **
(18:3)	18.7 ± 1.0	16.0 ± 0.4 *	202.7 ± 0.3	203.1 ± 0.4	43.6 ± 1.9	44.1 ± 3.4	65.1 ± 9.8	75.5 ± 9.3
(20:0)	8.7 ± 0.2	8.9 ± 0.2	178.5 ± 0.7	183.4 ± 1.8 *	48.8 ± 2.1	49.0 ± 4.1	13.9 ± 0.8	14.4 ± 0.4
(20:1)	11.1 ± 0.2	14.0 ± 0.5 ***	179.2 ± 0.6	185.1 ± 1.9 *	64.2 ± 2.6	72.1 ± 5.6	29.7 ± 1.9	30.2 ± 1.2
(20:2)	17.1 ± 0.8	25.5 ± 1.3 #	178.1 ± 0.5	180.7 ± 0.8 *	66.0 ± 2.2	79.3 ± 5.6 #	24.3 ± 1.4	36.6 ± 1.9 ***
(20:3)	44.4 ± 5.1	33.7 ± 2.1	178.4 ± 0.5	178.1 ± 0.2	42.0 ± 1.9	44.0 ± 3.2	56.9 ± 3.4	139.1 ± 16.9 **
(20:4)	170.5 ± 16.1	157.8 ± 4.9 **	185.0 ± 3.0	185.8 ± 1.2	94.0 ± 6.6	95.8 ± 5.7	78.1 ± 5.6	161.4 ± 20.2 **
(20:5)	16.1 ± 1.3	9.8 ± 0.3 **	NQ	NQ	33.8 ± 2.7	34.4 ± 3.3	5.9 ± 1.0	6.6 ± 1.1

(22:5)	24.4 ± 2.0	15.6 ± 0.5 **	170.6 ± 0.3	169.9 ± 0.1 *	36.5 ± 2.6	37.4 ± 2.8	10.4 ± 0.8	12.7 ± 1.4
(22:6)	66.9 ± 6.5	41.4 ± 2.6 **	174.2 ± 1.1	172.1 ± 0.5 #	45.0 ± 1.7	45.5 ± 3.5	57.7 ± 4.2	96.2 ± 11.4 **
Lyso-PEs							
(16:0)	73.3 ± 4.2	61.6 ± 2.8 *	62.2 ± 10.5	165.1 ± 28.2 *	499.6 ± 40.4	489.9 ± 29.2	1,080 ± 101	728.8 ± 44.6 **
(16:1)	7.0 ± 0.2	6.0 ± 0.1 **	ND	ND	18.8 ± 3.6	13.5 ± 1.5	24.3 ± 2.7	18.4 ± 3.4
(18:0)	65.5 ± 1.6	70.3 ± 4.2	270.1 ± 42.1	940.4 ± 191.9 *	1,093 ± 72	1,304 ± 88.6 #	1,671 ± 104	1,347 ± 57.2 *
(18:1)	34.8 ± 2.4	35.1 ± 1.7	51.8 ± 4.8	138.7 ± 21.7 *	450.5 ± 52.6	512.3 ± 33.8	666.7 ± 63.5	646.7 ± 82.6
(18:2)	40.4 ± 2.9	42.0 ± 2.0	ND	ND	99.7 ± 23.9	94.3 ± 11.6	56.5 ± 5.2	122.6 ± 14.1 ***
(18:3)	4.3 ± 0.1	4.1 ± 0.0 *	ND.	ND	6.5 ± 1.7	5.0 ± 0.6	7.0 ± 0.9	9.9 ± 1.3
(20:1)	4.2 ± 0.1	4.4 ± 0.1 #	ND	ND	8.3 ± 0.9	10.7 ± 0.7 #	7.2 ± 0.4	11.2 ± 0.5 ***
(20:2)	3.7 ± 0.1	3.9 ± 0.0 #	ND	ND	6.9 ± 0.9	9.0 ± 0.7 #	2.2 ± 0.2	4.1 ± 0.3 ***
(20:3)	5.3 ± 0.3	4.7 ± 0.1	ND	ND	31.4 ± 4.8	37.2 ± 4.9	3.3 ± 0.4	9.8 ± 1.6 ***
(20:4)	19.9 ± 1.4	20.3 ± 0.6	ND	ND	194.1 ± 22.5	260.8 ± 22.0 #	33.0 ± 3.6	95.6 ± 14.2 ***
(22:4)	3.7 ± 0.1	3.7 ± 0.0	ND	ND	ND	ND	0.4 ± 0.1	1.6 ± 0.3 **
(22:5)	6.1 ± 0.3	4.9 ± 0.1 **	ND	ND	24.1 ± 2.6	29.2 ± 2.0	7.5 ± 1.0	13.0 ± 1.7 *
(22:6)	14.0 ± 1.8	9.1 ± 0.4 *	ND	ND	48.8 ± 5.5	53.7 ± 4.9	24.4 ± 3.4	37.3 ± 4.2 *

Hamsters were fed *ad libitum* either LFD or HFD during 30 days. The LFD contained 10% energy from fat and the HFD contained 21% energy from fat and 0.9 g per kg cholesterol. Samples were obtained on the last day of dietary intervention after a 6 h fast. Lysophosphatidylcholine (Lyso-PC) and lysophosphatidylethanolamine (Lyso-PE) levels were determined using UHPLC-QqQ analysis. For each animal group, the data are presented as the mean ± SEM (n = 8). Concentrations below the method limit were indicated as NQ (not quantified) or ND (not detected). Significance regarding the LFD-fed group (control animals) was measured using independent Student's t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Trends to increase or decrease were also indicated: # $p < 0.1$.
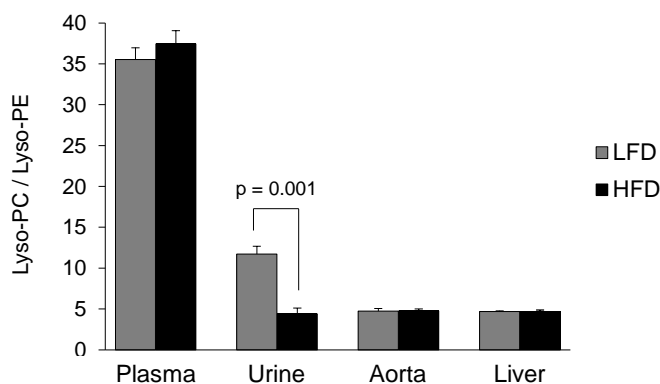
Figure 1. Proportion of total Lyso-PLs in hamsters fed low- (LFD) or high-fat diet (HFD).
The samples were obtained on the last day of intervention after a 6 h of fasting. The amount
of lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) was
determined using UHPLC-QqQ analysis. The data are given as the mean ± SEM (n = 8). The
significance between both groups was measured by conducting independent Student's t-test
(p < 0.05).

In this context, we proceeded to determine the overall effect of the HFD on the
lysophospholipidome performing PCA modelling of plasma and liver samples. Thus, when
the scores of the first two principal components were represented, two distinct clusters were
observed according to the type of diet administered, explaining about 61% and 70% variance
(R^2X) in plasma (**Fig. 2A**) and liver (**Fig. 2E**), respectively. In comparison with the hepatic
tissue, 95% confidence regions obtained examining Lyso-PLs in plasma were narrower for
both experimental groups, while the separation between clusters was complete. Moreover,
no outlier samples were identified in any of these tissues, suggesting a homogeneous
response profile of Lyso-PCs and Lyso-PEs to the dietary intervention.

**Figure 2. Multivariate analysis of the Lyso-PL levels in plasma and liver of hamsters
fed low-fat diet (LFD) or high-fat diet (HFD) over 30 days.** (**A, E**) Two-dimensional scores
plot of the PCA and (**B, F**) PLS-DA models calculated on the within-sample matrix derived
from the UHPLC-MS/MS quantification. The ellipses represent the 95% confidence interval
(CI) for each group. (**C, G**) Predictive accuracies using different number of Lyso-PLs. (**D**)
ROC curve using the 5 Lyso-PLs with highest relevance in the classification of plasma
samples. (**H**) ROC curve using the 31 Lyso-PLs identified in liver samples. The area under
the curves (AUCs) and corresponding 95% CIs are reported in the inset.
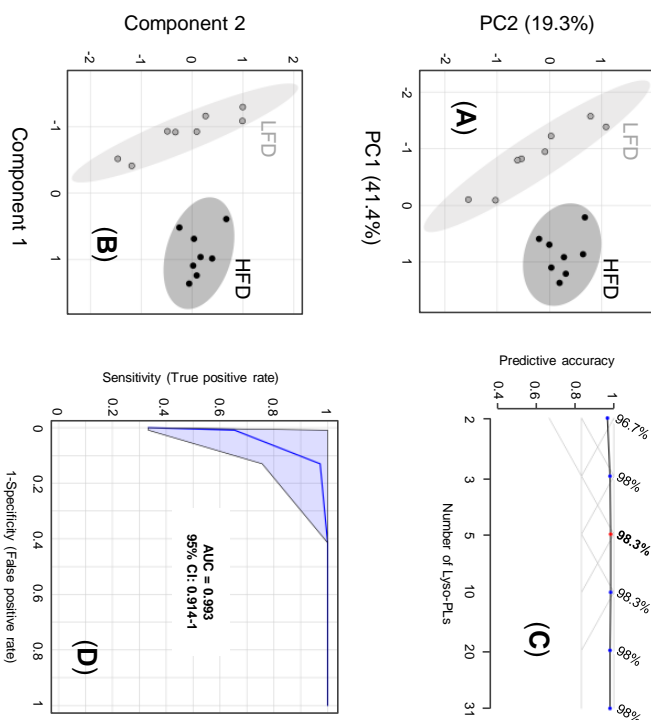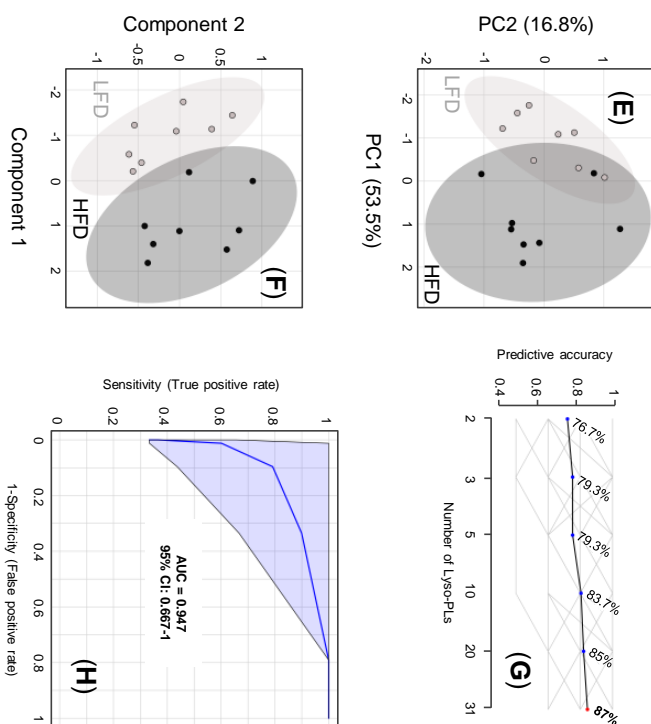
The MS data of the thirty-one Lyso-PLs were also used to establish PLS-DA models to classify normal and dyslipidemic hamsters. The quality parameters of the PLS-DA models are summarized in **Table 4**.

Table 4. Parameters for assessment of the quality of the PLS-DA models in hamsters

Tissue	LV	R^2X	R^2X(cum)	R^2Y	R^2Y(cum)	Q^2Y	Q^2Y(cum)
Plasma	1	40.6	40.6	90.0	90.0	81.6	81.6
	2	19.8	60.4	4.5	94.5	3.0	84.6
Liver	1	53.3	53.3	73.5	73.5	65.9	65.9
	2	12.4	65.7	8.7	82.2	7.9	58.0

The quality parameters are expressed in %. R^2X and R^2Y are the variance explained by each latent variable (LV) in the descriptor matrix (X) and response matrix (Y), respectively. Q^2Y is the predicted variation in the class response.

According to the cross-validation, one component was sufficient to achieve a suitable modelling between the descriptor matrix (X-variables) and the class response (Y-variables). Nevertheless, the first two components were represented to allow score plotting (**Fig. 2B and 2F**) and thus maximize the separation among groups (classes) obtained with the unsupervised PCA. According to this, it was the first component of the X-scores plot the main responsible for the discrimination between classes, resulting in a greater cumulative performance in plasma (R^2Y = 94.5%, Q^2Y = 84.6%) than liver (R^2Y = 82.2%, Q^2Y = 58.0%). Whereas R^2Y represents the extent of fit of the data, Q^2Y estimates the predictive ability of the model and in both tissues this predictor was clearly over the threshold of 40% stipulated for biological models [28]. To ensure no overfitting of the models to the metabolomics data, PLS-DAs were validated by random permutation of the class labels (n = 2,000), providing satisfactory *p-values* of 0.036 in plasma and 0.006 in hamster liver. In addition, ROC curves were also created by plotting *sensitivity* against *1-specificity* using different combinations of the most important Lyso-PLs in the classification of hamster groups. Thus, we conducted comparison between the arising biomarker models by calculating the accuracy of each of them. In plasma, the maximal accuracy (of 98.3%) was achieved using the five most important metabolites (**Fig. 2C**), presenting an AUC of 0.993 with a narrow 95% confidence interval (CI) ranging from 0.914 to 1 (**Fig. 2D**). Interestingly, molecular species belonging to the Lyso-PC class were exclusively found among the circulating metabolites that most contributed to the separation of the animal groups (see **Supplementary Material** for detailed information). Instead in liver, the thirty-one Lyso-PLs were necessary to reach a predictive

accuracy of 87% which corresponded to an AUC of 0.947 and 95% CI between 0.667 and 1 (**Fig. 2G-H**). These findings indicated that the whole families of Lyso-PCs and Lyso-PEs are involved in the metabolic pathways disrupted in liver as a consequence of the chronic intake of HFD.

3.3. The HFD administration increases the steatotic profile in the liver of hamsters

As suggested by the histological analysis of the liver samples (**Fig. 3A**), the profound modification undergone by the lysophospholipidome could be related to the advancement of hepatic steatosis in the animals of the present study. Even though the relative liver weight of both groups was comparable (**Fig. 3B**), hematoxylin-eosin staining of liver sections demonstrated that more than 60% of the animals fed LFD had normal liver structure with well-arranged hepatic cords and sinusoids, whereas ~90% of the biopsies from the HFD-fed group exhibited a marked steatotic profile with centrolobular distribution. As **Fig. 3A** shown, the diet-induced steatosis was defined by hepatocytes distended by multiple microvesicles filled of lipids that not displaced the nucleus of the cell, but no evidences were found of macrovesicular fat accumulation. We also examined the score of steatosis according to the percentage of hepatic parenchyma compromised, revealing that HFD-fed hamsters had significantly higher scores than LFD-fed animals (**Fig. 3C**). Importantly, many of the HFD-fed hamsters that developed steatosis also presented inflammation signs with prevalent moderate intensity and lobular arrangement (**Fig. 3D**). None of the biopsies exhibited signs of ballooning degeneration or fibrosis of the hepatocytes. As a result, based on the histological features, the metabolic changes observed in liver in relation to Lyso-PLs may be associated to the benign progression of hepatic steatosis in hamsters fed diets rich in fats.

3.4. The Lyso-PL levels in plasma and liver are strongly correlated

To investigate the specific circulating changes able to predict the early development of hepatic steatosis, we decided to perform a correlation study with the thirty-one Lyso-PLs identified in plasma and liver of dyslipidemic hamsters. As shown in **Fig. 4**, Lyso-PL content in both tissues was highly correlated predominantly in a negative sense, pointing that certain Lyso-PLs could be transported to the hamster liver for metabolism. Among Lyso-PLs, Lyso-PCs were the circulating metabolites involved in the strongest negative correlations. Specifically, Lyso-PCs containing (14:0), (15:0), (16:1), (17:1), (18:3), (20:5), (22:5) and (22:6) acyl chains; as well as Lyso-PEs (16:1) and (22:6) were associated with the greater number of negative correlations ($r > 0.5$). Although plasma levels of these Lyso-PLs were decreased in HFD-fed animals, only the Lyso-PLs with the acyl chain (22:6) were significantly accumulated in liver (**Table 3**). By contrast, hepatic Lyso-PC (14:0), (15:0), (16:1) and (17:1)

levels were significantly reduced as a consequence of the treatment with HFD. Consequently, the multicompartmental analysis of the lysophospholipidome of HFD-fed hamsters evinced a possible intrahepatic modification of Lyso-PLs by successive elongation of the acyl chain and addition of double bonds (desaturation) in a process similar to the formation of the precursors of eicosanoids. In fact, the same reverse trend between plasma and liver of dyslipidemic animals was observed solely in other Lyso-PLs with long chain (LC)-PUFAs, namely Lyso-PC (20:4) and Lyso-PE (22:5). Therefore, the excess of fat of the diet caused, in addition to a higher incidence of steatosis in the animals, an intensification of the hepatic accumulation of LC-PUFAs-containing Lyso-PLs, thus leaving an indicative trait in plasma.
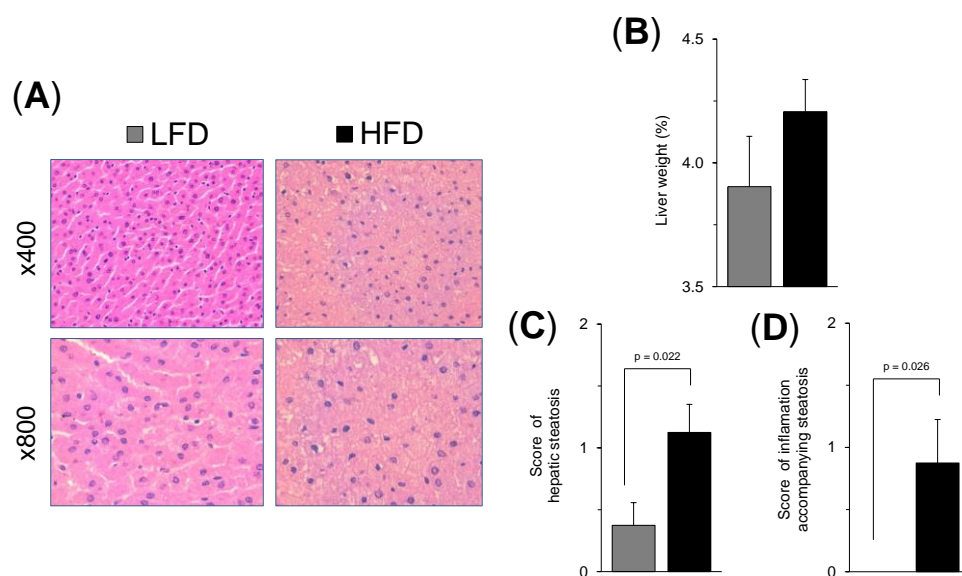
Figure 3. Effect of the thirty-day administration of low-fat diet (LFD) and high-fat diet (HFD) on the hamster liver. (**A**) Histological analysis of liver tissue showing representative images of the study. Liver sections were stained with hematoxylin-eosin and examined under a light microscope (n = 8 per group). The liver of HFD-fed animals shows hepatocellular vacuolization with microvesicular fat accumulation. (**B**) Relative liver weight in both animal groups, which was calculated following the formula (liver weight / body weight * 100) and expressed as % of the total body weight. Data are expressed as mean ± SEM. (**C**) Score of hepatic steatosis. The score ranged from 0 to 3 and were determined estimating the % of hepatocytes containing lipid droplets. (**D**) Score of inflammation (0-3) associated with steatosis. The significance was measured by conducting independent Student's t-test (p < 0.05).
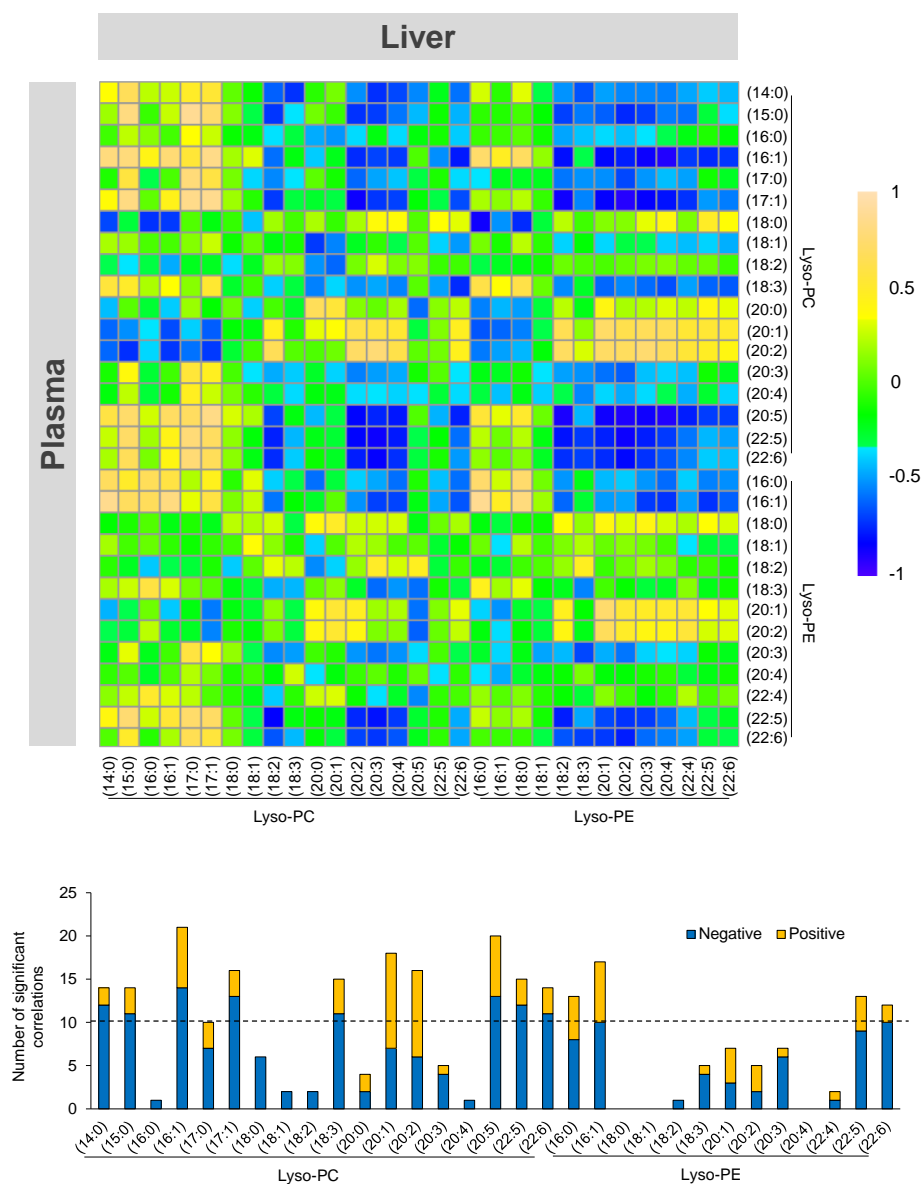
Figure 4. Effect of the HFD on the metabolic correlations across plasma and liver Lyso-PL content. The normalized abundance of Lyso-PLs was correlated across both biological compartments using Pearson's correlation testing. The outcoming correlation coefficients (*r*) are displayed on a color-coded matrix 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.

3.5. Lyso-PL evaluation in humans confirm their suitability as biomarkers

In a similar way that the results obtained in animals, a reduction in certain Lyso-PLs was observed in the plasma of adult subjects whose transaminase levels were higher (**Table 5**). Thus, the MS-based metabolomics evaluation of human samples showed that the AT subjects had, in addition to higher cholesterol levels, a considerable decreased content of Lyso-PCs (20:2), (20:3), (20:5), (22:5), (22:6) and Lyso-PEs (16:0), (18:0), (18:2), (20:5), (22:6) in plasma, as compared to the control group. Furthermore, the magnitude of the change was greater in the polyunsaturated species. Therefore, our results suggested that the mechanisms responsible for the alteration of circulating Lyso-PLs could be similar in hamsters and humans.

Table 5. Determination of the circulating Lyso-PL levels altered in humans

Circulating metabolite	FC (%)	*p-value*
Lyso-PC		
(20:2)	-10.3	*0.062*
(20:3)	-17.8	*0.025*
(20:5)	-44.9	*0.014*
(22:5)	-13.1	*0.070*
(22:6)	-22.4	*0.003*
Lyso-PE		
(16:0)	-18.9	*0.003*
(18:0)	-16.1	*0.010*
(18:2)	-13.3	*0.073*
(20:5)	-29.9	*0.014*
(22:6)	-14.9	*0.029*

The circulating levels of lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) were determined using UHPLC-MS/MS analysis. For each group of subjects, the data are presented as the mean ± SEM (n = 24). Fold-change (FC) was calculated following the formula: ((AT abundance – C abundance) / C abundance)* 100. Two-tailed *p-values* were calculated using independent Student's t-test.

4. Discussion

In the present study we have shown that a 30-day consumption of the HFD induces isolated hypercholesterolemia and promotes the development of microsteatosis in hamster liver. This 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 [29–32]. However, in our model, the circulating transaminases were unaltered, the hepatic tissue did not present significant hepatomegaly and the histological examination did not reveal hepatocyte balloon degeneration or fibrosis (**Fig. 3**). Consequently, the steatosis degree induced by the pro-dyslipidemic diet in this study was still incipient and the liver has not experienced substantial injuries that could affect the functionality of the organ [33]. In fact, while the development of insulin resistance is a common pathological feature of steatohepatitis (the progressive form of hepatic steatosis), we have not observed signs of hyperglycemia in the animals that presented an increased steatotic profile (**Table 2**).

Interestingly, the high content in fats of the diet elicited a drastic alteration on the lysophospholipidome promoting an overall reduction of the Lyso-PL loading in plasma (**Table 3**). Our results are in line with previous studies describing that the biologically active Lyso-PLs are targeted *via* portal circulation to restrict fatty acid oxidation in rodent liver and thereby reduce energy expenditure [16,17]. Thus, selective attenuation in the absorption of these lipid compounds through enterocytes could function as a coping mechanism to the chronic exposure of dietary fats. Inhibition of luminal phospholipid hydrolysis through the pancreatic phospholipase A_2 (PLA2G1B) also suppresses cholesterol absorption but only partially since additional enzymes are able to catalyze phospholipid digestion without contributing to the release of Lyso-PLs in the intestinal tract [34]. In the current study, we showed significant declines in the circulating levels of almost 50% of the Lyso-PL species identified, including Lyso-PCs and Lyso-PEs, in association with the development of hepatic steatosis in hamsters. This metabolic condition, which may be useful for the subclinical non-invasive diagnosis of fatty liver diseases, was also related to excessive removal of Lyso-PEs from the body through urine. According to our observations in hamsters, previous investigations carried out with steatotic mice had already described a lowering effect of the diet on the circulating Lyso-PL levels associated with an increment in the inflammation markers of hepatocytes [18,35].

Our results further revealed an interesting and, at first sight, contradictory response of the hamster liver to the long-term administration of HFD. In this way, although the diet was enriched especially in saturated and monounsaturated fats, we have observed an intrahepatic replacement of saturated and monounsaturated acyl lyso forms by longer highly

unsaturated acyl Lyso-PLs. In this context, several studies on free fatty acids described sequential processes of desaturation and elongation in rodent liver [36–38]. Through this mechanism, dietary saturated fats, in particular myristic acid (C14:0), would contribute to the metabolism of α-linolenic acid (C18:3 ω-3) in hepatocytes and therefore regulate the bioavailability of ω-3 LC-PUFAs [39,40]. In this context, Dabadie *et al.* observed beneficial effects in the circulating lipid profile of humans which were associated with a moderate intake of saturated fats. These effects were attributed to myristic acid by promoting the conversion of α-linolenic acid into ω-3 LC-PUFAs that significantly improved docosahexaenoic acid (DHA; C22:6 ω-3) levels in circulating phospholipids and cholesteryl esters [41]. However, when increasing the dietary C14:0 from 1.2% to 1.8% energy the incorporation of DHA and docosapentaenoic acid (DPA; C22:5 ω-3) into glycerophospholipids decreased. In relation to this, another study investigating the modulation of LC-PUFAs bioavailability by myristic acid showed dose-dependent accumulation of eicosapentaenoic acid (EPA; C20:5 ω-3) but also dihomo-γ-linolenic acid (C20:3 ω-6) in rat liver as a function of dietary C14:0 [40]. Therefore, saturated fats appear to be able to regulate biosynthesis of both ω-3 and ω-6 essential fatty acids according to physiological requirements. Our results showed that while the plasma levels of Lyso-PLs containing the fatty acid precursor C18:3 decreased substantially in the HFD-fed hamsters, the hepatic accumulation (and excretion in urine) did not become significant in comparison with control animals (**Table 3**). However, the boost in dietary fats promoted both removal from bloodstream and hepatic build-up of intermediates in the conversion pathway of C18:3 into ω-3 LC-PUFAs in form of Lyso-PL. As **Table 3** shown, this statement is supported by the significantly altered levels of Lyso-PLs possessing C22:5 and C22:6 hydrocarbon chains identified in plasma and liver of both animal groups. These molecular species of Lyso-PLs were in addition among those who undergone a greater reduction of the circulating levels along with EPA-containing Lyso-PC. Consequently, intracellular biosynthesis appeared to be contributing to the accumulation of DHA in hamster hepatocytes, the final product of the ω-3 conversion pathway [42]. The main role of free DHA is the inhibitory effect on inflammatory eicosanoid and cytokine production reducing the magnitude of the inflammatory response [43]. Besides in liver, DHA appears to possess hepatoprotective effects preventing DNA damage and oxidative stress and reducing substantially the tissue inflammation [44].

Furthermore, saturated fatty acids (SFAs) have been associated with lipoapoptotic events as well as inhibition of the VLDL secretion by hepatocytes [36,45]. *In vitro* studies revealed that the apoptotic effect of palmitic acid (C16:0) in hepatocytes is mediated by the intracellular conversion to the corresponding Lyso-PC and subsequent activation of specific GPCRs that control cellular integrity [46]. In addition, previous research on Lyso-PCs (16:0) and (18:0) have defined saturated acyl Lyso-PLs as pro-inflammatory compounds [47–50].

Therefore, through stimulation of the synthesis of ω-3 LC-PUFAs, liver would be able to protect itself against the progression of lipoapoptotic incidents and attenuate the inflammation induced by the pro-inflammatory lipids [51,52]. Thus, along with other saturated and monounsaturated acyl Lyso-PCs, Lyso-PC (16:0) has been proved to be an effective pro-inflammatory compound, whose action can be antagonized *in vivo* by the activities of Lyso-PCs (20:4) and (22:6) [51,53,54]. Moreover, substitution of the Lyso-PC moiety by a phosphorylethanolamine in Lyso-PEs (20:4) and (22:6) do not affect biological activity and the polyunsaturated acyl Lyso-PEs conserve potent anti-inflammatory actions [55]. Consequently, the opposite effects that Lyso-PLs have on several immune cell types have been identified as a regulatory mechanism to manage the onset and resolution of inflammation [56,57]. Interestingly, the HFD-fed hamsters of the present study exhibited greater levels of Lyso-PC (20:4) and (22:6), as well as of the corresponding Lyso-PEs, suggesting that liver is triggering anti-inflammatory processes to resolve local inflammation caused by the accumulation of lipid substances in hepatocytes. Consistent with these findings, histological examination indicated that the HFD-induced steatosis was accompanied by a slight extent of lobular inflammation in liver (**Fig. 3D**).

As with the generalized lowering of plasma Lyso-PL levels, the hepatic response seems to reflect an adaptation mechanism to counteract the detrimental effects that the prolonged consumption of fats has on the organism. Our observations are in accordance with the reported implications that bioactive Lyso-PLs have on the absorption of other lipids, β-oxidation of fatty acid molecules, apoptosis, as well as development and resolution of the inflammatory process. Furthermore, while we observed an enhanced accumulation of lipid drops in the liver of HFD-fed hamsters, the latest studies have associated this event with the mechanism by which long-chain unsaturated fatty acids added extracellularly are able to antagonize the detrimental effects of SFAs in diverse cell lines [58,59]. Interestingly, Plötz *et al.* demonstrated that gondoic acid (C20:1 ω-9) is especially involved in the protection against SFA-inducing lipotoxicity by stimulating intracellular formation of fat vesicles which trap SFAs after being esterified in triglycerides. The incorporation into Lyso-PLs promotes micellar conformation due to the typical structure of these molecules, which resembles an inverted cone [60]. Therefore, the isolated increase of Lyso-PC (20:1) that we have identified in the plasma of HFD-fed animals as compared to the controls (p < 0.001) could be contributing to the early development of hepatic steatosis in the present study. Interestingly enough, lipid droplets located intracellularly are also being recognized as intracellular loci for intensification of the eicosanoid response during inflammation [59].

Altogether, the results of our study support a model in which the increased intake of fats is transported into the hamster liver inducing development of mild microsteatosis and

accumulation of LC-PUFAs-containing Lyso-PLs after 30 days of intervention. Importantly, this pathological event was associated with a drastic modification of the lysophospholipidome while the circulating transaminases were unaltered. Thus, the early development of diet-induced steatosis appears to be characterized by a general drop in the circulating Lyso-PL levels, exacerbated excretion of Lyso-PEs through urine and intrahepatic modification of the lysophospholipidome in favour of polyunsaturated forms. Furthermore, our results in humans confirm these findings, indicating that the mechanisms involved in the early development of steatosis could be comparable in both mammal species. Consequently, the results presented in this study are of considerable interest as they could be applied in the subclinical diagnosis of steatosis, although further research must be conducted on human samples.

5. Acknowledgements

6. Supplementary material

Table S1. Differences in the extraction conditions depending on the tissue and Lyso-PLs identified

Tissue	Sample volume (μL)	MeOH volume (μL)	Concentration step	Identified metabolites	
Plasma	5	75	-	Lyso-PCs	(14:0), (15:0), (16:1), (17:0), (17:1), (18:3), (20:0), (20:1), (20:2), (20:3), (20:4), (20:5), (22:5), (22:6)
				Lyso-PEs	(16:0), (16:1), (18:0), (18:1), (18:2), (18:3), (20:1), (20:2), (20:3), (20:4), (22:4), (22:5), (22:6)
	2	198	-	Lyso-PCs	(16:0), (18:0), (18:1), (18:2)
Urine	40	200	-	Lyso-PCs	(14:0), (15:0), (16:0), (16:1), (17:0), (18:0), (18:1), (18:2), (18:3), (20:0), (20:1), (20:2), (20:3), (20:4), (22:5), (22:6)
				Lyso-PEs	(18:0), (18:1)
Aorta [1]	40	200	-	Lyso-PCs	(14:0), (15:0), (16:0), (16:1), (17:0), (17:1), (18:0), (18:1), (18:2), (18:3), (20:0), (20:1), (20:2), (20:3), (20:4), (20:5), (22:5), (22:6)
				Lyso-PEs	(16:0), (16:1), (18:0), (18:1), (18:2), (18:3), (20:1), (20:2), (20:3), (20:4), (22:5), (22:6)
Liver [2]	50	110	-	Lyso-PCs	(16:0), (16:1), (17:0), (18:0), (18:1), (18:2), (18:3), (20:3), (20:4)
				Lyso-PEs	(16:0), (18:0), (18:1)
	395	5	360 → 60 μL	Lyso-PCs	(14:0), (15:0), (17:1), (20:0), (20:1), (20:2), (20:5), (22:5), (22:6)
				Lyso-PEs	(16:1), (18:2), (18:3), (20:1), (20:2), (20:3), (20:4), (22:4), (22:5), (22:6)

Table S1. To determine the Lyso-PL content, different ratios of sample/MeOH were homogenized by vortexing (1 min) and ultrasonication (30 s). Mixtures were then incubated on ice (10 min) and centrifuged (20,000 g, 10 min). If appropriate, constant volumes of supernatant were dried under nitrogen flow and re-dissolved in the indicated volume of water/isopropanol/acetonitrile (4:3:3 v/v/v) prior to injection. Otherwise, supernatants were directly analyzed by UHPLC-+ESI-MS/MS [25].

*[1] Prior to metabolite extraction, aortic tissues were homogenized in 100 µL of cold PBS buffer.

*[2] Freeze-dried liver powder (20 mg) was homogenized in 1 mL of cold MeOH containing BHT (1 µM) as antioxidant.

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Table S2. Matrix effect percentages of tissues on different Lyso-PLs

Tissue	Spiked concentration (µg/L)	Lyso-PCs			Lyso-PEs		
		(16:0)	(18:0)	(20:0)	(16:0)	(18:0)	(18:1)
Plasma	10^2	-	-	110	97	91	84
	10^5	-	106	112	113	107	105
Urine	10^{-1}	129	122	120	122	109	113
Aorta	10^5	115	121	122	104	104	101
Liver	10^2	-	-	111	128	92	104
	10^5	128	132	125	123	120	118

Matrix effect was determined by spiking pooled samples of hamsters (n = 16) with standard Lyso-PLs at different concentrations. The constitutive concentrations of Lyso-PCs (16:0) and/or (18:0) in plasma and liver were too high to perform the stimation, especially at low concentration levels.
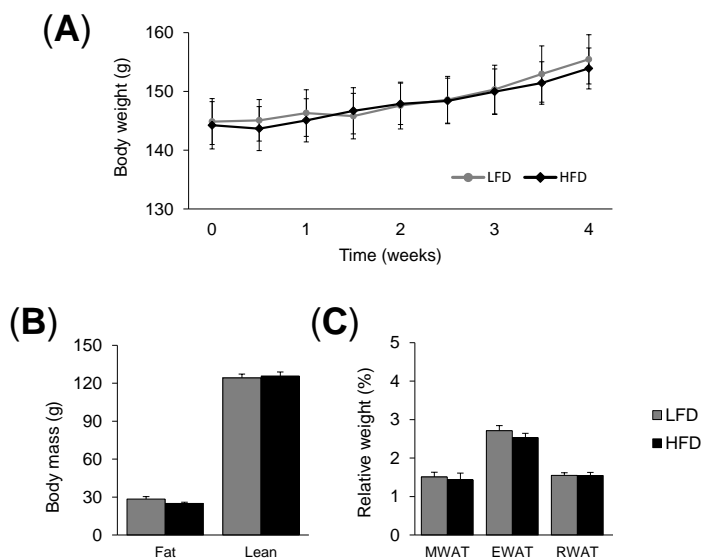
Figure S1. Body weight and adiposity-related parameters in hamsters fed low-fat diet (LFD) or high-fat diet (HFD) over 30 days. (**A**) Evolution of the body weight of the animals, which was recorded twice a week along the study. Data are the means with their SEM (n = 8 per group) represented by vertical bars. (**B**) Body mass composition measured at the end of the experiment on live unanaesthetized animals using quantitative magnetic resonance. (**C**) Relative weights of the white adipose tissue depots calculated following the formula (tissue weight / body weight * 100) and expressed as % of the body weight.
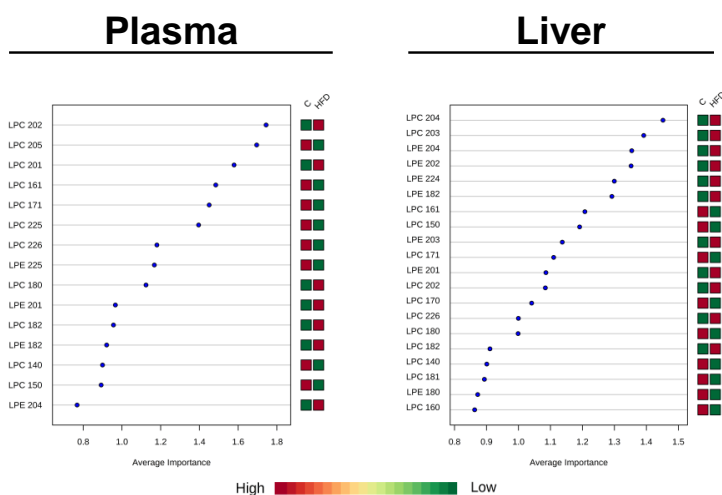
Figure S2. Most important Lyso-PLs ranked according to ROC curve analysis. The scale from red to green represents the relative abundance of each metabolite in the two animal groups. LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; C, animals fed low-fat diet (control group); HFD, animals fed high-fat diet.

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4. GENERAL DISCUSSION

4. General discussion

Lipids are a diverse group of compounds that fulfil important biological functions for the organism. Although these substances are generally hydrophobic and soluble in organic solvents, included in this group are also amphipathic molecules which, due to their particular behaviour in aqueous solutions, are indispensable for the formation of biological membranes [1]. Among them, glycerophospholipids [2], particularly phosphatidylcholines, are the major structural components in the membrane of eukaryotic cells. Their hydrophobic portion is a diacylglycerol that contains saturated and unsaturated acyl chains of varying lengths. Attached to *sn3*-position of glycerol is a phosphate associated with the corresponding head group, constituting the hydrophilic moiety of phospholipids. The hydrophobic portions tend to cluster to thereby minimize contact with water, whereas the hydrophilic moieties interact with the outer environment, as well as between them, resulting in the formation of laminar structures with two molecules of thickness known as lipid bilayers.

These small lipids of relatively simple structure, besides controlling the activity of transmembrane proteins, thus regulating the transport of substances between the different biological compartments [3], also determine the capacity of membrane for cleavage and fusion, allowing the cells to carry out processes of cell division, reproduction and vesicular transport [4]. Besides fulfilling an indispensable structural function, these polar lipids can act as bioactive molecules intervening in cellular recognition processes, acting as mediators in the transduction of intracellular signals and interacting extracellularly with G-protein-coupled receptors (GPCRs) [5]. The signalling function has been mainly attributed to lysophospholipids (Lyso-PLs), as they are much more hydrophilic and versatile compounds than the phospholipids from which they derive [1] (**Fig. I**).
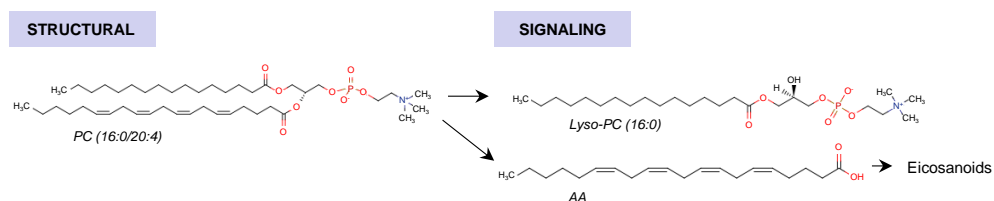
Figure I. Membrane lipids and signalling molecules. The main structural components of the eukaryotic membrane are phosphatidylcholines (PC). Through degradation of membrane PCs, the signal is divided in two that can be transmitted along the membrane through the hydrophobic portions of the molecules or propagate in the cytoplasm or in the extracellular environment where they are also soluble. Thus, breakdown products of PCs, including lysophosphatidylcholine (Lyso-PC) and arachidonic acid (AA), act as signalling molecules, but also as precursors for the synthesis of important bioactive molecules.

Therefore, Lyso-PLs are deacylated forms of phospholipids, which can be ubiquitously generated under phospholipase activity in the remodelling pathway, also called Lands' cycle [2]. Alternatively, it has been shown that saturated acyl Lyso-PC can be generated in an enzyme-independent manner by a mechanism involving spontaneous deacylation of an oxidized PC intermediate [6]. However, Lyso-PL levels are tightly controlled, since there are also acyltransferases and lysophospholipases responsible for restricting the availability of these bioactive lipids [7,8].

Thus, glycerol-based Lyso-PLs are involved in a wide range of physiological and pathological processes, such as reproduction [9], vascular development [10], carcinogenesis [11], neural differentiation [12], apoptosis [13], immunity [14–16] and advancement of metabolic diseases [17,18]. Due to the dramatic progression that omics sciences have undergone in the last years, metabolic alterations in the Lyso-PLs patterns are being identified as biomarkers of very diverse pathologies. Individual species belonging mostly to Lyso-PC and lysophosphatidylethanolamine (Lyso-PE) categories have been proposed as putative biomarkers of diabetes [19], atherosclerosis [20,21], obesity [22–25] and several types of cancer [26,27] in humans. Thus, implementation of mass spectrometry-based metabolomics for the analysis of plasma Lyso-PLs appears to have notable potential in the diagnosis of lipid disorders and could be used to evaluate the therapeutic effects of anti-dyslipidemic drugs.

In accordance with the literature, the metabolic studies carried out in the present doctoral thesis identified circulating Lyso-PLs as suitable descriptors of the metabolic state of animals fed high-calorie diets and/or subject to periodic physical training. Interestingly, the results of the **Manuscript 3**, which are focused on the lysophospholipidome, provided an overall view of the rat serum metabolome similar to that obtained with the non-targeted perspective (**Manuscript 1, Fig. 5**). Furthermore, with the summarised predictive model of **Manuscript 3** (**Fig. 4**) it was possible to achieve even greater reliability to discern between dietary groups. Therefore, our results indicated that the targeted metabolomics evaluation of the circulating lysophospholipidome holds remarkable potential in the diagnosis of lifestyle-related diseases.

The sterol-activated nuclear receptor, also known as the liver X receptor (LXR), is a nutriable target able to interact with cholesterol oxidation products, namely oxysterols, playing an essential role as sensor of the dietary cholesterol [28]. LXR regulates the expression of lysophosphatidylcholine acyltransferase 3 (LPCAT3), which is involved in the remodelling of membrane phospholipids in liver [29,30]. It is known that the activity of this transmembrane protein generates preferentially phosphatidylcholine species that contain *sn2*-polyunsaturated acyl chains [31]. Because *sn1*-saturated acyl Lyso-PLs (and to a lesser

extent monounsaturated) serve as substrate for LPCAT3, the resulting phospholipids possess an asymmetric structure, which provides fluidity to the intracellular membranes thus enabling vesicle trafficking and the fulfilment of functions of crucial importance for the cell [32]. As shown in **Fig. II**, in hepatocytes, the assembly and secretion of VLDLs is influenced by the membrane phospholipid composition of endoplasmic reticulum (ER) and, when there is a lack of polyunsaturated forms, the particles are smaller and mobilize lipids inefficiently [33].
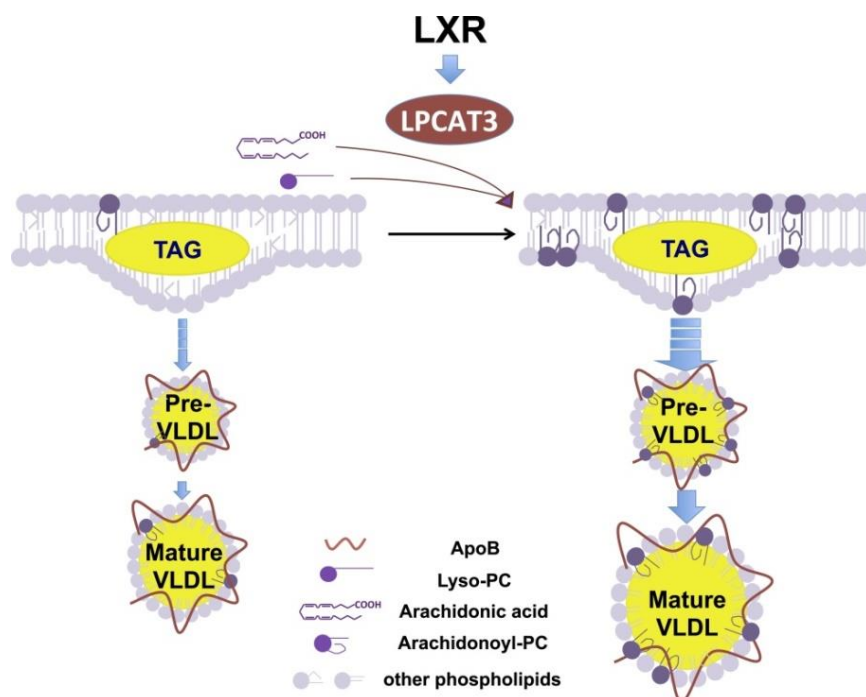
Figure II. Role of LXR-LPCAT3 pathway in the modulation of membrane phospholipid composition in ER and lipidation of VLDL particles. In liver, the activation of LXR promotes the incorporation of polyunsaturated fatty acids (PUFAs), especially arachidonic acid (C20:4), into phosphatidylcholines (PCs) of intracellular membranes, through LPCAT3 activity. The modification in membrane composition of ER generates a dynamic membrane environment that facilitates the transfer of triglycerides (TAG) to nascent apoB-containing lipoproteins, leading to the efficient lipidation of these particles. The figure was obtained from [33].

Therefore, the promotion of LPCAT3 activity ameliorates ER stress and inflammation induced by saturated fatty acids (SFAs) both by increasing the bioavailability of eicosanoid precursors and by preventing the activation of inflammatory kinases [29,34,35]. In relation to this, up-regulation of LPCAT3 has been demonstrated in stearoyl-CoA desaturase

1 (SCD1) knockdown cells [36]. The activity of SCD1 led to desaturation of palmitic (C16:0) and stearic (C18:0) acids, resulting in the corresponding monounsaturated fatty acids (MUFAs) and thereby determining the SFA / MUFA balance in membrane phospholipids. Overexpression of LPCAT3 and SCD1 was related to the development of fatty liver disease in both mice [37] and humans [38].

According to this mechanism, our *in vivo* studies showed increased LPCAT3 activity in hamster liver as a consequence of the intake of a diet moderately high in fat (HFD). Thus, although the hepatic expression remained unchanged (**Fig. 4c**, **Manuscript 2**), the targeted metabolomics analysis of Lyso-PLs identified lower levels of saturated and monounsaturated forms in the plasma and liver of HFD-fed hamsters as compared to animals fed the low-fat diet (LFD) (**Table 3**, **Manuscript 4**). Concretely in liver of those hamsters, the 30-day treatment with HFD also induced significant increases in the individual levels of polyunsaturated acyl Lyso-PLs, namely Lyso-PCs (18:2), (20:2), (20:3), (20:4), (22:6) and Lyso-PEs (18:2), (20:2), (20:3), (20:4), (22:4), (22:5), (22:6), as well as in the total content, as can be observed in **Fig. III**. As previously observed in **Manuscript 3**, the overall behaviour of the two types of Lyso-PLs investigated, Lyso-PCs and Lyso-PEs, was consistent with that of total Lyso-PLs (data not shown) since it depends on the acyl chain rather than the head group.
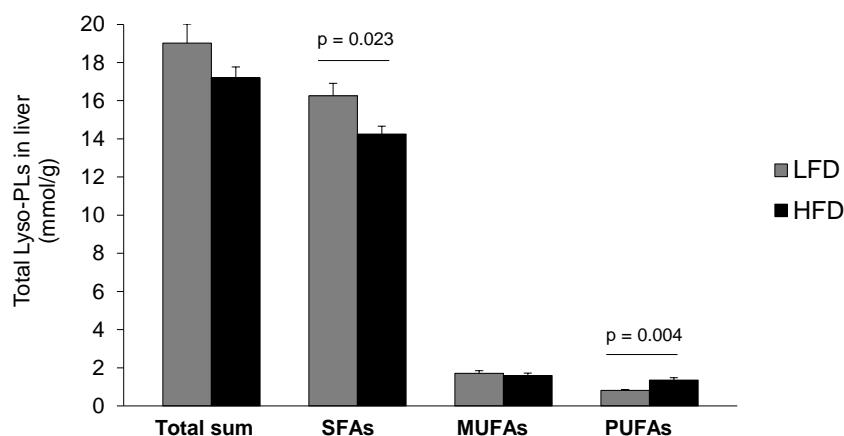
Figure III. Total levels of hepatic Lyso-PLs containing different saturation degrees in the *sn1*-acyl chain. The results derived from **Manuscript 4** where adult hamsters were fed low-fat diet (LFD; n=8) or high-fat diet (HFD; n=8) over 30 days. After treatment, the individual levels of thirty-one Lyso-PLs were determined in liver by targeted metabolomics. The statistical comparison between groups was performed using independent Student's t-test ($p < 0.05$).

In fact, although Lyso-PCs and Lyso-PEs can be produced in blood by the hydrolytic action that several secreted enzymes (e.g. LCAT, sPLA$_2$ and lipases) exert on circulating phospholipids (see **Manuscript 2** for further information), direct hepatic secretion is an important source of lyso forms [39,40], particularly of those that contain PUFAs. These polar lipids are discharged into the bloodstream and transported to peripheral tissues, bound to albumin and lipoproteins, more rapidly than free fatty acids [39,41]. Thus, the transport of Lyso-PLs of hepatic origin is especially important in tissues with a high demand for PUFAs, as well as for choline and ethanolamine (e.g. brain). This group of Lyso-PLs, unlike those containing SFAs, seems to result from phospholipase activity of type 1 (PLA$_1$), whereby the acyl chains located at the *sn1*-position of the hepatic phospholipids are cleaved, releasing SFAs into the organ [42].

The detrimental effects of saturated fats on hepatocytes have been widely described [43–45]. Among them, SFAs have been associated with lipoapoptotic incidents and inflammation, as well as inhibition of the VLDL secretion by hepatocytes. Therefore, when SFAs are consumed in excess, as in the case of the HFD used for our hamster studies, significant amounts are accumulated in liver [46] thereby promoting the development of steatosis in the animals (**Fig. 3, Manuscript 4**). We have shown how numerous lipid droplets filled the cytoplasm of the hepatocytes when the HFD feeding was extended to 30 days, while a slight infiltration of inflammatory cells occurred predominantly in the lobular zone, but in none of the biopsies have we found signs of necrosis or fibrosis. Based on the histological features, the metabolic changes observed in liver that involve the lysophospholipidome may be associated to the early progression of hepatic steatosis in HFD-fed hamsters.

In vitro studies revealed that the apoptotic effect of SFAs in cells is facilitated by esterification with Lyso-PC moieties and successive activation of GPCRs that control cellular integrity [47]. In **Manuscript 4**, we demonstrated, for the first time in hamsters, that SFAs once esterified in liver to the glycerol backbone of Lyso-PLs could be subjected to the action of LPCAT3 as an adaptive mechanism to the unbalanced intake of fats. Thus, LPCAT3 but also PLA$_1$ activity would led to a marked increase of polyunsaturated glycerophospholipids that, incorporated into the membranes, favor dynamic processes that are of vital importance for the preservation of the lipid homeostasis. As shown in **Fig. IV**, the insertion of Lyso-PLs in lipid bilayers further promotes micellar conformation due to the shape of inverted cones [48]. Furthermore, it has recently been demonstrated that polyunsaturated acyl Lyso-PCs and Lyso-PEs, specifically the Lyso-PLs (20:4) and (22:6), exhibit anti-inflammatory properties that are able to counteract the pro-inflammatory effects of saturated acyl Lyso-PLs [16,49]. In relation to this, we have observed that Lyso-PLs containing (20:4) and (22:6) acyl chains were increased in the plasma (**Table 6, Manuscript 2**) and liver (**Table 3, Manuscript 4**)

from the studies carried out with dyslipidemic hamsters. The accumulation of PUFAs-containing glycerophospholipids in membranes therefore promotes the intrahepatic formation of lipid droplets which serve as reservoirs of lipids potentially toxic to the cell [33]. Interestingly enough, these organelles are also being recognized as intracellular loci allocated to the amplification of the eicosanoid response during inflammation [50].
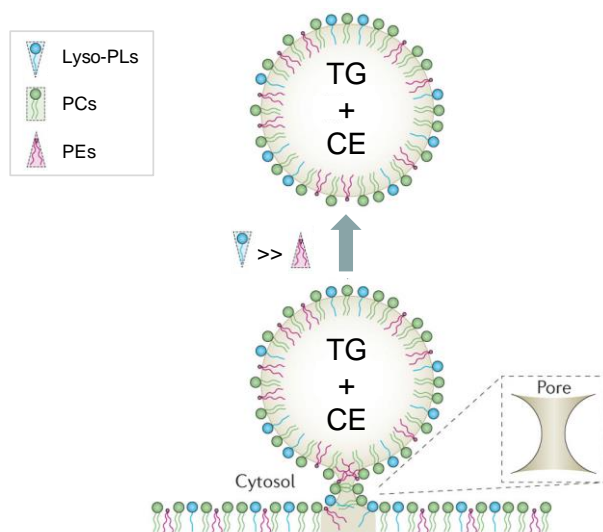

Figure IV. Formation of a lipid droplet in the cellular cytosol. A droplet that contains triglycerides (TG) and cholesterol esters (CE) and is covered with different phospholipids forms a pore in the outer monolayer of a bilayer (e.g. in the ER membrane). If at the place of the pore the monolayer is rich in positively curved lipids, such as Lyso-PLs, the pore will tend to close giving rise to the cytosolic lipid droplet. The figure was adapted from Thiam *et al.* 2013 [48].

It is also known that hepatic accumulation of Lyso-PCs inhibits β-oxidation of fatty acids [51] and raises apoB-containing lipoprotein production in liver along with plasma levels of triglycerides [30]. Therefore, reducing the absorption of Lyso-PLs from intestinal lumen, energy expenditure is increased and hypertriglyceridemia largely avoided. According to this assumption, we have demonstrated a generalized lowering effect of the HFD in the plasma Lyso-PL levels (**Table 3, Manuscript 4**), while the circulating triglycerides were unaffected (**Table 2, Manuscript 4**). This metabolic pattern is also profiling the adaptation of hamster metabolism to the intake of fats, preventing the total build-up of Lyso-PLs in liver (**Fig. III**) and the suppression of the lipid catabolism associated with the development of fatty liver diseases. Our findings are in agreement with previous researches carried out on mice with diet-induced dyslipidemia [37,25]. Like us, Tanaka *et al.* do not point to modifications in

circulating lysophospholipidome have a predominant plasmatic origin (e.g. deterioration of LCAT activity), but demonstrated that it is rather intrahepatic and is associated with an increased inflammatory response and disruption of bile acid metabolism as a consequence of the progression of nonalcoholic steatohepatitis (NASH) in mice. Furthermore, substantial increments in the faecal excretion of Lyso-PCs have recently been identified in patients with hepatic cirrhosis [52].

For the absorption of dietary fats, which occurs in the small intestine, cholesterol is mixed in a lipid emulsion with triglycerides and phospholipids [53]. The hydrolysis of phospholipids that are located at the surface of the emulsion particle is carried out by pancreatic phospholipase A_2 and it is required for the release of cholesterol (as well as fatty acids and Lyso-PLs) and subsequent incorporation into bile acid micelles to be absorbed through the enterocytes [54,55]. In relation to this, a deterioration of the exocrine pancreatic function has been described in patients with chronic liver diseases [56]. In addition, it is noteworthy that, in our first study, we identified significant declines in the serum levels of bile acids in response to chronic consumption of a cafeteria diet (CAF) in rats (**Table 1, Manuscript 1**). Interestingly, this type of diet has also been associated with the development of fatty liver in different animal models [57,58].

Therefore, alterations in the circulating levels of both Lyso-PLs and hydroxylated bile acids could have a common link and be due to adjustments in the digestion and absorption of dietary lipids in rodents whose liver function is compromised. Our findings are also supported by previous investigations that identified metabolites belonging to both lipid families as proper indicators of liver injury in both rodents [37,27,59–61] and humans [61–65]. Taken together, these observations suggest that similar mechanisms may operate in rodents and humans. Additionally, we have observed that when the animals practice aerobic exercise with assiduity, the diet-induced alterations in bile acids are accentuated (**Fig. 6, Manuscript 1**), suggesting that physical activity is also involved in the modulation of fat absorption [66]. As reported in the literature, bile acids are not only important for lipid absorption and cholesterol metabolism, but also decisive signalling molecules [67–69]. Thus, these metabolites of amphipathic nature are physiological ligands for farnesoid X receptors (FXRs) that act as intracellular bile acid sensors to maintain cholesterol homeostasis by means of the transcriptional regulation of CYP7A1, the rate-limiting enzyme in the classic pathway of bile acid synthesis in liver [70,71]. Furthermore, while we found evidence of systemic (see **Manuscript 1** for detailed information) and hepatic (**Fig. 3d, Manuscript 4**) inflammation in rodents fed high-calorie diets, other authors have indicated that inflammatory cytokines may inhibit CYP7A1 expression [72]. Interestingly, it is also through the interaction with FXR that intrahepatic bile acids are able to regulate the enterohepatic transport [71]. Altogether, our

results support a model in which SFAs-induced inflammation is driving important metabolic processes for the animals, such as the fluidization of intracellular membranes thereby minimizing the harmful effects of fatty infiltration in the liver, as well as the disruption of the conversion of cholesterol into bile acids and the malabsorption of lipids from the diet.

The development of fatty liver is also associated with an impairment of the lipoprotein production [73], as evidenced by the comparative expression analyses carried out on hyperlipidemic hamsters. Thus, we have observed that the hepatic expression of endothelial lipase (*Lipg* locus) was largely inhibited by the chronic intake of HFD as compared to the hamsters fed LFD (**Fig. 4a, Manuscript 2**) and, interestingly, this fact is related to the high circulating levels of HDL found in diet-induced dyslipidemic animals (**Table 4, Manuscript 2**) [74]. The prevalent phospholipase activity of the endothelial lipase mediates phospholipid modulation in plasma HDL particles enhancing cholesterol removal from peripheral tissues and promoting HDL uptake in liver [75,76]. By reducing the expression of the enzyme, circulating levels of Lyso-PLs also decrease thereby preventing the detrimental accumulation of both Lyso-PLs and cholesterol in hamster liver [77]. Moreover, the circulating HDL particles become poorly lipidated which makes them susceptible to clearance by the kidneys [75,78]. Since endothelial lipase acts predominantly on phosphatidylethanolamines, the transcriptional modifications could lead to increased excretion of Lyso-PEs through urine, as we demonstrated in the last study (**Table 3, Manuscript 4**).

In summary, the liver of both animals and humans with nonalcoholic fatty liver disease (NAFLD) develops a central metabolic phenotype represented by a strong dysregulation in the homeostasis of phospholipids, in which bile acids are involved. As **Fig. V** shows, the worsening of fatty liver progresses through different phases as liver function becomes increasingly compromised [79]. The literature indicates that, according to our studies in hamsters, the attenuation of Lyso-PC levels in plasma is a prevalent metabolic profile that remains constant along the various stages of the hepatic disease in humans [63,80,81]. However, metabolic investigations that associate alterations in circulating Lyso-PEs with the onset of hepatic disorders are fairly scarce besides revealing contradictory results [23,27,59,82,83]. These findings, added to those found in our hamster studies, reveal that metabolism of Lyso-PEs is especially dependent of etiological factors. Consequently, when liver injury is induced through the administration of drugs, Lyso-PE levels raise within the bloodstream [27,82,83], as we observed in the hamsters chronically treated with Poloxamer 407 (P407) (**Fig. 5, Manuscript 2**). This may be due to reduced expression of phosphatidylethanolamine N-methyltransferase (PEMT), whose activity catalyses the methylation of phosphatidylethanolamine to form phosphatidylcholine in liver membranes

when dietary choline is insufficient [84]. Interestingly, it has also been shown that its gene expression is strongly repressed in human hepatocellular carcinomas [85]. In addition, our results revealed that the hepatic expression of secreted phospholipase A_2 (sPLA$_2$), a well-known pro-inflammatory enzyme which has predominant affinity towards phosphatidylethanolamines [86], is extremely up-regulated in P407-injected animals, but not in those fed the HFD (**Fig. 4b, Manuscript 2**). This effect is comparable to that reported by other studies investigating sPLA$_2$-mediated progression of liver damage in rodents treated with hepatotoxic agents [87]. Therefore, plasma Lyso-PEs appears to be attractive non-invasive biomarkers of liver injury caused by drugs. Since so far no research on P407 had been performed by untargeted evaluation, the results presented in **Manuscript 2** are also relevant for the pharmacological industry [88].
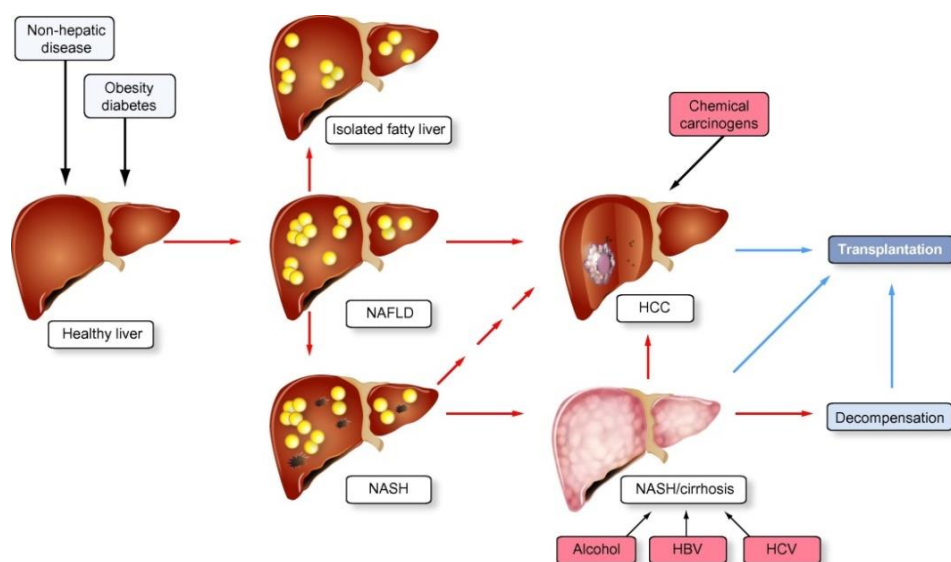
Figure V. Schematic illustration of the progression from the healthy liver to hepatocellular carcinoma (HCC) through the different phases of nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), and hepatic cirrhosis. NAFLD may progress to NASH but ~80% of cases remain as isolated fatty liver [89]. The figure, obtained from Beyoglu *et al.* [79], also shows other etiological factors apart from the diet. HBV, hepatitis B virus; HCV, hepatitis C virus.

Importantly, the generalized decrease in the plasma levels of Lyso-PCs and Lyso-PEs that we have identified in **Manuscript 4 (Table 3)** occurs in a very incipient state of steatosis, when traditional biomarkers of hepatic disorder such as transaminases are not altered. Furthermore, in order to investigate the adequacy of the Lyso-PLs as biomarkers of early pathology, a preliminary study was conducted in a small cohort of humans grouped

according to their transaminase levels, but within the range considered as healthy (**Table 1, Manuscript 4**). The metabolomics analysis of plasma samples (**Table 5, Manuscript 4**) confirmed the findings obtained in hamsters, indicating that the mechanisms involved in the early progression of steatosis could be similar in both species.

Although further research must be conducted on humans, the results of the present doctoral thesis are of considerable interest since they indicate that the plasma determination of Lyso-PLs could be applied in the subclinical non-invasive diagnosis of hepatic steatosis.

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5. CONCLUSIONS

Conclusions

1. **Non-targeted metabolomic reveals Lyso-PLs as biomarkers of chronic metabolic disorders (CHAPTER 1)**

   1.1. **An unhealthy diet has a strong impact on the metabolome that is difficult to revert with the practice of running, even when maintained daily (MANUSCRIPT 1)**

   - The chronic consumption of cafeteria diet causes a generalized alteration of the circulating lipidome which can be source of biomarkers of phenotypic flexibility.

   - The routinely practice of physical exercise at the intensities evaluated modifies the circulating lipidome in a slight manner.

   - The impact of cafeteria diet in the metabolome is greater than the exerted by the physical activity.

   - Animals fed a control diet are more susceptible to exercise-induced changes in metabolites than animals with metabolic syndrome.

   - In a situation of unhealthy diet, the practice of moderate intensity of exercise is able to partially reverse the disturbance in the metabolome. However, a higher intensity of exercise is less effective at the global level.

   1.2. **The analysis of Lyso-PLs provides information for the diagnosis and management of lipid disorders providing information regarding etiology and disease degree (MANUSCRIPT 2)**

   - Both types of treatments, high-fat diet and the drug Poloxamer 407, produce an overall disturbance in the circulating levels of Lyso-PLs, confirming the suitability of this group of molecules in the evaluation of metabolic disorders.

   - Regarding Lyso-PL category, Lyso-PEs are associated with progression of the disorder induced by the pharmacological treatment, while Lyso-PCs are more specific biomarkers for the dyslipidemia from dietary origin.

- The dose of 50mg/kg of Poloxamer 407 administered by i.p injections every 3 days effectively produces an steady condition of moderate dyslipidemia in hamsters, as compared with the exerted by higher doses.

- Depending on the etiology, the dyslipidemia acts through different hepatic mechanisms. Lipolysis is inhibited by the diet, while inflammation processes predominate in the hamsters treated with Poloxamer 407.

2. **The exhaustive analysis of the metabolic patterns of Lyso-PLs confirm these molecules as suitable biomarkers of chronic metabolic disorders (CHAPTER 2)**

   2.1. **The targeted metabolomics evaluation of the lysophospholipidome confirm these metabolites as plasma biomarkers of homeostatic disturbance (MANUSCRIPT 3)**

- The lysophospholipidome effectively describes the general status of the metabolome in rats fed either control or cafeteria diet and that practice exercise at different intensities.

- Lyso-PEs are the Lyso-PL category primarily influenced by the practice of physical exercise, specially in animals with metabolic syndrome and moderate exercise intensity.

- A multi-composed biomarker that includes a selected group of Lyso-PLs reveals as an adequate tool to evaluate the metabolic response to different challengues, according to the multivariate analysis.

   2.2. **The determination of plasma Lyso-PL levels is useful for the subclinical non-invasive diagnosis of fatty liver diseases (MANUSCRIPT 4)**

- In an early state of hepatic disorder in which liver function is not compromised, a generalized decrease of the circulating Lyso-PL levels is associated with intrahepatic microvesicular deposition of fats.

- Plasma succesfully reflects changes in hepatic metabolism, as shown by the decline in plasmatic Lyso-PLs which is inversely correlated with the content of polyunsaturated acyl Lyso-PLs in liver.

- The drastic modification of the hepatic lysophospholipidome in response to the high-fat diet indicates that liver is an essential organ in the metabolism of Lyso-PLs.

3. **The evaluation of the circulating lysophospholipidome in humans reveals similar alterations to those identified in animals, thus confirming the interest of this family of compounds (MANUSCRIPT 4)**

Conclusions

1. **Els anàlisis de metabolòmica no dirigida revelen els Lyso-PLs com a biomarcadors de trastorns metabòlics crònics (CAPÍTOL 1)**

   1.1. **Una dieta poc saludable té un fort impacte en el metaboloma que és difícil de revertir amb la pràctica d'exercici, fins i tot quan es realitza diàriament (MANUSCRIT 1)**

   - El consum crònic de la dieta de cafeteria causa una alteració generalitzada dels lípids circulants que pot ser font de biomarcadors de flexibilitat fenotípica.

   - La pràctica habitual de l'exercici físic a les intensitats avaluades modifica d'una manera lleugera el lípidoma circulant.

   - L'impacte de la dieta de cafeteria en el metaboloma és més gran que l'exercit per l'activitat física.

   - Els animals alimentats amb una dieta control són més susceptibles als canvis induïts per l'exercici en els metabòlits que els animals amb síndrome metabòlica.

   - En una situació de dieta poc saludable, la pràctica d'intensitat moderada de l'exercici és capaç de revertir parcialment la pertorbació del metaboloma. Tanmateix, una major intensitat d'exercici és menys efectiva a nivell global.

   1.2. **L'anàlisi dels Lyso-PL proporciona informació per al diagnòstic i la gestió de trastorns lipídics, proporcionant informació sobre l'etiologia i el grau de la malaltia (MANUSCRIT 2)**

   - Els dos tractaments, dieta alta en greixos i la droga Poloxàmer 407, produeixen una pertorbació general en els nivells circulants dels Lyso-PL, confirmant la idoneïtat d'aquest grup de molècules en l'avaluació de trastorns metabòlics.

   - Pel que fa a la categoria dels Lyso-PL, els Lyso-PEs estan associats amb la progressió del trastorn induït pel tractament farmacològic, mentre que els Lyso-

PCs són biomarcadors més específics per a la dislipidèmia a partir d'origen dietètic.

- La dosi de 50 mg/kg de Poloxàmer 407 administrada per injeccions i.p cada 3 dies produeix un estat estable de dislipidèmia moderada en hàmsters, en comparació amb el que exerceixen dosis més altes.

- Depenent de l'etiologia, la dislipidèmia actua a través de diferents mecanismes hepàtics. La lipòlisi està inhibida per la dieta, mentre que els processos d'inflamació predominen en els hàmsters tractats amb Poloxàmer 407.

2. **L'anàlisi exhaustiu dels patrons metabòlics dels Lyso-PL confirma aquestes molècules com a biomarcadors adequats de trastorns metabòlics crònics (CAPÍTOL 2)**

2.1. **L'avaluació per metabolòmica dirigida del lisofosfolipidoma confirma aquests metabòlits com a biomarcadors plasmàtics del trastorn homeostàtic (MANUSCRIPT 3)**

- El lisofosfolipidoma descriu eficaçment l'estat general del metabolome en rates alimentades amb dieta control o cafeteria i que practiquen exercici a diferents intensitats.

- Els Lyso-PEs són la categoria de Lyso-PL principalment influenciada per la pràctica de l'exercici físic, especialment en animals amb síndrome metabòlica i intensitat moderada d'exercici.

- Un biomarcador multicompost que inclou un grup seleccionat de Lyso-PL es revela com una eina adequada per avaluar la resposta metabòlica a diferents "challenges", d'acord amb l'anàlisi multivariant del resultats.

2.2. **La determinació dels nivells dels Lyso-PL de plasma és útil per al diagnòstic subclínic no invasiu de malalties del fetge gras (MANUSCRIT 4)**

- En un estat precoç de trastorn hepàtic en el qual la funció hepàtica no està compromesa, una disminució generalitzada dels nivells de Lyso-PL circulants està associada amb la deposició microvesicular intrahepàtica de greixos.

- El plasma reflecteix amb èxit els canvis en el metabolisme hepàtic, tal com demostra la disminució de Lyso-PLs plasmàtics, que correlaciona inversament amb el contingut d'acyl Lyso-PLs poliinsaturats en el fetge.

- La modificació dràstica del lisofosfolipidoma hepàtic en resposta a la dieta alta en greixos indica que el fetge és un òrgan essencial en el metabolisme de Lyso-PLs.

3. **L'avaluació del lisofosfolipidoma circulant en humans revela alteracions similars a les identificades en animals, confirmant així l'interès d'aquesta família de (MANUSCRIT 4)**

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Una prueba más, superada, y una lección enorme, aprendida. ¡A por la siguiente!

ANNEX

Manuscript 5

Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum

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Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum

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Abstract

Recent investigations based on non-targeted metabolomics have proposed lysophospholipids (Lyso-PLs) as biomarkers of different diseases. In particular, lysophosphatidylcholines (Lyso-PCs) and lysophosphatidylethanolamines (Lyso-PEs) have been associated with serious lipid pathologies. Methods to determine the different molecular species in a biological sample and to quantify even less abundant species are required for the evaluation of the Lyso-PL pattern as a novel comprehensive biomarker of dyslipidemia. This study describes the development and validation of an ultra-high-performance liquid chromatography coupled to tandem mass spectrometry assay for the determination of a large number of Lyso-PCs and Lyso-PEs in biological samples. The method was validated in rat serum using two simple methanol-based extractions with low sample volumes (5 - 50 µL) that covered the wide concentration range of these metabolites. In total, thirty-one Lyso-PLs were separated and quantified with low method limits of detection and quantification, reaching values of 0.2 and 0.8 nM, respectively. The method was subsequently applied in the identification of Lyso-PL-related changes produced by the chronic intake of a cafeteria diet. The results showed alterations in the majority of Lyso-PCs and Lyso-PEs in rat serum. Furthermore, multivariate analysis indicated that the comprehensive evaluation of serum Lyso-PLs could be an excellent indicator of the nutritional phenotype associated with an increased risk of lipid disorders.

Keywords: ultra-high-performance liquid chromatography-tandem mass spectrometry; glycerophospholipid; lysophosphatidylcholine; lysophosphatidylethanolamine; biomarker; cafeteria diet

Highlights

- UHPLC-+ESI-MS/MS method for glycerol-based lysophospholipid determination
- Lysophosphatidylcholine and lysophosphatidylethanolamine characterization in serum
- Separation of individual species within both classes of lipids
- Quantification of low-abundance lysophospholipid species
- Lysophospholipids as biomarkers of nutritional phenotype in cafeteria-fed rats

1. Introduction

Glycerol-based lysophospholipids (Lyso-PLs) are structural components of cellular membranes that can also act as signaling molecules in a wide range of physiological and pathological events including inflammation, reproduction, nervous and vascular system development and carcinogenesis [1]. The molecular structure of Lyso-PLs is very simple and consists of a hydrophobic acyl chain attached to the *sn1-* or *sn2-* position of the glycerol backbone and a hydrophilic phosphate head group in the *sn3*-position whose nature determine the diverse classes of Lyso-PLs.

Among circulating lyso forms, those containing a choline group, namely lysophosphatidylcholines (Lyso-PCs), are the most abundant, with serum levels of several hundred micromolar [2,3]. Lyso-PCs have been mainly related to inflammatory diseases and atherosclerosis [4–7]. Conversely, lysophosphatidylethanolamines (Lyso-PEs) exhibit a smaller head group than Lyso-PCs and are present at lower circulating levels, reaching a maximum concentration of several hundred nanomolar [8,9]. Information regarding the biological significance of Lyso-PE in serum is scarce compared to Lyso-PCs, although both classes of Lyso-PLs have been postulated as biomarkers of the progression of different pathologies. Lipid profiling of patients with different development statuses of atherosclerotic plaques showed that certain modifications in the circulating levels of Lyso-PCs and Lyso-PEs were associated with disease severity [10]. Continuing with a holistic view, a recent study noted the importance of both Lyso-PLs as serum indicators of hepatocarcinogenesis progression in humans [11]. *In vivo* investigations demonstrated that dysregulation of serum and hepatic levels of several Lyso-PCs and Lyso-PEs occurs in rodents fed high-calorie diets [12–14], while differential patterns of secreted Lyso-PLs have been observed in a cellular model of steatosis [9]. Therefore, the examination of circulating levels of Lyso-PLs holds remarkable potential in the diagnosis of lipid disorders and could be used to assess the therapeutic effects of drugs. Since the pathways leading to the synthesis of Lyso-PCs and Lyso-PEs are related [15], it would be of interest to quantify them in a high-throughput analysis with sufficient specificity to differentiate Lyso-PLs containing different side chains.

A variety of methodologies, including coupled techniques based on chromatography, have been developed for the evaluation of Lyso-PCs and Lyso-PEs in biological samples. One of the most common techniques involves separation by thin-layer chromatography (TLC) on silica gel plates [16]. However, once Lyso-PLs are isolated, they must be extracted for further analysis of the fatty acid composition, and thus this methodology is laborious and time-consuming [17,18]. Moreover, the quantification of molecular species present in low abundance using this approach is very difficult. In recent years, chromatographic techniques

have become more robust and specific. The use of reverse phase high-performance liquid chromatography (HPLC) coupled to fluorescence [19], ultraviolet (UV) [20], radioactive [21], phosphorus [22], and evaporative light-scattering [23] detection permits enhanced separation of Lyso-PLs containing different acyl chains from complex mixtures with high resolution. However, these quantitative techniques have low sensitivity and poor selectivity for Lyso-PLs. By contrast, electrospray ionization mass spectrometry (ESI-MS) stands out as suitable technique due to the easily ionizable nature of the polar head group of Lyso-PLs [24]. The soft ionization achieved using ESI-MS offers a series of advantages that make it an ideal technique for the analysis of biological samples. ESI-MS detects intact molecules with very high sensitivity, and ESI-tandem MS (ESI-MS/MS) permits the determination of the structural composition of the relevant ions. Thus, HPLC-ESI-MS/MS can lead to real and simultaneous identification and quantification of the many compounds present in a biological sample. As a result, this approach is currently used in studies evaluating the Lyso-PL content of experimental rodent and human samples [2,25,26]. The recent emergence of ultra-HPLC (UHPLC) with sub-2-µm column particles has enabled much greater operating pressures compared with HPLC, thus reducing analysis times without sacrificing efficiency [27,28]. Such properties are essential for clinical research and the evaluation of biomarkers since these studies require the evaluation of a large number of samples. Because Lyso-PEs are the Lyso-PLs more recently postulated as biomarkers in mammals, most of the present quantitative methods which are focused on Lyso-PLs have considered few or none molecular species within the lipid subclass.

Therefore, the main objective of the present study was to develop a rapid, sensitive and reproducible methodology based on UHPLC-ESI-MS/MS for the exhaustive characterization of Lyso-PCs and Lyso-PEs as part of the evaluation of the comprehensive biomarker in serum. First, circulating members belonging to the two lipid subclasses were identified in a pooled serum sample using a UHPLC coupled to a quadrupole time-of-flight mass spectrometer (UHPLC-Q-TOF). Second, a quantitative method was applied using two simple methanol-based procedures (to cover the entire concentration range of the metabolites of interest) and coupling of the UHPLC to a triple quadrupole mass spectrometer (UHPLC-QqQ). To validate the developed method, several quality parameters were determined by spiking the rat serum with different Lyso-PL standards. The suitability of the quantitative methodology was demonstrated by comparison of the circulating levels of Lyso-PLs found in two groups of rats with different diets. Subsequent multivariate data analysis of all identified Lyso-PLs was also conducted in both animal groups to evaluate the efficiency of the biomarker in serum.

2. Material and methods

2.1. Chemicals and reagents

Methanol (Scharlab, Barcelona, Spain), acetonitrile (Millipore, Darmstadt, Germany), isopropanol, glacial acetic acid (Panreac Applichem, Barcelona, Spain), chloroform and 7.5 M ammonium acetate solution (Sigma-Aldrich, St. Louis, MO, USA) were of the highest grade commercially available. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain). Lyso-PL standards were all of > 99% purity and were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Lyso-PC standards included 1-tridecanoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (13:0); 1-palmitoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (16:0); 1-stearoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (18:0); and 1-arachidoyl-*sn*-glycero-3-phosphocoline, Lyso-PC (20:0). Lyso-PE calibrators were 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine, Lyso-PE (16:0); 1-stearoyl-*sn*-glycero-3-phosphoethanolamine, Lyso-PE (18:0); and 1-oleoyl-*sn*-glycero-3-phosphoethanolamine, Lyso-PE (18:1). The standards contained a small proportion of the corresponding *sn2*-Lyso-PL isomer. The standards were individually dissolved in methanol/chloroform/water (65:35:8 v/v/v) at 2 mg/mL and stored in dark-glass vials at -20°C prior to use. On the day of LC-MS/MS analysis, a mixed standard solution with a concentration of 100 mg/L was prepared using methanol and diluted with water/isopropanol/acetonitrile (4:3:3 v/v/v) to the desired concentrations. Lyso-PC (13:0) was separately handled similarly to the standards for use as internal standard (IS). Butylated hydroxytoluene (BHT; Sigma-Aldrich, St. Louis, MO, USA) was used as an antioxidant.

2.2. Animal procedure

Serum samples were obtained from female Sprague-Dawley rats (3-month-old). The animals were maintained under standard conditions of temperature (22 ± 1°C) and relative humidity (50 ± 10%) with a light/dark period of 12 h. The rats were randomly divided into two groups (n = 5 per group) with free access to food and water. For 8 weeks, the animals were fed *ad libitum* either standard chow (ST) or a highly palatable diet rich in fat and carbohydrates known as the cafeteria diet (CAF) [14]. The CAF was renewed daily and consisted of the followings components (quantity per rat/day): bacon (8-12 g), biscuits with pâté (12-15 g) or cream cheese (10-12 g), sweet roll (8-10 g), carrot (6-8 g), milk with sugar (220 g/L; 50 mL) and ST chow. After overnight fasting, ST- and CAF-fed rats were alternately sacrificed by beheading to avoid interference due to chemical drugs, and total blood was collected in falcon tubes. Serum samples were obtained by allowing blood to clot at room temperature for 30 min. The samples were then centrifuged at 2,000 g and 4°C for 15 min, aliquoted and stored at -80°C until metabolite extraction and LC-MS/MS quantification. All

procedures were performed in accordance with the European Communities Council Directive regarding the protection of experimental animals (86/609/EEC).

2.3. Identification of Lyso-PCs and Lyso-PEs in rat serum

Serum volumes of 10 µL from each animal were pooled (n = 10) for determination of the specific members of Lyso-PCs and Lyso-PEs in rat serum. Metabolites were extracted from serum using a hydroalcoholic solution. Briefly, 900 µL of methanol/water (8:1 v/v) was added to 100 µL of pooled sample, vortexed (30 s) and ultrasonicated (30 s). The mixture was incubated on ice for 10 min and centrifuged (20,000 g, 10 min, 4°C). The supernatants were dried under nitrogen flow and re-dissolved in 200 µL of water/methanol (1:1 v/v) prior to injection.

The exhaustive characterization of the two circulating subclasses of lipids was performed using a UHPLC 1290 coupled to a Q-TOF 6550 Series mass spectrometer equipped with a Dual ESI source using Agilent Jet Stream Technology (AJS ESI) (Agilent Technologies, Palo Alto, CA, USA). The chromatographic section comprised a degasser, a binary pump, a thermostatted autosampler (held at 4°C) and a thermostatted column compartment. The mobile phase was 0.2% (v/v) acetic acid in water (solvent A) and 0.2% (v/v) acetic acid in methanol (solvent B). The flow rate was 0.6 mL/min. An injection volume of 2 µL was loaded onto a Zorbax SB-Aq (1.8 µm particle size, 2.1 mm internal diameter x 50 mm length) analytical column held at 60°C and equipped with a Zorbax SB-C8 Rapid Resolution Cartridge (3.5 µm, 2.1 x 30 mm) guard column, also from Agilent Technologies. Chromatographic separation was performed using a linear gradient of 2-98% B over 13 min, followed by a 3-min solvent B hold and 1 min to return to initial conditions. Thereafter, a post-time of 5 min was applied. To identify the greatest possible number of Lyso-PLs, the mass spectrometer was operated in both positive (+ESI) and negative (-ESI) ionization modes. The ESI source settings were as follows: nebulizer pressure 45 psi; desolvation gas flow rate 11 L/min at 325°C; source temperature and gas flow rate, 200°C and 14 L/min, respectively; capillary voltage 4 kV; and fragmentor 140 V. Data were acquired using MassHunter Data Acquisition (Agilent Technologies, version 6.0) over the 100 - 1200 m/z range at a rate of 1.5 spectra/s. Dynamic mass axis calibration was accomplished through continuous infusion of a reference mass solution: 121.0509 and 922.0098 m/z for +ESI and 119.0363 and 980.0164 m/z for -ESI. Molecules were tentatively identified by comparison of the exact mass with published data. The confirmation of the metabolites as Lyso-PC and Lyso-PE molecular species was achieved with the spectrometer operating in MS and MS/MS modes and by applying collision energies of 10, 20 and 40 V. In addition, six commercially available

standards were analyzed to verify the identity of the lipids. All identified serum Lyso-PLs were subsequently quantified by UHPLC-QqQ.

2.4. Sample preparation for exhaustive Lyso-PL quantification

After determination of the target Lyso-PLs, the sample treatment process was optimized. Lyso-PLs are found in a broad range of concentrations in the circulation. Consequently, it was necessary to use two slightly different procedures (named *Low Sample Volume* and *High Sample Volume*) to treat the serum samples and quantify all compounds.

The *Low Sample Volume* (LSV) procedure was developed to quantify the most abundant circulating Lyso-PLs. Briefly, 5 µL of serum and 4 µL of IS (20 µM) were added to 71 µL of cold methanol containing BHT (1 µM) as an antioxidant. The mixture was homogenized by vortexing (1 min) and ultrasonication (30 s). The samples were then incubated on ice for 10 min and centrifuged at 20,000 g and 4°C for 10 min. The supernatants were collected in dark-glass vials and analyzed immediately.

Additionally, *High Sample Volume* (HSV) extraction was developed for the quantification of the remaining Lyso-PLs. This procedure yielded serum extracts with higher concentrations of the desired analytes than the LSV method. In summary, 50 µL of serum and 2.5 µL of IS (20 µM) were added to 197.5 µL of cold methanol containing BHT (1 µM), vortexed (1 min) and ultrasonicated (30 s). Then, the samples were placed on ice for 10 min and centrifuged (22,000 g, 10 min, 4°C). The supernatants were dried under nitrogen flow and re-dissolved in 50 µL of water/isopropanol/acetonitrile (4:3:3 v/v/v) prior to injection.

2.5. Lyso-PL quantification: UHPLC-+ESI-MS/MS

Quantitative analysis of the target compounds was performed by coupling the UHPLC 1290 to a QqQ 6490 Series mass spectrometer operating in AJS +ESI (Agilent Technologies, Palo Alto, CA, USA). Solvent A consisted of water/isopropanol/acetonitrile/500 mM ammonium acetate (89:5:5:1 v/v/v/v), whereas solvent B was isopropanol/acetonitrile/500 mM ammonium acetate (50:49:1 v/v/v). A sample volume of 2 µL was applied to a reversed-phase column (Acquity UPLC BEH C8, 1.7 µm, 2.1 x 150 mm) (Waters Corporation, Milford, MA, USA) held at 50°C with a 0.3 mL/min flow of 30% B. The analytes were separated using linear gradient elution to 75% B over 20 min, followed by a linear increase in solvent B to 100% over 1 min and isocratic 100% B for 4 min. The chromatographic system was returned to the initial conditions in 2 min, followed by a 2-min equilibration prior to the subsequent injection. The total run time was 29 min. The ionization source parameters were optimized using MassHunter Optimizer (Agilent Technologies,

version 6.0) as follows: nebulizer gas (nitrogen) with a pressure of 25 psi; a gas flow of 12 L/min at 240°C; a sheath gas flow of 12 L/min at 350°C; a capillary voltage of 4.5 kV; and a nozzle voltage of 500 V. To obtain the highest abundances of the selected product ions, the fragmentor and cell accelerator voltages were also optimized and set to 380 and 5 V, respectively. The other operating parameters for the targeted quantification of Lyso-PLs are described in **Table 1**, including the optimal collision energies for each transition, which were determined by direct injection of standard solutions with 0.5 mL/min flow of solvent A / solvent B (1:1 v/v). $[M+H]^+$ ions were used as parent ions. For each analyte, the most abundant transition was selected for the subsequent quantification; the other two transitions were used for qualitative purposes. To further increase the sensitivity of the method, dynamic Multiple Reaction Monitoring (MRM) was selected as acquisition mode. The MRM transition list was dynamically created using a window of 1 min around the expected retention times (RTs). The cycle time was fixed to 750 ms, and the dwell time ranged from 27.50 to 373.92 ms.

Standard calibration curves were established for the quantitative analysis of endogenous Lyso-PLs. Increasing concentrations of the standards were diluted with constant final volumes of water/isopropanol/acetonitrile (4:3:3 v/v/v) in the presence of the IS. The standard concentrations ranged from 0 to 5 mg/L, whereas the IS was added at a final concentration of 450 or 325 µg/L depending on the later procedure of extraction. Then, the mixtures were extracted and analyzed using the same procedure as for the serum samples. Peak detection and integration were performed using MassHunter Quantitative Analysis (Agilent Technologies, version 6.0). Calibration curves for each standard were generated by plotting the peak abundance ratios (analyte / IS) versus the concentration ratios (analyte / IS) and fitting to a linear regression. As described in **Table 1**, the concentration of each circulating Lyso-PLs was calculated by using the closest related calibration curve, according to a previously described procedure [24]. For the two described extractions of the sample, the method detection and quantification limits (MDL and MQL, respectively) were calculated based on the respective instrumental limits of detection (3 times the signal / noise ratio) and quantification (10 times the signal / noise ratio).

2.6. Quality parameters

Other quality parameters were determined to validate and evaluate the suitability of the developed quantitative method. The percentages of recovery, precision, repeatability, accuracy and matrix effect were determined by spiking pooled serum samples from ST- or CAF-fed animals with standard solutions. Moreover, two different concentration levels of Lyso-PLs (0.1 and 1 mg/L) were studied. The recovery percentages were calculated by

comparing the resulting abundances of a standard added before or after the extraction of the pooled samples. The method variation was analyzed in triplicate and considered as the relative standard deviation (% RSD) of the concentration in spiked serum samples that were randomly distributed intraday (precision) and on three different days (repeatability). The method accuracy was assessed by comparing the mean concentration of three spiked, pooled, pre-treated samples with the value of the commercial standard at this concentration. Finally, the matrix effects were determined as the ratio of the difference between the abundances obtained by spiking pooled samples after extraction and the blank pooled sample versus the abundance of the diluted standard at the same concentration.

2.7. Statistical evaluation

Statistical analysis was performed with Statistical Package for Social Sciences (IBM SPSS Statistics, version 19.0). The serum concentration of Lyso-PLs is expressed as the mean ± standard error (SEM). The differences among the two animal groups were assessed using Student's t-test. A two-tailed value of $p < 0.05$ was considered statistically significant. Principal component analysis (PCA) and multivariate biomarker validation using receiver operating characteristic (ROC) curves were performed using the online software MetaboAnalyst 3.0 [29].

Table 1. Target lysophospholipids (Lyso-PLs) and abbreviations, molecular formula and weight, retention time, transitions and optimal collision energies used for UHPLC-QqQ analysis

Compound	Lipid abbreviation	Molecular formula	Molecular weight	RT (min)	Parent ion (m/z)	Daughter ions (m/z)	Collision energies (V)
Lysophosphatidylcholines							
1-tridecanoyl-sn-glycero-3-phosphocholine [IS]	Lyso-PC (13:0)	$C_{21}H_{44}NO_7P$	453.55	8.9	454.3	184.0*, 104.1, 86.0	24, 28, 40
1-myristoyl-sn-glycero-3-phosphocholine [A]	Lyso-PC (14:0)	$C_{22}H_{46}NO_7P$	467.58	10.3	468.3	184.0*, 104.1, 86.0	24, 28, 40
1-pentadecanoyl-sn-glycero-3-phosphocholine [A]	Lyso-PC (15:0)	$C_{23}H_{48}NO_7P$	481.60	11.6	482.3	184.0*, 104.1, 86.0	24, 28, 40
1-palmitoyl-sn-glycero-3-phosphocholine [A]	Lyso-PC (16:0)	$C_{24}H_{50}NO_7P$	495.63	13.0	496.3	184.0*, 104.1, 86.0	24, 28, 40
1-palmitoleyl-sn-glycero-3-phosphocholine [A]	Lyso-PC (16:1)	$C_{24}H_{48}NO_7P$	493.61	11.1	494.3	184.0*, 104.1, 86.0	24, 28, 40
1-heptadecanoyl-sn-glycero-3-phosphocholine [B]	Lyso-PC (17:0)	$C_{25}H_{52}NO_7P$	509.66	14.3	510.3	184.0*, 104.1, 86.0	32, 28, 40
1-heptadecenoyl-sn-glycero-3-phosphocholine [B]	Lyso-PC (17:1)	$C_{25}H_{50}NO_7P$	507.64	12.4	508.3	184.0*, 104.1, 86.0	32, 28, 40
1-stearoyl-sn-glycero-3-phosphocholine [B]	Lyso-PC (18:0)	$C_{26}H_{54}NO_7P$	523.68	15.5	524.3	184.0*, 104.1, 86.0	32, 28, 40
1-oleoyl-sn-glycero-3-phosphocholine [B]	Lyso-PC (18:1)	$C_{26}H_{52}NO_7P$	521.67	13.7	522.3	184.0*, 104.1, 86.0	32, 28, 40
1-linoleoyl-sn-glycero-3-phosphocholine [B]	Lyso-PC (18:2)	$C_{26}H_{50}NO_7P$	519.65	11.9	520.3	184.0*, 104.1, 86.0	32, 28, 40
1-linolenoyl-sn-glycero-3-phosphocholine [B]	Lyso-PC (18:3)	$C_{26}H_{48}NO_7P$	517.64	10.7	518.3	184.0*, 104.1, 86.0	32, 28, 40
1-arachidoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (20:0)	$C_{28}H_{58}NO_7P$	551.74	17.8	552.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosenoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (20:1)	$C_{28}H_{56}NO_7P$	549.72	15.9	550.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosadienoyl-sn-glycero-3-phosphocholine [C]	LysoPC (20:2)	$C_{28}H_{54}NO_7P$	547.70	14.2	548.3	184.0*, 104.1, 86.0	28, 28, 40
1-eicosatrienoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (20:3)	$C_{28}H_{52}NO_7P$	545.69	12.9	546.3	184.0*, 104.1, 86.0	28, 28, 40
1-arachidonoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (20:4)	$C_{28}H_{50}NO_7P$	543.67	11.9	544.3	184.0*, 104.1, 86.0	28, 28, 40

Compound	Abbreviation	Formula	Mass	RT	Precursor	Transitions	CE
1-eicosapentaenoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (20:5)	$C_{28}H_{48}NO_7P$	541.66	10.4	542.3	184.0*, 104.1, 86.0	28, 28, 40
1-docosapentaenoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (22:5)	$C_{30}H_{52}NO_7P$	569.71	13.1	570.3	184.0*, 104.1, 86.0	28, 28, 40
1-docosahexaenoyl-sn-glycero-3-phosphocholine [C]	Lyso-PC (22:6)	$C_{30}H_{50}NO_7P$	567.69	11.7	568.3	184.0*, 104.1, 86.0	28, 28, 40
Lysophosphatidylethanolamines							
1-palmitoyl-sn-glycero-3-phosphoethanolamine [D]	Lyso-PE (16:0)	$C_{21}H_{44}NO_7P$	453.55	13.3	454.3	313.3*, 436.3, 62.0	16, 12, 12
1-palmitoleoyl-sn-glycero-3-phosphoethanolamine [D]	Lyso-PE (16:1)	$C_{21}H_{42}NO_7P$	451.53	11.3	452.3	311.3*, 434.3, 62.0	16, 12, 12
1-stearoyl-sn-glycero-3-phosphoethanolamine [E]	Lyso-PE (18:0)	$C_{23}H_{48}NO_7P$	481.60	15.8	482.3	341.3*, 464.3, 62.0	16, 12, 12
1-oleoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (18:1)	$C_{23}H_{46}NO_7P$	479.59	14.0	480.3	339.3*, 462.3, 62.0	16, 12, 12
1-linoleoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (18:2)	$C_{23}H_{44}NO_7P$	477.57	12.2	478.3	337.3*, 460.3, 62.0	16, 12, 12
1-linolenoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (18:3)	$C_{23}H_{42}NO_7P$	475.56	10.6	476.3	335.3*, 458.3, 62.0	16, 12, 12
1-eicosenoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (20:1)	$C_{25}H_{50}NO_7P$	507.64	16.1	508.3	367.3*, 490.3, 62.0	16, 12, 12
1-eicosadienoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (20:2)	$C_{25}H_{48}NO_7P$	505.62	14.5	506.3	365.3*, 488.3, 62.0	16, 12, 12
1-eicosatrienoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (20:3)	$C_{25}H_{46}NO_7P$	503.61	13.1	504.3	363.3*, 486.3, 62.0	16, 12, 12
1-arachidonoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (20:4)	$C_{25}H_{44}NO_7P$	501.59	12.2	502.3	361.3*, 484.3, 62.0	16, 12, 12
1-docosatetraenoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (22:4)	$C_{27}H_{48}NO_7P$	529.65	14.1	530.3	389.3*, 512.3, 62.0	16, 12, 12
1-docosapentaenoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (22:5)	$C_{27}H_{46}NO_7P$	527.63	13.4	528.3	387.3*, 510.3, 62.0	16, 12, 12
1-docosahexaenoyl-sn-glycero-3-phosphoethanolamine [F]	Lyso-PE (22:6)	$C_{27}H_{44}NO_7P$	525.61	11.9	526.3	385.3*, 508.3, 62.0	16, 12, 12

[IS] Internal Standard.

[A, B, C, D, E, F] Serum concentration was calculated with Lyso-PC (16:0), Lyso-PC (18:0), Lyso-PC (20:0), Lyso-PE (16:0), Lyso-PE (18:0), Lyso-PE (18:1) calibration curve, respectively.

* Transitions for quantification.

3. Results

3.1. Development of an optimized methodology for the evaluation of serum Lyso-PLs

After characterization of the lysophospholipidome in rat serum, eighteen Lyso-PCs and thirteen Lyso-PEs were tentative identified. The chromatographic peaks eluted within a range of 10.8 - 12.7 min (data not shown). The Lyso-PCs were identified in the +ESI analysis with *m/z* values between 468.3048 and 570.3512, whereas the Lyso-PEs were detected in both ionization modes. During the process of Lyso-PE identification, we observed that one of the major product ions in the +ESI fragmentation corresponded to the loss of the phosphorylethanolamine group, with a mass of 141 ($C_2H_8NO_4P$). In –ESI, although the polar head of Lyso-PEs is easily ionizable, with an *m/z* 140, the acyl chain is the predominant anionic fragment [8,30]. Thus, different ions from the fragmentation of Lyso-PEs can be observed in each ionization mode. As an example, **Fig. 1** shows the MS/MS spectra of a Lyso-PE species at a given collision energy in both ionization modes. Due to the ability of Lyso-PEs and their characteristic fragments to be ionized in both modes, we decided to quantify all identified Lyso-PLs in a unique +ESI-MS/MS analysis performed by UHPLC-QqQ.
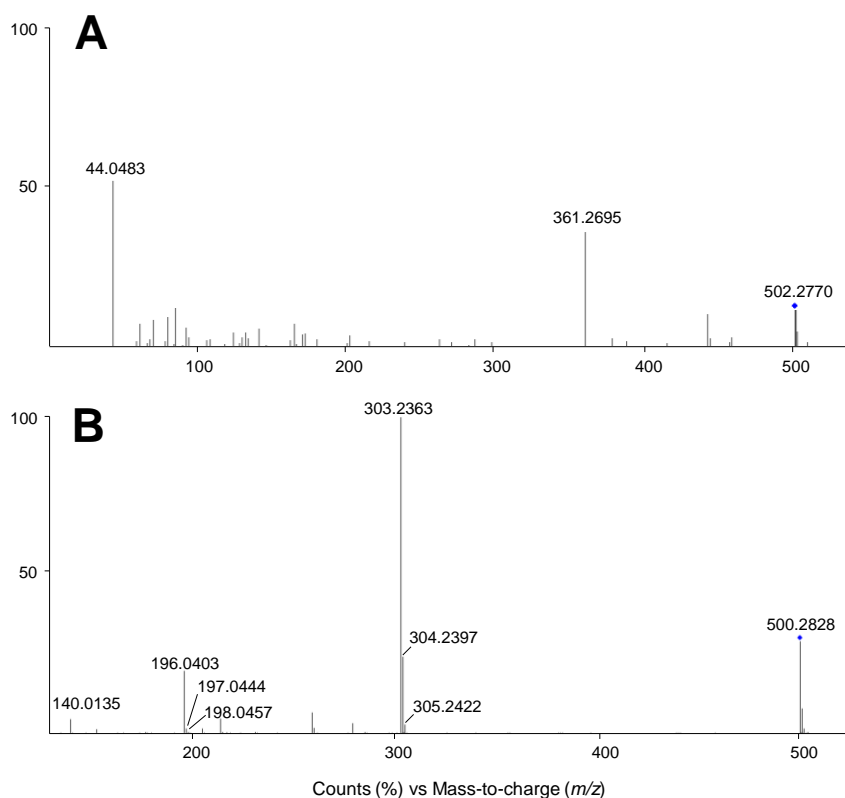
Figure 1. ESI-MS/MS spectra acquired by UHPLC-Q-TOF corresponding to Lyso-PE (20:4) at a collision energy of 20 V. (**A**) In positive ionization mode, the major peaks identified were the parent ion (m/z 502), the ethylamine ion (m/z 44) and the ion corresponding to loss of the phosphorylethanolamine head group (m/z 361). (**B**) In negative mode, the parent ion (m/z 500), the arachidonic acid side chain (m/z 303), the released phosphorylethanolamine group (m/z 140) and the propylene glycol phosphorylethanolamine ion (m/z 196) were identified.

The development of the quantitative method started with the determination of the most abundant Lyso-PL transitions and their collision energies (**Table 1**). For all analytes, [M+H]$^+$ ions were selected as precursor ions. Consistent with the observations of Liebisch *et al.*, the fragmentation of Lyso-PCs generated common product ions with the following *m/z* values: 184.0 (the phosphocholine ion); 104.1 (the choline ion); and 86.0 (the choline subsequently fragmented) [24]. By contrast, the optimal fragmentation patterns of Lyso-PEs required lower collision energies and differed from each other since they tend to lose the phosphorylethanolamine group and a molecule of water [30]. Only *m/z* 62.0 (the ethanolamine ion) was preserved in all Lyso-PEs because it is the only ion that does not come from the radical chain, which has a variable mass. As **Table 1** indicates, the abundant transitions *[M+H]$^+$ > 184* and *[M+H]$^+$ > [M-141+H]$^+$* were used for the quantification of Lyso-PCs and Lyso-PEs, respectively. By monitoring these quantitative transitions in a mixture of four standards, the solvent gradient was optimized using a 0.3 mL/min flow held at 50°C (**Fig. 2**). Starting with the chromatographic conditions described in Donovan *et al.* [31] (**Fig. 2A**), the proportion of solvents (dashed line) was modified to achieve baseline separation between the different standards and distinction of less abundant isomeric forms (**Fig. 2C**). In that final chromatogram, all of the peaks displayed suitable efficiency and symmetry. Lyso-PC (16:0) eluted first, followed by Lyso-PE (18:1) and Lyso-PCs (18:0) and (20:0) in that order. Hence, the elution of the Lyso-PLs depended on the length and number of double bonds of the acyl chain rather than the nature of the polar head group. In addition, the *sn1*-Lyso-PLs were retained by the column longer than the *sn2*-isomers, as shown previously [25]. For further analysis of multiple samples, the 100% B hold was extended to 4 min to ensure clearance of the chromatographic column. In addition, the MRM scan type was switched to dynamic mode. Thus, the transitions were monitored only around the expected RT allowing to reach a suitable sensitivity for the quantification of the multiple molecular species of Lyso-PLs identified in serum. The RT selected for the acquisition of each analyte was the RT of the second peak ± 0.5 min, that is, *sn1*-Lyso-PL. Representative chromatograms from the Lyso-PL analyses of both a standard solution and a serum sample are shown in **Figs. 3 and 4**, respectively.
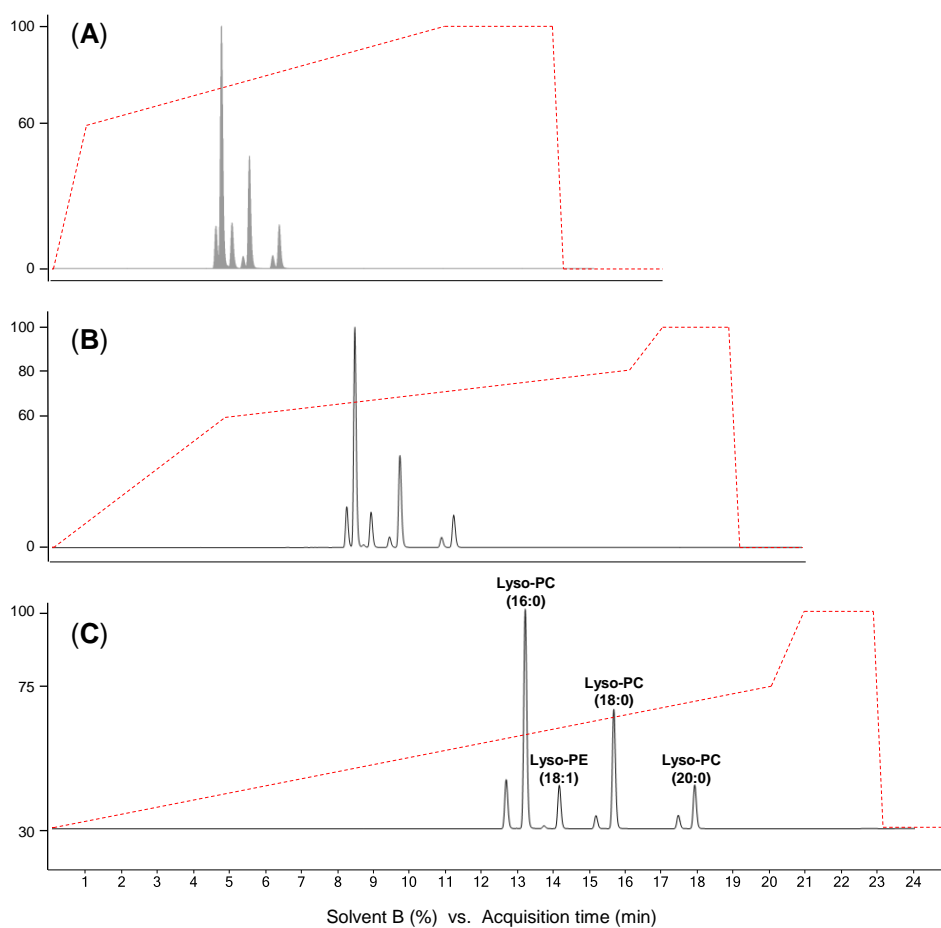
Figure 2. Optimization of the solvent gradient. Total ion chromatograms from UHPLC-QqQ analysis of Lyso-PL standards at different run times and proportions of the solvents. (**A**) Chromatographic conditions obtained from Donovan *et al.* (**B**) Intermediate conditions. (**C**) Optimized conditions.

As shown in **Fig. 3**, the sensitivity levels were greater for the analysis of Lyso-PCs than for Lyso-PEs. The signals from Lyso-PEs were approximately 25% of those from the Lyso-PCs. This difference is related to the highest values of slope obtained in the calibration curves of Lyso-PCs and is in agreement with the limits of the UHPLC-+ESI-MS/MS method (**Table 2**), which are generally greater for Lyso-PE quantification than for Lyso-PC. The slopes of the Lyso-PC and Lyso-PE standard curves were very similar, including that of the unsaturated Lyso-PE (18:1), and displayed a variation coefficient of 6% and 17%, respectively. The calibration curves for the Lyso-PLs were linear over the concentration ranges analyzed (**Table 2**), and all equations displayed excellent data fit ($R^2 > 0.999$). The samples could not be prepared in the biological matrix since the constitutive levels of the serum compounds were too high. Consequently, the parameters describing method performance (**Table 3**) could not be evaluated for the most concentrated circulating forms, Lyso-PCs (16:0) and (18:0) (**Fig. 4A**).

After testing various proportions of sample and methanol (data not shown), 16- and 1.25-fold dilutions with respect to the initial serum volume were selected to cover the whole range of circulating levels of Lyso-PLs. **Table 4** displays the specific identified Lyso-PLs analyzed from each different extraction. Lyso-PCs were mainly quantified in LSV extracts, and Lyso-PEs were mainly quantified after the HSV procedure. The recoveries, intra and interday variations, accuracies and matrix effects of the quantitative method are shown in **Table 3**. For the LSV extraction, all analyzed parameters gave satisfactory results, and no considerable differences were noted between the pooled sera of ST- and CAF-fed rats. The recoveries varied between 98 and 117%, and the % RSD ranged from 0.8 to 9.4 and 2.7 to 6.4 for intraday and interday studies, respectively. The optimum recoveries (98 - 105%) and the largest variations were observed for the highest spiked concentration of 1 mg/L, but the method accuracy remained nearly 100% at this concentration, and the matrix effect was minimal for both levels of concentration. The performance of the Lyso-PE (18:1) standard could be determined for the HSV treatment only because this form represents the minor circulating Lyso-PL among the used standards. Consequently, its quality results should be extrapolated to the analysis of the other, even less abundant Lyso-PEs in quantification. Whereas the recovery and variations of Lyso-PE (18:1) were similar in both extractions, the percentages of accuracy and, in particular, the matrix effect were worse for HSV than for the LSV procedure. Therefore, the HSV extraction must be used only to assist the complete quantification of Lyso-PLs by enabling the measurement of those with the lowest levels, mainly Lyso-PEs. Thus, the MDL and MQL for Lyso-PEs were approximately 0.3 and 1.1 nmol/L for the HSV procedure and 1.3 and 4.3 for LSV, respectively (**Table 2**).
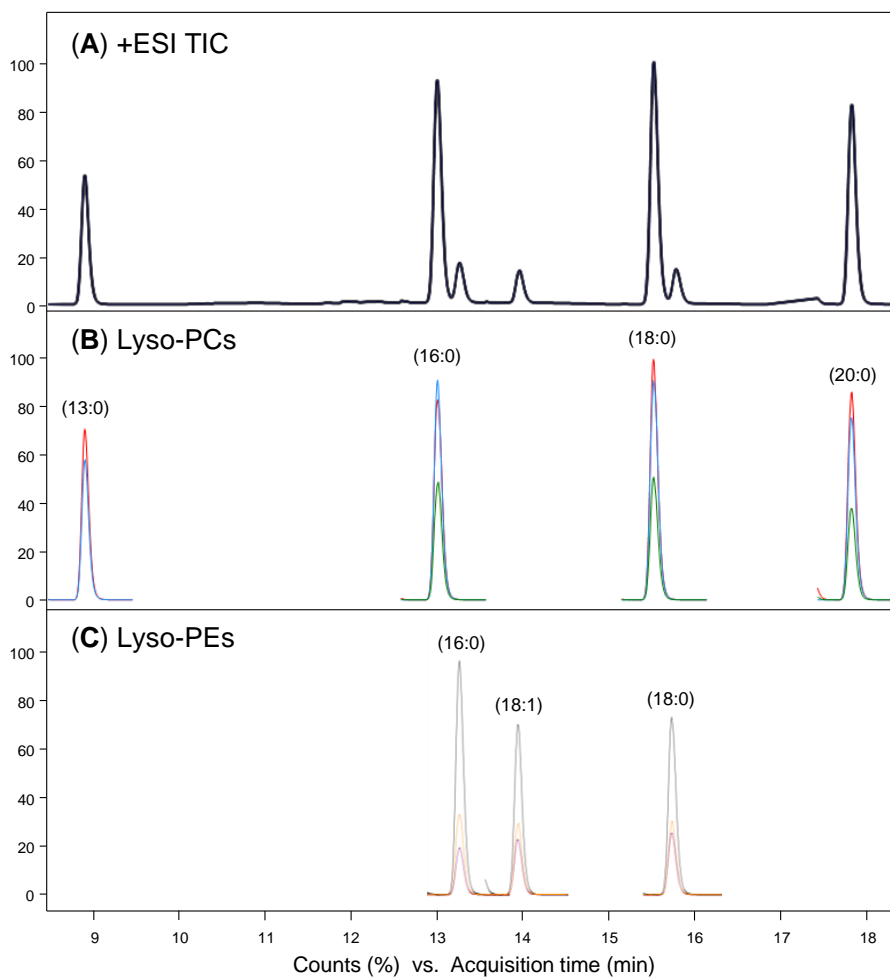
Figure 3. Transition abundances at an intermediate point of the standard curve of Lyso-PLs. (**A**) Total ion chromatogram and (**B**, **C**) extracted ion chromatograms of Lyso-PCs and Lyso-PEs, respectively. The standards were diluted with water/isopropanol/acetonitrile (1 mg/L) in the presence of the internal standard (0.45 mg/L) and processed similarly to the samples. The acquisition mode was dynamic MRM with a RT window of 1 min.

Table 2. Calibration curves, regression coefficients (R^2), working linear ranges and method limits of detection and quantification (MDL and MQL) for the analysis of Lyso-PCs and Lyso-PEs

Compound[a]		Calibration curve	R^2	Working linear range (nM)	MDL[b] (nM)	MQL[b] (nM)
Lyso-PC	**(16:0)**	y = 0.7295x - 0.0135	0.999	0.1 – 10,088	0.48	1.60
	(18:0)	y = 0.7595x + 0.0090	1.000	0.1 – 9,548	0.30	1.01
	(20:0)	y = 0.6729x + 0.0064	0.999	0.3 – 9,062	1.50	5.00
Lyso-PE	**(16:0)**	y = 0.2005x + 0.0007	1.000	0.2 – 6,615	1.06 / 0.24	3.53 / 0.81
	(18:0)	y = 0.1524x + 0.0007	1.000	0.2 – 6,229	1.11 / 0.35	3.69 / 1.15
	(18:1)	y = 0.1495x - 0.0001	0.999	0.3 – 4,170	1.67 / 0.38	5.56 / 1.26

[a] Lipid nomenclature: Monoacylglycerophospholipids such as Lyso-PCs and Lyso-PEs are composed of acyl chains of different lengths and unsaturation degrees. The first value shown in parentheses represents the number of carbon atoms of the acyl chain, whereas the second value denotes the number of double bonds.

[b] Method detection and quantification limits are expressed in nmol/L of fresh sample. The limits were calculated for the quantification of Lyso-PCs and Lyso-PEs from 5 µL of serum (LSV procedure). Low abundant circulating Lyso-PEs were quantified starting from 50 µL of fresh sample (HSV procedure). Lyso-PE limits from HSV extracts are shown after those from LSV (LSV / HSV).

Table 3. Quality parameter percentages of the quantitative method used for the determination of Lyso-PCs and Lyso-PEs

Compound	Spiked concentration (mg/L)	Recovery		Precision[a]		Repeatability[b]	Accuracy		Matrix effect	
		ST	CAF	ST	CAF	CAF	ST	CAF	ST	CAF
Lyso-PC (20:0)	**0.1**	114	109	0.8	3.1	3.0	94	86	89	88
	1	98	101	9.1	6.2	6.4	105	99	81	85
Lyso-PE (16:0)	**0.1**	113	115	2.7	4.5	3.7	111	115	84	75
	1	101	103	7.5	5.8	4.5	113	99	77	82
Lyso-PE (18:0)	**0.1**	117	117	2.9	2.6	2.8	118	116	67	61
	1	105	105	8.0	6.5	5.2	120	115	70	78
Lyso-PE (18:1)	**0.1**	109	112	2.3	3.0	2.7	115	105	83	74
	1	99 / 93	102 / 98	9.4 / 8.1	6.1 / 6.2	4.9 / 6.2	96 / 71	87 / 79	75 / 50	80 / 57

The percentages of recovery, precision, repeatability, accuracy and matrix effect were determined by spiking pooled serum samples from standard (ST)- or cafeteria (CAF)-fed animals with standard Lyso-PLs. Two concentration levels (0.1 and 1 mg/L) were assessed. The constitutive concentrations of Lyso-PCs (16:0) and (18:0) in serum were too high to calculate any parameters. For similar reasons, the method quality parameters were evaluated only for the LSV pre-treatment of the sample, except in the Lyso-PE (18:1) analysis, for which the estimation of the HSV procedure could also be performed at the highest level of the standard concentration (LSV / HSV).

[a] RSD (%, n = 3) of intraday variation.

[b] RSD (%, n = 9) of interday variation (3 days).
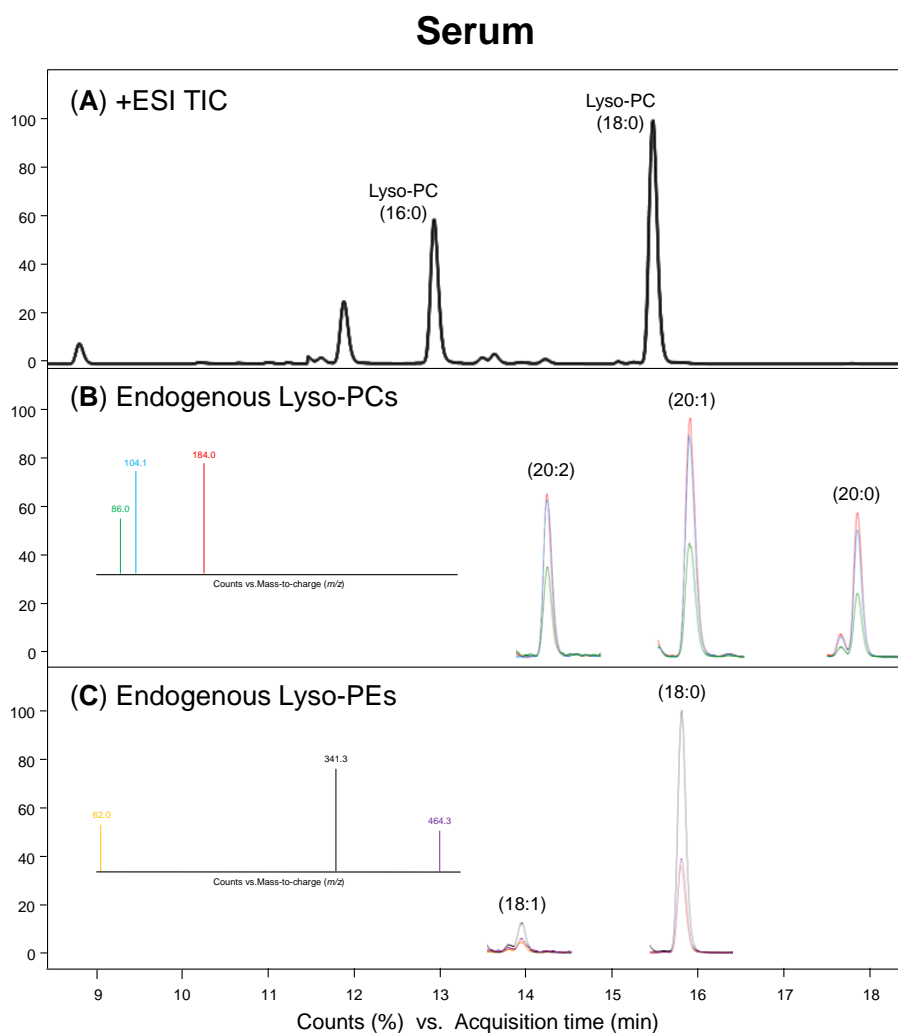
Serum

Figure 4. Transition abundances of circulating Lyso-PLs. (**A**) Total ion chromatogram from Lyso-PL analysis of a serum sample showing the most concentrated forms. (**B**, **C**) Extracted ion chromatograms of some endogenous Lyso-PCs and Lyso-PEs, respectively. The daughter ions from the joint fragmentation of Lyso-PCs and the spectra of a specific Lyso-PE containing the (18:0) acyl chain are also shown. The acquisition mode was dynamic MRM with a RT window of 1 min.

Table 4. Circulating levels of the identified Lyso-PLs expressed in micromolarity (µM)

Lyso-PL class	Acyl chain	Animal group (n = 5)			Lyso-PL class	Acyl chain	Animal group (n = 5)		
		ST	CAF	p-value			ST	CAF	p-value
	(14:0)	2.076 ± 0.056	2.907 ± 0.222	0.007					
	(15:0)	1.503 ± 0.100	1.237 ± 0.060	0.052		(16:0)	4.561 ± 0.279	4.615 ± 0.057	0.860
	(16:0)	154.173 ± 0.529	151.653 ± 2.456	0.345		(16:1) HSV	0.030 ± 0.002	0.047 ± 0.002	< 0.001
	(16:1)	2.302 ± 0.099	4.281 ± 0.462	0.003					
	(17:0)	3.322 ± 0.223	2.605 ± 0.171	0.034					
	(17:1) HSV	0.111 ± 0.007	0.254 ± 0.008	< 0.001					
	(18:0)	221.317 ± 15.612	231.646 ± 11.577	0.609		(18:0)	9.198 ± 0.380	10.508 ± 0.509	0.073
	(18:1)	10.806 ± 0.211	21.415 ± 1.665	< 0.001		(18:1)	1.111 ± 0.060	1.320 ± 0.060	0.039
	(18:2)	31.985 ± 1.111	20.759 ± 0.266	< 0.001		(18:2)	1.059 ± 0.026	0.616 ± 0.097	0.006
Choline	(18:3)	0.413 ± 0.011	0.374 ± 0.083	0.665	Ethanolamine	(18:3) HSV	0.023 ± 0.001	0.009 ± 0.002	< 0.001
	(20:0)	0.506 ± 0.019	0.293 ± 0.020	< 0.001					
	(20:1)	1.328 ± 0.121	0.833 ± 0.070	0.008		(20:1) HSV	0.048 ± 0.003	0.053 ± 0.009	0.599
	(20:2)	0.569 ± 0.028	0.163 ± 0.017	< 0.001		(20:2) HSV	0.033 ± 0.003	0.016 ± 0.003	0.002
	(20:3)	0.806 ± 0.104	1.586 ± 0.099	0.001		(20:3) HSV	0.027 ± 0.001	0.038 ± 0.003	0.028
	(20:4)	48.629 ± 2.998	58.608 ± 1.804	0.021		(20:4)	1.059 ± 0.046	1.626 ± 0.144	0.006
	(20:5)	0.129 ± 0.046	0.274 ± 0.051	0.069					
	(22:5)	0.536 ± 0.125	0.738 ± 0.058	0.197		(22:4) HSV	0.050 ± 0.002	0.041 ± 0.004	0.089
	(22:6)	6.587 ± 1.235	6.176 ± 0.355	0.763		(22:5) HSV	0.041 ± 0.004	0.054 ± 0.008	0.155
						(22:6)	0.346 ± 0.043	0.343 ± 0.011	0.937

Rats were fed standard chow (ST) or cafeteria diet (CAF) for 2 months. The serum levels of Lyso-PLs were quantified at the end of the experiment after an overnight fast (12 h). The quantitative analysis of Lyso-PLs required the use of 5 µL of serum for the LSV procedure. As indicated in the table, the determination of minor Lyso-PLs required the use of 50 µL of sample for the HSV extraction. The data are presented as means ± SEM (n = 5 per group). Statistical comparisons between the two groups of animals were performed using Student's t-test. A two-tailed value of p < 0.05 was considered statistically significant.

3.2. Circulating levels of Lyso-PLs found in rats fed different diets

After validation, the analytical method was used for the quantification of Lyso-PL levels in rat serum. **Table 1 and Fig. 4** show the chromatographic and spectrometric results of the quantitative method in a rat serum sample. Three transitions were monitored for each analyte. Endogenous Lyso-PLs eluted in increasing order of carbon atoms, and Lyso-PLs of identical length eluted by decreasing number of double bonds in the side chain. Because of the association between endogenous Lyso-PLs and the intake of diets rich in fats [12,13], circulating levels were compared between animals fed two different diets (ST or CAF) (**Table 4**). Following the order of abundance, the rat serum was particularly rich in Lyso-PCs containing the acyl chains (18:0) > (16:0) >> (20:4) > (18:2) > (18:1). In general, the circulating amounts of Lyso-PEs were lower, but the distribution of their abundances was similar to that of Lyso-PCs, and the saturated Lyso-PEs with 18 and 16 carbon atoms were by far the most abundant species. The results also showed numerous significant changes in Lyso-PCs and Lyso-PEs among both dietary groups. It is remarkable that the two subclasses of lipids exhibited similar trends depending on the structure of the hydrocarbon chain. This trend could be clearly observed in the significantly increased (16:1), (18:1), (20:3) and (20:4) and decreased (18:2) and (20:2) Lyso-PL levels in CAF- compared to ST-fed rats. These results indicated that the developed UHPLC-+ESI-MS/MS method can be successfully applied for the quantification of endogenous changes in Lyso-PL levels in response to chronic intake of CAF.

3.3. Lyso-PL examination as a suitable biomarker of nutritional phenotype

To simplify the large amount of data, multivariate analysis based on PCA and ROC curve evaluation including the thirty-one quantified Lyso-PLs was performed (**Fig. 5**). The unsupervised PCA analysis revealed clear clustering between the two dietary groups that explained 70% of the variance when the scores of the first two principal components were employed (**Fig. 5.A**). Furthermore, the predictive capacity of the method was assessed using ROC curve analysis (**Fig. 5.B**). The results indicated excellent accuracy (100%) of the model, with an area under the curve (AUC) of 1. An expanded version of the multivariate analysis can be found in the **Supplementary material**. Our results demonstrated that the comprehensive examination of circulating Lyso-PL levels represents an excellent biomarker of the nutritional phenotype of CAF-fed rats, thus highlighting the need for a method specifically developed to quantify Lyso-PLs.

Score plot

ROC curve
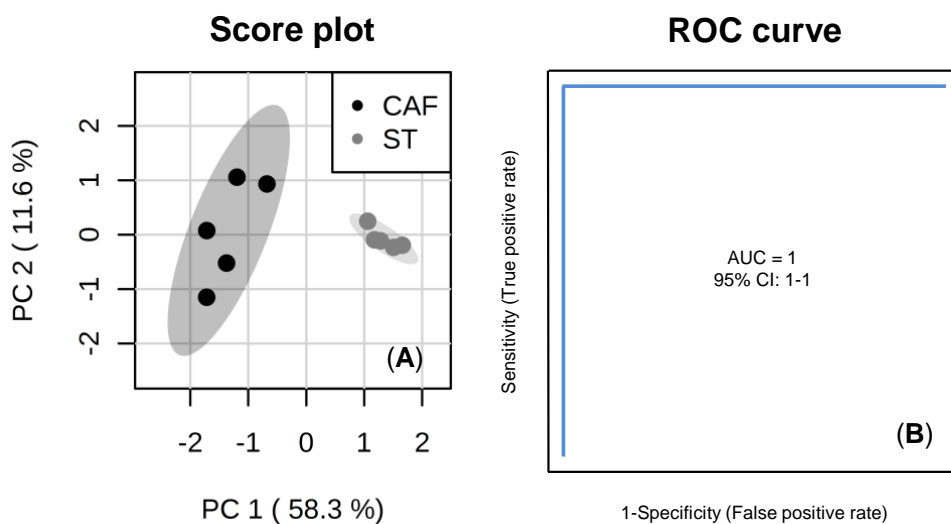
Figure 5. Multivariate analysis of the Lyso-PL biomarker model. (**A**) PC1/PC2 scores plot from principal component analysis of rats fed standard chow (ST) or cafeteria diet (CAF) (n = 5 per group). The percentages in parentheses indicate the variance explained by each component. The ellipses mark the 95% confidence interval (CI). (**B**) ROC curve analysis exploring the suitability of the multivariate biomarker. The precision of the model was measured using the area under the curve (AUC) and the corresponding 95% CI.

4. Discussion

Direct flow injection coupled to mass spectrometry has been applied by many researchers for the quantification of lyso compounds [17,24,32]. Most of them use TLC before sample injection to separate the lipids into classes [17,24], but this methodology has certain disadvantages. The use of chromatographic columns allows more efficient separation of lipid compounds and, consequently, high peak resolution [33–35]. The UHPLC-+ESI-MS/MS method described here assisted the separation and quantification of eighteen Lyso-PCs and thirteen Lyso-PEs of endogenous origin, minimizing ion suppression between different molecular species and matrix components. This is particularly important in the analysis of circulating Lyso-PLs, which are present in a wide concentration range. The optimized parameters used in our UHPLC system even allowed baseline separation of isomeric forms. Although this work focused on the determination of 1-acyl-Lyso-PLs (*sn1*-Lyso-PLs), it would be possible to apply the method to quantitative studies of both isomers because the *sn2*-isomer always elutes first. In agreement with the observations of Lee *et al.*, the Lyso-PLs eluted according to the length of the acyl chain and the degree of saturation rather than the nature of their polar group [30].

In our study, Lyso-PLs of the same family had similar slopes, particularly those containing acyl chains of equal length, regardless of the number of double bonds (**Table 2**). This is in accordance with the results obtained by Liebisch *et al.* when comparing Lyso-PCs of different chain lengths [24]. Based on these findings and the lack of commercially available standards for some endogenous Lyso-PLs, circulating unsaturated Lyso-PLs were quantified using the closest saturated standard curve. Other researchers have conducted detailed evaluations of Lyso-PLs in rodent serum [8,25], but the present method permits the quantification of a greater number of Lyso-PCs and Lyso-PEs. The disadvantages of those methods mainly pertained to lyso forms containing ethanolamine as polar group, which are often present in lower circulating amounts than Lyso-PCs, with serum levels of several hundred micromolar [2,25]. According to Bollinger *et al.*, the most abundant serum Lyso-PCs are those with saturated acyl chains of 18 and 16 carbons, as well as those with (18:2), (18:1) and (22:6) side chains, albeit in much lower quantities [8]. However, we also observed that Lyso-PC (20:4) was further concentrated in serum than (18:2), probably due to differences between rodent species (**Table 4**). Another metabolomics study conducted in rats showed that the serum level of Lyso-PC (20:4) is similar to that of Lyso-PC (18:1) and decreased when the diet was supplemented with a natural product with hypocholesterolemic effects [2]. This finding suggests that Lyso-PC (20:4) could be particularly influenced by the diet composition. In our study, Lyso-PC (20:4) was one of the quantified Lyso-PCs whose serum levels increased significantly in rats fed the pro-dyslipidemic CAF diet. In fact, CAF induced dysregulations in the levels of the majority of Lyso-PCs and Lyso-PEs in the present study. These results are comparable to those obtained in a previous work in which we identified several metabolites, including Lyso-PLs, whose circulating levels differed between rats fed ST and CAF [14]. These targeted and non-targeted studies are consistent since the serum amounts of Lyso-PCs containing (16:1) and (17:1) acyl chains increased and those with (20:1) and (20:2) acyl chains decreased in CAF-fed animals. The present methodology enables the identification of many more significant changes related to Lyso-PLs compared with non-targeted techniques.

The solvent chosen for treating the serum samples, methanol, was effective in the extraction of the majority of polar lipids present in biological samples and was compatible for ESI-MS/MS analysis [33,34]. Other procedures commonly used for lipid extraction include modifications of the Folch [36] or Bligh and Dyer [37] methods. These traditional techniques involve the formation of two organic phases and promote the increased enrichment of the chloroform phase with hydrophobic lipids in contrast to Lyso-PLs. Alternative methodologies have recently emerged, such as the methyl-*tert*-butyl ether (MTBE) [17] or butanol-methanol (BUME) [38] procedures, but these chloroform-free lipid extractions are more time-consuming than the methanol treatment. The recoveries of nearly 100% indicate that our procedure is

adequate for the analysis of choline and ethanolamine containing Lyso-PLs without requiring a tedious extraction process and using low sample volumes (5-50 µL). In addition, the simplicity of the procedure allowed good reproducibility between analyses, which is essential for the quantitative evaluation of multiple samples.

The validation study also showed excellent accuracies and slight matrix effects in the spiked samples, indicating that this methodology enabled real quantification of lipids in serum. The lack of difference in quality parameters between ST and CAF pooled samples suggests that this method is also useful for accurate comparisons of different dietary groups and for the evaluation of Lyso-PLs as biomarkers of nutrition and disease. The results obtained in sera of ST- and CAF-fed rats demonstrated that the overall sensitivity and precision obtained with the developed method allowed the quantification of endogenous changes in circulating Lyso-PLs. Furthermore, multivariate analysis showed that Lyso-PL examination is an excellent biomarker for the prolonged intake of a diet rich in fat and carbohydrates in rats. Interestingly, this type of diet has been associated with the development of metabolic syndrome in rodents and humans [39,40]. Thus, serum Lyso-PL evaluation may be regarded as a quantitative measure of the nutritional phenotype of CAF-fed rats, a holistic concept that integrates the effects of the diet with health status [41].

We conclude that the simple methodology for the collection and treatment of serum samples, which does not require lipid pre-fractionation, along with the great results for the quality parameters of the technique support the use of UHPLC-+ESI-MS/MS analysis of Lyso-PCs and Lyso-PEs for accurate comprehensive diagnosis of lipid disorders. The present method is expected to be further convenient for the quantitative profiling of Lyso-PLs in other interesting biological fluids and tissues.

5. Acknowledgments

6. Supplementary material

Figure S1. Principal component analysis of the thirty-one lysophospholipids identified in rat serum. (**A**) Two-dimensional scores plot explaining the maximal variance of the data. Animals fed standard chow (ST) are represented as grey spots and animals fed cafeteria diet (CAF) as black spots (n = 5 per group). The ellipses mark the 95% confidence interval. (**B**) Three-dimensional scores plot. (**C**) Two-dimensional loadings plot showing the underlying lysophospholipids responsible for the separation of both dietary groups. LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine. (**D**) Variance explained for each principal component (PC) (*blue line*) and accumulated variance with the increase in the number of PCs in consideration (*green line*).
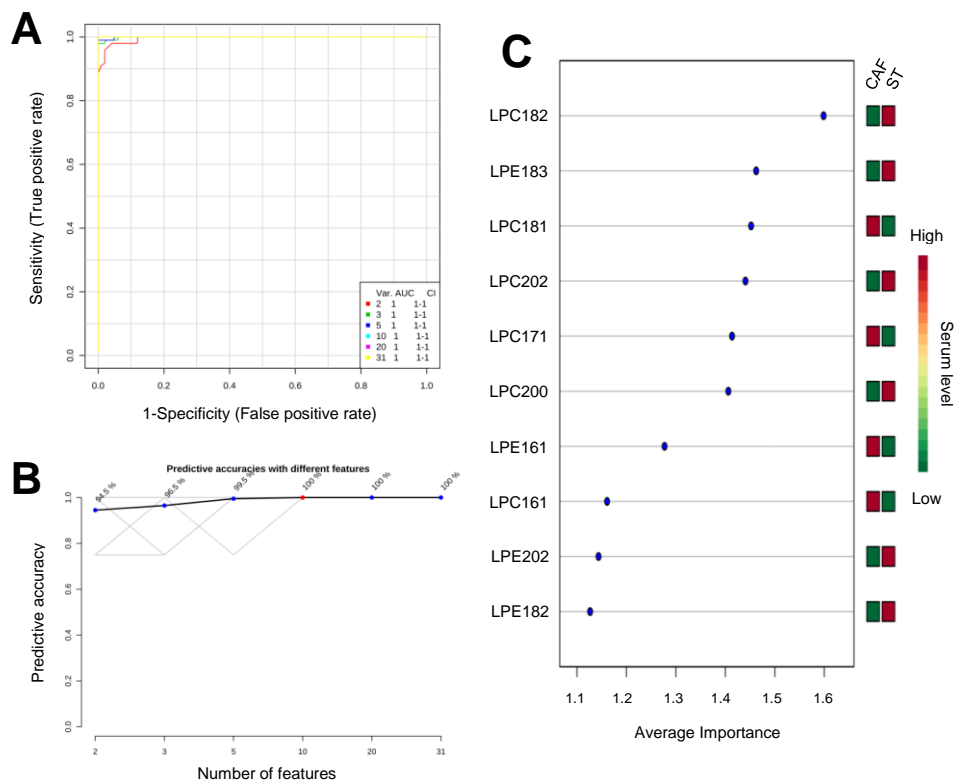
Figure S2. Receiver operating characteristic (ROC) curve analysis for the evaluation of the multivariate biomarker. (**A**) ROC curves using the two, three, five, ten, twenty and thirty-one serum lysophospholipids with the highest variable importance in the discrimination of both dietary groups. The areas under the curve (AUC) and the corresponding confidence intervals (CI) are reported in the inset. All the curves showed optimal sensitivity and specificity. (**B**) Predictive accuracies achieved with the biomarker model using different number of serum lysophospholipids. (**C**) Importance values of the ten lysophospholipids providing 100% accuracy to our model. The comparison between the circulating levels of rats fed the standard chow (ST) and the cafeteria diet (CAF) is also shown. LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

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UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL BIOMARKERS OF ALTERED HOMEÒSTASIS
Susana Suárez García

LIST OF PUBLICATIONS

- **Suárez-García S,** del Bas JM, Caimari A, Escorihuela RM, Arola L, Suárez M. "*Impact of a cafeteria diet and daily physical training on the rat serum metabolome*". PLoS One. 2017;12:e0171970

- **Suárez-García S,** Caimari A, del Bas JM, Suárez M, Arola L. "*Serum lysophospholipid levels are altered in dyslipidemic hamsters*". Sci. Rep. Nature Publishing Group; 2017

- **Suárez-García S**, Arola L, Pascual-Serrano A, Arola-Arnal A, Aragonès G, Bladé C, Suárez M. "*Development and validation of a UHPLC-ESI-MS/MS method for the simultaneous quantification of mammal lysophosphatidylcholines and lysophosphatidylethanolamines in serum*". J. Chromatogr. B. 2017;1055:86–97

- Pascual-Serrano A, Arola-Arnal A, **Suárez-García S**, Bravo FI, Suárez M, Arola L, Bladé C. "*Grape seed proanthocyanidin supplementation reduces adipocyte size and increases adipocyte number in obese rats*". Int. J. Obes. Nature Publishing Group; 2017

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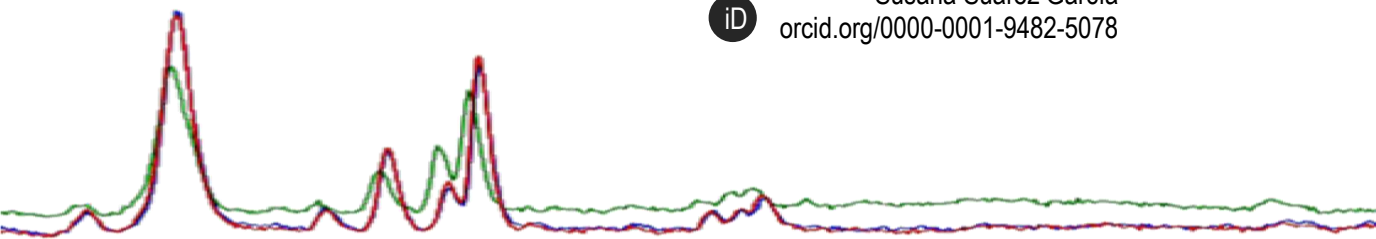# LIST OF TABLES

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UNIVERSITAT ROVIRA I VIRGILI
IDENTIFICATION OF NOVEL BIOMARKERS OF ALTERED HOMEÒSTASIS
Susana Suárez García

Poor eating habits exert a direct influence on the incidence of chronic diseases of high global prevalence, such as cardiovascular and fatty liver diseases. Nevertheless, the beneficial effects of a correct nutrition are principally effective in the opening phase of the metabolic disorder, being able to prevent and even reverse its development. By contrast, current biomarkers have a delayed response, contributing to the late diagnosis of the disease and encouraging the use of pharmacological therapies. These treatments can also be expensive and lead to serious side effects for the patients. Therefore, it is essential to find new risk markers that assist on early diagnosis. Only with these novel biomarkers able to determine the primarily metabolic disturbances that lead into a plethora of diseases, nutrition can be efficient in disease therapy. The recent emergence of the omics sciences along with the improvement of the equipment and tools for the processing of large volumes of data, provide the adequate support for biomarker search.

In this context, the present doctoral thesis was aimed to identify novel early biomarkers useful in the diagnosis of metabolic disorders derived for altered nutrition and lifestyle. For this purpose, non-targeted metabolomics was applied to the analysis of plasma samples from rodents fed different diets and subject to behavioural intervention. Once the biomarkers were selected, their distribution and metabolism were further studied. Finally, the adequacy of the putative biomarkers was determined in humans. Our results revealed circulating lysophospholipids as early predictors of chronic disorder. The metabolic assessment of the lysophospholipidome was relevant for the subclinical non-invasive diagnosis of fatty liver. Furthermore, the findings in humans seemed to confirm this assumption. As a conclusion, we propose lysophospholipids, including lysophosphatidylcholines and lysophosphatidylethanolamines, as good candidates for biomarkers of risk of fatty liver in humans.

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