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Ano 2021

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Análise do fosfolipidoma plasmático revela diferenças entre o perfil de crianças com fenilcetonúria e de crianças saudáveis

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, especialização Bioquímica Clínica, realizada sob a orientação científica da Doutora Ana Sofia Pereira Moreira, Investigadora do Departamento de Química da Universidade de Aveiro, e da Professora Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

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Dedico este trabalho à minha avó Júlia e ao meu avô Hélder, pelo incansável apoio durante todo meu percurso académico e também na minha vida.

o júri

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palavras-chave

Erros inatos do metabolismo, fenilcetonúria, alterações lipidícas, *stress* oxidativo, lipidómica, espetrometria de massa

resumo

A fenilcetonúria (PKU) é o erro inato mais prevalente no metabolismo de aminoácidos. A PKU é caracterizada pela deficiência de uma enzima do metabolismo da fenilalanina (Phe), a fenilalanina hidroxilase, a qual é responsável pela conversão de Phe em tirosina (Tyr). A deficiência desta enzima causa a acumulação de Phe, podendo causar um comprometimento cognitivo grave. Assim, o diagnóstico precoce através do rastreio neonatal e uma rápida implementação do tratamento são essenciais para evitar sequelas irreversíveis. A abordagem terapêutica usada na PKU baseia-se no controlo rigoroso do aporte de Phe através uma dieta restrita em alimentos ricos em proteínas que deve ser mantida durante toda a vida. As elevadas restrições na dieta podem levar a desequilíbrios noutros nutrientes, nomeadamente em lípidos.

Estudos anteriores com plasma/soro e glóbulos vermelhos de pacientes com PKU com dieta restrita em Phe revelaram alterações lipídicas, nomeadamente nos componentes de lipoproteínas e no perfil de ácidos gordos. Apesar das alterações já reportadas, são necessários mais estudos para compreender em detalhe as alterações no perfil lipídico de pacientes com PKU, nomeadamente a nível dos fosfolípidos, os quais também têm importantes funções de sinalização e regulação. Assim, o principal objetivo deste trabalho foi estudar o fosfolipidoma de fenilcetonúricos, a fim de identificar a ocorrência de alterações no perfil lipídico. Para tal, extratos plasmáticos enriquecidos em fosfolípidos obtidos a partir de crianças com PKU e de crianças saudáveis (grupo controlo, CT) foram analisados por HILIC-MS/MS e GC-MS.

Deste modo, foram identificadas 187 espécies lipídicas pertencentes a 9 classes diferentes de fosfolípidos e 3 ceramidas. A análise de componentes principais do conjunto de dados das espécies lipídicas mostrou uma distinção entre os grupos PKU e CT. A análise univariada revelou que 146 espécies de fosfolípidos foram significativamente diferentes entre os dois grupos. As espécies com maior variação incluíram principalmente fosfatidilcolinas, contendo ácidos gordos polinsaturados (PUFA), estando estas mais abundantes no grupo PKU. Para doentes com uma dieta suplementada com PUFA, os níveis elevados de espécies contendo PUFA podem estar relacionados com a suplementação. Este estudo foi o primeiro a comparar o lipidoma polar plasmático de crianças com PKU e de crianças saudáveis, realçando que o fosfolipidoma de crianças com PKU está significativamente alterado quando comparado com crianças saudáveis.

Em suma, a abordagem lipidómica usada neste estudo permitiu a análise e identificação do perfil fosfolipídico de crianças com PKU e de crianças saudáveis. Contudo, mais estudos são necessários para esclarecer se alterações identificadas são específicas de crianças fenilcetonúricas, nomeadamente daquelas que fazem suplementação com PUFA.

keywords **Inborn erros of metabolism, phenylketonuria, lipid changes, oxidative stress, lipidomics, mass spectrometry**

abstract

Phenylketonuria (PKU) is the most prevalent innate error in amino acid metabolism. PKU is characterized by the deficiency of a phenylalanine (Phe) metabolism enzyme, phenylalanine hydroxylase, which is responsible for the conversion of Phe into tyrosine (Tyr). Deficiency of this enzyme causes the accumulation of Phe, which may cause severe cognitive impairment. Early diagnosis through neonatal screening and a rapid therapeutic implementation are essential to prevent irreversible sequelae. Therapeutic approach for PKU is based on strict control of Phe intake through a lifelong diet restricted in protein-rich food. High dietary restrictions can lead to imbalances in other nutrients, notably lipids.

Previous plasma/serum and red blood cells studies of PKU patients with Phe-restricted diet revealed lipid changes, namely in lipoprotein's components and fatty acid profile. Despite the changes already reported, further studies are needed to understand in detail the changes in the lipid profile of PKU patients, particularly at the level of phospholipids, which also have important signalling and regulatory functions. Thus, the main aim of this work was to study the phenylketonuric phospholipidome in order to identify the occurrence of changes in lipid profile. To this end, the plasma phospholipid-enriched extracts obtained from PKU and healthy children (control group, CT) were analysed by HILIC-MS/MS and GC-MS.

Using this approach, 187 lipid species belonging to 9 different phospholipid classes and 3 ceramides were identified. Principal component analysis of lipid species dataset showed a distinction between PKU and CT groups. Univariate analysis revealed that 146 phospholipid species were significantly different between groups. The species with major variation included phosphatidylcholines (PC), bearing polyunsaturated fatty acids (PUFA), which were more abundant in the PKU group. For patients with diet supplemented with PUFA, the higher level of PUFA-containing lipid species may be related with such supplementation. This study was the first report comparing the plasma polar lipidome of PKU and healthy children, highlighting that the phospholipidome of PKU children is significantly altered when compared with healthy children.

In conclusion, the lipidomic approach used in this work allowed the analysis and identification of the phospholipid profile of PKU and healthy children. However, further studies with larger cohorts are needed to clarify if the changes identified are specific to the phenylketonuric children, namely those with PUFA supplementation.

Publications and communications in scientific meetings

Articles in international journals with referees	I.M.S. Guerra, H.B. Ferreira, B. Neves, T. Melo, L.M. Diogo, M.R. Domingues, A.S.P. Moreira, Lipids and phenylketonuria: Current evidences pointed the need for lipidomics studies, Archives of Biochemistry and Biophysics. 688 (2020) 108431. https://doi.org/10.1016/j.abb.2020.108431 I.M.S. Guerra, H L. Diogo, M. Pinho, T. Melo, P. Domingues, M.R. Domingues, A.S.P. Moreira, Plasma phospholipidomic profile differs between children with phenylketonuria and healthy children, *Submitted* (30 December 2020).
Communications in poster	I.M.S. Guerra, H L. Diogo, M. Pinho, T. Melo, P. Domingues, M.R. Domingues, A.S.P. Moreira, Lipidomics as a tool to decode changes in lipid profile of children with phenylketonuria, One Health Day – Emerging Diseases, CESAM University of Aveiro, (11 November 2020)

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Abbreviations

AA - Arachidonic acid

ACN - Acetonitrile

ALA - Alpha-linolenic acid

Apo B - Apolipoprotein B

BBB - Blood-brain barrier

BH_4 - Tetrahydrobiopterin

CE - Cholesterol esters

Cer - Ceramide

CT - Control

DHA - Docosahexaenoic acid

Dim - Dimension(s)

EPA - Eicosapentaenoic acid

FA - Fatty acid(s)

FAME - fatty acid methyl ester(s)

FID - flame ionization detector

GC - Gas chromatography

GSHPx - Glutathione peroxidase

HDL-C – High-density lipoprotein cholesterol

HFA - Hyperphenylalaninemia

HILIC - Hydrophilic interaction liquid chromatography

HPLC - High-performance liquid chromatography

IEM - Inborn error(s) of metabolism

LA - Linoleic acid

LC - Liquid chromatography

LDL-C - Low-density lipoprotein cholesterol

LNAA - Large neutral amino acid(s)

LPC - Lysophosphatidylcholine

LPE - Lysophosphatidylethanolamine

LPG - Lysophosphatidylgylcerol

MDA - Malondialdehyde

MS - Mass spectrometry

MS/MS - Tandem mass spectrometry

MUFA- Monosaturated fatty acid(s)

P - Phosphorus

PAH - Phenylalanine hydroxylase

PC - Phosphatidylcholine

PCA - Principal component analysis

PE - Phosphatidylethanolamine

PG - Phosphatidylglycerol

Phe - L-Phenylalanine

PI - Phosphatidylinositol

PKU - Phenylketonuria

PL - Phospholipid(s)

PS - Phosphatidylserine

PUFA - Polyunsaturated fatty acid(s)

Q10 - Ubiquinone-10

RBC - Red blood cells

ROS - Reactive oxygen species

SAA - Serum amyloid A

Se - Selenium

SFA - Saturated fatty acid(s)

SM - Sphingomyelin

SPE - Solid phase extraction

TAG - Triacylglycerols

TC - Total cholesterol

TLC - Thin layer chromatography

TXB2 - Thromboxane B2

TXB3 - Thromboxane B3

Tyr - L-Tyrosine

VLDL-C - Very low-density lipoprotein cholesterol

I. Introduction

The text and figures/tables in Sections I.1-I.3 of this chapter were integrally published in the following article:

I.M.S. Guerra, H.B. Ferreira, B. Neves, T. Melo, L.M. Diogo, M.R. Domingues, A.S.P. Moreira, Lipids and phenylketonuria: Current evidences pointed the need for lipidomics studies, Archives of Biochemistry and Biophysics. 688 (2020) 108431. https://doi.org/10.1016/j.abb.2020.108431.

I.1. INBORN ERRORS OF METABOLISM (IEM)

IEM are a phenotypically and genetically heterogeneous group of disorders caused by alteration of a specific chemical reaction in metabolism [1]. IEM are individually rare (some with a birth prevalence less than 1 per 100 000), but collectively they account for a significant proportion of life-threatening and/or chronic illnesses, particularly in children [2].

The pathogenesis of an IEM can be attributed to loss or, more rarely, gain of function of a mutant protein (usually an enzyme or a transporter), and thus, the disease is generally associated with an altered metabolite flux through the involved pathway [1,2]. Pathological consequences can be due to: 1) direct toxicity of accumulating upstream metabolites, 2) deficiency of downstream products beyond the block of the metabolic pathway, 3) activation of alternative metabolic pathways leading to unusual metabolite production, and/or 4) feedback inhibition or activation by the substrate(s) or the product(s) on the same or different pathways (Figure I.1) [1,3].

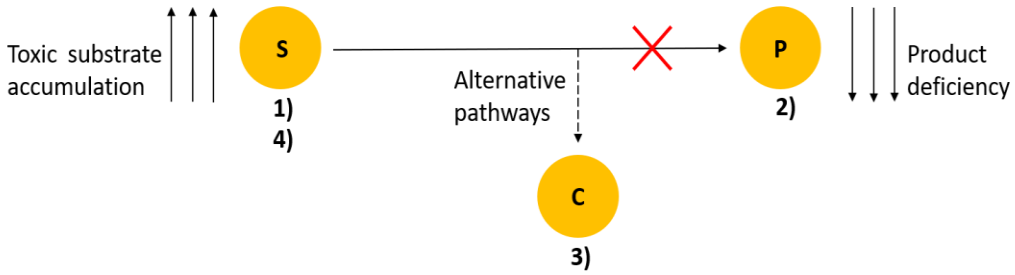

Figure I.1. Pathogenic mechanism in inborn errors of metabolism (IEM). The enzyme deficiency leads to accumulation of substrate (S) and intermediary metabolites proximal to the blockage and the formation of alternative products (C) and may also lead to deficiencies of products downstream of the blockage (P). Pathological consequences can be due to: 1) direct toxicity of accumulating upstream metabolites, 2) deficiency of downstream products beyond the blockage, 3) activation of alternative metabolic pathways leading to alternative metabolite production, and 4) feedback inhibition or activation by the substrate on the same or different pathway. Adapted from Lanpher *et al* [1] and Dixon *et al* [4].

A subgroup of IEM, in which phenylketonuria (PKU) is included [3], is due to malfunction of the catabolic pathways of specific amino acids, with accumulation of the substrates of the defective enzyme and/or production of an alternative product. Those disorders, caused by "endogenous" intoxication, are amenable to dietary intervention with restriction of the accumulated amino acids and supplementation of deficient metabolites.

I.2. PHENYLKETONURIA (PKU)

PKU is the most prevalent disorder of amino acid metabolism. Its occurrence varies among ethnic groups and geographic regions worldwide. In Europe, the mean birth prevalence is 1 in 10 000 [5]. In Portugal, the estimated frequency was 1 in 10 867, in 2018 [6]. PKU is characterized by elevated levels of L-phenylalanine (Phe) in the blood, a condition known as hyperphenylalaninemia (HFA). In more than 98% of the patients, HFA is due to deficiency of the liver enzyme phenylalanine hydroxylase (PAH) that converts Phe into L-tyrosine (Tyr) [7]. Accumulation of Phe is associated with central nervous system toxic effects, leading to progressive intellectual impairment, autism, microcephaly, seizures, and motor deficits. Some patients are blond and present eczematous rash due to Tyr (and melanin) deficiency. In order to prevent possible irreversible complications, the early diagnosis and implementation of treatment is essential [8,9].

Phe is an essential amino acid, which is mainly metabolised in the liver. When Phe is in excess in the body, and is not used for protein synthesis, it is hydroxylated into Tyr by PAH, with tetrahydrobiopterin (BH_4) as cofactor, as well as iron and molecular oxygen (Figure I.2) [10]. PAH deficiency results in a total or partial inability to convert Phe, from the diet or derived from catabolism of proteins in the body, into Tyr, leading to an increase of Phe concentration in the blood [7]. Beyond enzymatic protein deficiency, PKU can also be caused by defects in the enzymatic cofactor (BH_4) synthesis or reduction [11]. These are extremely rare, have specific pathogenic mechanisms and clinical phenotype and will not be further discussed.

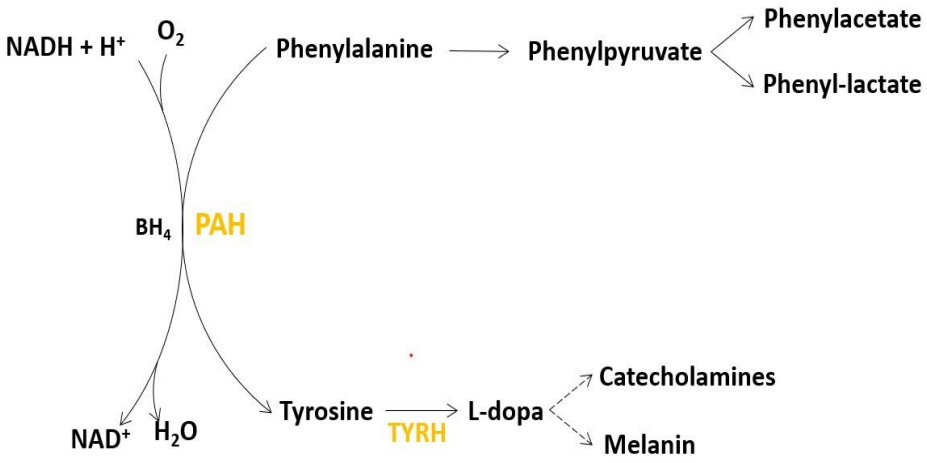

Figure I.2. Phenylalanine metabolism. BH_4: tetrahydrobiopterin; PAH: phenylalanine hydroxylase; TYRH: tyrosine hydroxylase; Adapted from Rocha and Martel [11] and van Wegberg *et al* [5].

PAH deficient activity leads to an increase of Phe plasma levels and high Phe/ Tyr ratio. Tyr concentration can be normal or low. Due to the high concentrations of Phe, an alternative pathway for Phe degradation, less active in healthy individuals, becomes prominent in PKU. In this pathway, Phe undergoes a transamination, leading to the formation of phenylpyruvate. This metabolite, together with Phe, is accumulated in the blood and excreted in the urine [12,13]. Phenylpyruvate molecules that are not excreted may be decarboxylated to phenylacetate, or reduced to phenyl-lactate [11] (Figure I.2). The excretion of excessive Phe and its metabolites can create a characteristic musty body odor [7,14].The decrease or absence of PAH activity may also lead to a deficiency of Tyr and its downstream products, which includes the decrease of melanin production (Figure I.2). As a consequence, PKU patients may have hair, skin and eye hypopigmentation [15].

The mechanisms by which high Phe concentrations disturb cerebral metabolism and cognitive function are not yet fully understood. However, several factors have been proposed as contributing to the neurotoxicity in PKU, including:

i) The effect of elevated Phe concentrations on the transport of other metabolites across the blood-brain barrier (BBB): Phe concentrations in the brain occur from its passage across the BBB. There is a competition between Phe and other large neutral amino acids (LNAA), such as Tyr, leucine, tryptophan, threonine, isoleucine, valine, methionine and histidine, for the transport at the BBB, which is mediated by the LNAA transporter 1 [16–19]. The increased Phe concentrations in blood lead to high

levels of Phe and a decrease of the other LNAA in the brain. This unbalance negatively influences the protein synthesis rate, causing the impairment of the dendritic projections and myelination (increase of myelination turnover and decrease of myelin production) [18];

ii) Tyr deficiency: In PKU, Tyr becomes a semi-essential amino acid. The reduced levels of Tyr in brain lead to impaired synthesis of catecholamines such as dopamine, norepinephrine and epinephrine (Figure I.2), which are important neurotransmitters [16,20,21];

iii) The effect of elevated Phe concentrations on the production of free radicals: The accumulation of Phe and its metabolites induces, directly or indirectly, the production of free radicals and/or depletion of the central nervous system antioxidant capacity, contributing to increased oxidative stress and neurological impairment [22];

All those factors contribute to the pathophysiology of the neurological impairment observed in PKU, especially in non-treated or late-treated patients.

I.2.1. DIAGNOSTIC

In Europe, PKU diagnosis is based on the criteria established by the European Society for Phenylketonuria and Allied Disorders Treated as Phenylketonuria [5]. Newborn screening is done in most European countries [8]. Blood is collected at a Guthrie card by pricking the baby's heel [5], as it has high density of blood capillaries and few nerves, causing less pain to the newborn. Also, the heel is easily accessible. Usual time collection is between the third and sixth day after birth to ensure that the newborn had, at least, 48 hours of feeding. Earlier blood collection can lead to false negative results, as there has not yet been time for diet Phe to reach diagnosable levels [23].

In the past, for PKU screening, two laboratory methods were used: 1) the Guthrie card bacterial inhibition assay, a time-tested, inexpensive, simple and reliable test, and 2) the fluorometric analysis, a reliable quantitative and automated test, which produces fewer false-positives test results than the bacterial inhibition assay [24]. Nowadays, the analysis of dried blood spots is based on tandem mass spectrometry (MS/MS). This is a very sensitive technique and, in a single analysis, it can measure Phe and Tyr concentrations [5,14,24], as well as of other metabolites related to other

IEM [25,26]. MS/MS is now used in newborn screening programs in all Europe. In case of a positive result, Phe is higher than 2.0 mg/dL [13], Tyr values may be normal or low, and Phe/Tyr ratio is three or above (normal Phe/Tyr ~1) [27]. If this happens, it is crucial to track down defects on biopterin metabolism [28], especially when normal plasma Tyr values are found, through the study of urinary biopterin and neopterin profiles and blood dihydrobiopterin reductase activity determination.

PKU phenotype determination is not always straightforward because Phe concentrations are measured in newborn babies when blood Phe might not have time to reach its highest value. Also, Phe tolerance levels change with age. Nevertheless, three forms of the disease, according to the Phe values found at screening can be considered: hyperphenylalaninemia (3-6 mg/dL), moderate or atypical PKU (6-20 mg/dL), and classic PKU (higher than 20 mg/dL) [13]. Once diagnosis is established, dietary treatment by Phe restriction is implemented as soon as possible, in order to prevent possible irreversible neurological sequelae.

I.2.2. THERAPEUTIC APPROACH IN PKU

Phe levels in the body are a result of a balance between diet, catabolism of endogenous proteins and conversion of Phe into other metabolites [19]. Therapeutic approach for PKU aims to normalise Phe and Tyr concentrations in the blood, preventing the development of neurological manifestations. PKU patients follow a Phe-restricted diet, which is achieved by lowering natural protein intake and supplementing with amino acid mixture free of Phe, as needed [29]. Blood Phe levels must be regularly evaluated and kept below 6 mg/dL until 12 years of age, as well as before and during pregnancy. In late adolescence and in adulthood, Phe levels under 8 mg/dL are recommended [13,29]. As Phe levels fall during the day and rise during the night until reaching a maximum value in the early morning, blood samples should be collected in the morning after fasting overnight [5].

A diet restricted in Phe should be implemented after PKU diagnosis and maintained for live [29]. The normal daily variation of blood Phe levels is lower than 50% in healthy individuals, but it can be much higher in PKU. Such variation in PKU patients may be influenced by the adherence to diet, but also changes in growth rate, illness and genotype [30].

In PKU diet, rich protein foods, such as eggs, milk, cheese, meat, fish and dried beans, are excluded [31]. Drinks containing aspartame are also avoided because, when metabolised, it releases Phe, L-aspartic acid and methanol [7]. The low-Phe diet provides a low amount of natural protein, which may not be enough for growth requirements. Thus, in order to provide a nutritionally adequate diet, in most PKU patients, it is necessary a semi-synthetic diet based on commercial formulas with Phe-free essential amino acids, which also contain minerals, vitamins and other nutrients [5,29]. In particular, the diet restrictions lead to a low dietary intake of polyunsaturated fatty acids (PUFA). To ensure that the dietary needs for essential PUFA are met, amino acid mixtures containing PUFA and PUFA supplements are prescribed to PKU patients [5]. However, some imbalance in the level of PUFA can occur, as described below (section I.2.3.2).

The dietary treatment is complex, demanding, and lifelong. It requires the patient's full understanding of the disease and cooperation. Therefore, any methods that relieve the dietary requirement are welcomed by the patients [32]. In this context, there has been a great interest in alternative and complementary therapeutic approaches for PKU.

BH_4 has recently been approved to treat PKU. Patients with high residual activity of PAH, but also a minority of patients with classical PKU, can benefit from this treatment. Some mutations are associated with BH_4-sensitive phenotype of PKU [33], in which giving pharmacological doses of exogenous BH_4 results in an increase in the activity of PAH that is enough to reduce circulating Phe to a therapeutically relevant extent [34,35].

Efficacy and safety of BH_4 has been demonstrated in children bellow the age of 4, which led to the European approval for BH_4 in this age category [36,37]. Nevertheless, few clinical studies are available to demonstrate long-term therapeutic efficacy, as well as the long-term neurocognitive consequences and the impact on behaviour and quality of life of PKU patients [38,39].

Because Phe competes with other LNAA for transport across the BBB, supplementation with LNAA (Phe-free) is another alternative therapeutic approach. As supported by quantitative magnetic resonance spectroscopy analysis, supplementation with LNAA reduces the influx of Phe into the brain, consistent with competitive inhibition of Phe transport due to increased plasma levels of other LNAA

[19]. LNAA supplementation is most effective in lowering plasma Phe concentrations in individuals who have difficulties in complying with the low-Phe diet [7,29]. However, this approach has some side effects at the gastrointestinal level, since a similar transporter for LNAA exists in the gut [29].

An alternative therapeutic approach for PKU, approved for adult patients, involves the oral administration of phenylalanine ammonia lyase (PAL), a bacteria-derived enzyme that catalyses the conversion of Phe to transcinnamic acid and ammonia in the intestinal lumen, preventing its absorption [40].

Despite the alternative approaches that have been developed, low-Phe diet remains the cornerstone for lifelong treatment of PKU. This type of diet favours a dietary intake rich in carbohydrates. The high carbohydrate intake has been pointed out, for example, as the cause for PKU patients are at risk of carbohydrate intolerance and insulin resistance [41]. However, there is currently no clear evidence that PKU patients may be at higher risk of developing diabetes mellitus and most studies only include children or young adults [42]. On other hand, dietary restrictions may lead to deficiencies, namely of PUFA, contributing for changes in the lipidome of PKU patients. Such changes reported to date are detailed below.

I.2.3. CHANGES IN THE LIPIDOME IN PKU

Lipids include a heterogeneous group, comprising a high number of structurally and functionally distinct molecules. They play a central role in almost aspects of biological life, either as structural components of cell membranes or regulatory and signalling molecules in many metabolic and hormonal pathways [43].

The uptake of lipids from diet is important for the maintenance of the lipidome stability [44]. The human lipid profile is regulated by a plethora of metabolic pathways and enzymes. Its disturbance can be associated with a disease state [45]. As detailed below, changes in lipid profile of PKU individuals have been reported from analysis of plasma and serum samples, as well as red blood cells (RBC).

In the plasma/serum, the most abundant lipid classes include phospholipids (PL), sterol lipids (including free cholesterol and respective esters), triacylglycerols (TAG) and minor non-esterified free fatty acids [46,47]. PL and sterol lipids are also the most abundant lipids in RBC. Cholesterol in RBC is mainly free, whereas in plasma/serum

it is mainly esterified with fatty acids (FA), being a component of cholesterol esters (CE) [48]. As they are insoluble in water, lipids in the plasma/serum are mostly associated with proteins, forming lipoproteins [46,47]. Studies carried out to date have reported changes in lipoprotein components, as well as in the FA profile of plasma/serum and RBC of PKU individuals.

I.2.3.1. CHANGES IN LIPOPROTEIN COMPONENTS

Disturbances in lipoproteins components have been reported from the analysis of serum and plasma of patients with PKU, even with good metabolic control (Table I.1). In some studies, PKU patients (age: 6 months to 50 years) on dietary therapy exhibited lower total cholesterol (TC) levels compared with healthy controls [49–53]. The low TC levels in treated PKU patients could be attributed to their diet, where one of the main sources of lipids is olive oil [49–52]. Moreover, these low TC levels may also be explained by the impairment of cholesterol synthesis due to down-regulated expression of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase. The reduced expression of this enzyme might derive from the increased levels of Phe and its metabolites in plasma [50–52]. However, one study showed that TC levels did not differ between PKU patients (9 months to 7 years) and healthy individuals [54], and another study revealed that TC levels were higher in PKU patients (18-47 years) [55].

Lower low-density lipoprotein cholesterol (LDL-C) levels were also observed in PKU patients on dietary treatment when compared with healthy controls [50–52]. However, this difference was not found in other studies [49,54]. Schulpis *et al* [51] reported an increase in LDL-C/apolipoprotein B (Apo B) ratio in phenylketonurics (5-8 years). Regarding the very low-density lipoproteins cholesterol (VLDL-C), both an increase [50,54] and a decrease [49,51] have been observed. In several studies, it was reported low levels of plasmatic high-density lipoprotein cholesterol (HDL-C) in PKU patients (6 months to 50 years) compared with controls [49,50,52,53,55]. However, one study showed no significant differences in plasma HDL-C between PKU patients and controls [54].

Concerning TAG plasma levels, most of the studies demonstrated that they were higher in PKU patients (9 months to 36 years) with a good metabolic control compared with the healthy group. This trend has been related to the high consumption of carbohydrates in PKU diet [49,50,53,54]. A decrease in plasma TAG levels was

reported in one study with PKU patients with a diet that included an additional supplementation with long-chain PUFA (fish oil) [56], but two other studies, with supplementation as well, reported no significant changes neither in serum TAG levels, nor in other lipoprotein components [57,58].

The alteration of lipoprotein profile is a key risk factor in the etiology of atherosclerosis and, thus, cardiovascular diseases. Some of the studies above mentioned reported increased levels of TC and TAG, and decreased HDL-C levels in PKU patients (9 months to 47 years). These changes are known to contribute to a higher risk of atherosclerosis and cardiovascular diseases [54,55]. A positive correlation between increased levels of HDL-C and serum amyloid A (SAA) was found in PKU patients, suggesting that HLD-C is enriched with SAA. This change may indicate an alteration in function of this lipoprotein, acquiring a pro-inflammatory phenotype [55]. However, the above mentioned increase of LDL-C/Apo B ratio suggests that PKU patients (6 months to 50 years) are not at risk of developing atherosclerosis [51,52]. High LDL-C/Apo B ratio is associated with the presence of larger and less atherogenic particles, which are less susceptible to oxidative damage than small LDL-C particles [51]. As pointed out by the literature cited, there is currently no consistent evidence that PKU patients exhibited an atherogenic lipid profile and, consequently, higher risk of developing atherosclerosis. More studies, with adequate data statistical analysis, are required to clarify the potential risk of PKU patients develop atherosclerosis and cardiovascular diseases. Due to the low prevalence of PKU, most of the reported studies analyse the lipid profile of children and adults without the separation by age range. The lipid profile changes with age [59], therefore children will not have a similar lipid profile to adult patients. The conjugation of children and adult patients with PKU in the same study, as if they had a similar lipid profile, may lead to non-significant or even odd results. Thus, in the future, it is important to perform studies with different age ranges and genders, separating children from adults, in order to obtain significant and representative results of PKU population.

Table I.1. Changes in lipoprotein components observed in serum and plasma of PKU patients, subjected to a Phe-restricted diet and after an additional long-chain PUFA supplementation, reported in published lipidomics studies with statistical analysis.

Reference	Type of sample (results expressed as)	Number of PKU patients	Age range	Results	
				↓Reduction	↑Increase
Phe-restricted diet					
Schulpi and Scarpelezou [49]	Serum (mg/dL)	20 (10F+10M)	1-10 yr	TC, HDL-C, VLDL-C	TAG
Colomé *et al* [50]	Serum (mmol/L)	61	1-36 yr	TC, HDL-C, LDL-C	TAG, VLDL-C
Schulpis *et al* [51]	Serum (mmol/L)	44	5-8 yr	TC, LDL-C, VLDL-C, Apo B	LDL-C/Apo B
Azabdaftari *et al* [55]	Serum (mmol/L)	23	18–47 yr	HDL-C	TC, LDL-C/HDL-C
Couce *et al* [52]	Plasma (mg/dL)	100 (53F+47M)	6 m-50 yr	TC, HDL-C, LDL-C, Apo B	-
Rocha *et al* [53]	Plasma (mg/dL)	89	7.8-21 yr	TC, HDL-C	TAG
LaVoie *et al* [54]	Plasma (μmol/L)	21	9 m-7 yr	-	TAG, VLDL-C
Effect of additional long-chain PUFA supplementation					
Agostoni *et al* [56]	Plasma (mmol/L)	21	5-10 yr	TAG	-

This data represents the result of the comparison of PKU individuals with healthy controls, except in the study of Couce *et al* [52] (PKU vs Hyperphenylalaninemia) and Agostoni *et al* [56] (PKU before vs after supplementation).

Apo B, apolipoprotein B; F, female; HDL-C, high-density lipoprotein cholesterol; M, male; m, months; TAG, triacylglycerol; TC, total cholesterol; VLDL-C, very low-density lipoproteins cholesterol; yr, years;

I.2.3.2. CHANGES IN FATTY ACID PROFILE

FA play multiple biological roles in human body, and they are structural components of different lipids [60,61]. If the diet is changed, as it happens with PKU patients, and consequently the FA uptake is dissimilar, it will lead to changes in lipid profile in membranes and in lipid signalling cascades that will affect lipid metabolism and their role and functionality at cell and organ level [61]. In fact, changes in FA profile of plasma and RBC, where FA occur mainly esterified with PL, TAG and CE [60], have been reported both in PKU patients maintained on a Phe-restricted diet with and without an additional long-chain PUFA supplementation (Table I.2).

The analysis of FA performed by gas chromatography (GC), in most of the studies using GC with flame ionization detector (GC-FID), requires the derivatization of FA. With the exception of one out of the fourteen studies analysed in this review (Table I.2), which converted FA into pentafluorobenzyl ester (PFBE), FA were converted to FA methyl ester (FAME) derivatives, prepared by either acid-catalysed methods (which allow the analysis of free and esterified FA) or basic-catalysed methods (which limits the analysis to esterified FA).

The FA composition (free and esterified FA) of total plasma/plasma PL fraction of PKU patients (age: 2 months to 42 years), who have been maintained on a Phe-restricted diet, was evaluated in different studies [62–67]. In three of the six cited studies, a significant reduction of both PUFA docasapentaenoic acid (DHA) (22:6*n*-3) and arachidonic acid (AA) (20:4*n*-6) levels in total plasma and plasma PL fraction was reported [62,63,65]. Different authors proposed diverse explanations for the plasmatic reduction of DHA and AA. Sanjurjo *et al* [65] suggested that the lower levels of plasma DHA were probably a consequence of the prohibited intake of fish (rich in *n*-3 PUFA).In fact, the low dietary intake of DHA should be the main cause for the decrease in DHA levels, as most of DHA comes from the diet and its endogenous synthesis is very low [68]. In the study of Sanjurjo *et al* [65], PKU patients also showed higher levels of linoleic acid (LA) (18:2*n*-6) in plasma than those observed in healthy controls, which was associated to the relatively high LA intake by PKU individuals[65]. In other two studies [62,63], it was suggested that the reduction of plasmatic DHA and AA could be associated with an impairment in the endogenous synthesis of these FA in patients on a Phe-restricted diet. This impaired synthesis could be the result of the inhibition of enzymatic processes by Phe derived metabolites, within which are phenyl-lactate and phenylpyruvate. Other three studies revealed no significant differences levels of AA in total plasma/plasma PL fraction [64,66,67]. Some authors also showed a significant decrease on plasma levels of eicosapentaenoic acid (EPA) (20:5*n*-3) [62–64,67], but only Gramer *et al* [69] demonstrated no significant differences in the plasma levels of DHA and EPA. Only Giovannini *et al* [66] revealed that PKU patients had a lower level of DHA in plasma, while other PUFA, such as AA and EPA, did not show significant differences between PKU patients and heathy controls.

The inconsistent results obtained in the different studies, relatively to the plasma FA profile, may probably be due to the use of either plasma PL fraction or total plasma. When the FA profile is analyzed in total plasma, FA are esterified with various lipid classes (TAG, CE and PL) which are components of lipoproteins or free, whereas when in plasma PL fraction, FA are only esterified with PL [60].

The FA composition (free and esterified FA) of total RBC/RBC PL fraction of phenylketonurics with a low-Phe diet was evaluated in five studies [62–65,70]. In four of these studies, a significant decrease of DHA (22:6*n*-3) and EPA (20:5*n*-6) levels were reported [63–65,70]. In the study of Stroup *et al* [70], PKU patients also showed, in total RBC, higher levels of alpha-linolenic acid (ALA) (18:3*n*-3), the precursor of DHA and EPA. This suggests that the significant decrease of DHA and EPA levels was due to the diminished efficiency in the conversion of ALA in these PUFA. However, Galli *et al* [62] found no significant differences for those PUFA (EPA and DHA) between PKU and healthy individuals. Moreover, Van Gool *et al* [64] also found a decrease in AA levels RBC PL fraction, besides that observed in DHA and EPA. Galli *et al* [62] found no significative differences in the RBC AA levels, in contrast to the decrease that was reported in plasma lipids of PKU patients. Thus, it was suggested that RBC can efficiently control their AA levels, even in the presence of AA deficits in plasma. The different results obtained from total RBC and respective PL fraction can be due to the lower amount of FA in PL fraction, where only esterified FA in PL are analysed [48].

AA, EPA and DHA, also classified as highly unsaturated FA, have important functions in the living systems. EPA and AA can be released from PL by the action of phospholipase A. Most of the biological functions of AA are mediated by the so-called eicosanoids [71,72]. Eicosanoids are the active end-products of arachidonate metabolism involving cyclooxygenase and lipoxygenase, and the mediators are implicated in inflammatory, coagulative and vasoactive responses, and are effectors of the homeostatic processes [73]. Platelets are the main producers of thromboxane from AA. Thus, Mütze *et al* [73] and Agostoni *et al* [74] hypothesized that, if plasma level of AA is lower in PKU patients, it could result in some changes in platelets arachidonate and it will cause alteration in the production of platelet-derived eicosanoids. To explore this hypothesis, both studies measured the levels of PUFA and eicosanoids metabolites in a group of PKU patients (23 to 37 years and 2 to 17

years). In the Mütze *et al* [73] study, the levels of AA and thromboxane B_2 (TXB_2) did not differ between PKU patients and controls. In contrast, Agostoni *et al* [74] found reduced levels of TXB_2 and AA. On the other hand, the levels of thromboxane B_3 (TXB_3), a metabolite of EPA, were significantly lower in the PKU group when compared with the controls [73]. This may indicate a reduction on *n*-3 PUFA metabolism in patients with PKU, although the amount of EPA and DHA was adequate [73]. Also, Mütze and coworkers [73] hypothesized that the dietary restriction of long-chain PUFA affect the platelet function, but no differences between the PKU patients and controls were found concerning the aggregation and platelet eicosanoid release.

As shown in the studies above mentioned, the long-chain PUFA status in PKU patients is often compromised. Considering that long-chain PUFA are important structural and functional constituents of all cell membranes and essential for the normal cognitive and visual development, it has been hypothesized that lipid metabolism in PKU can be improved by dietary long-chain PUFA supplementation. In seven studies performed with PKU patients submitted to long-chain PUFA supplementation, either through fish oil or other supplements, it was noticed a significant increase of DHA in total plasma/plasma PL fraction and in the incorporation into PL in the RBC membranes [56–58,75–78]. Two of these studies showed an increased level of EPA in plasma [56,77]. In most of the studies, no modifications were observed in AA levels [56–58,75,76]. However, Beblo *et al* [77] observed a decrease in AA concentrations and Koletzko *et al* [78] observed an increase in AA levels, after the supplementation (Table I.2).

Despite the differences in results between studies, the great majority of the studies on the effect of the PUFA supplementation in FA profile of PKU patients reported an increase in the *n*-3 levels and a decrease in the *n*-6 levels, as well as a higher *n*-3/*n*-6 ratio. These changes may have positive impacts in the health of patients, as *n*-3 PUFA have been associated with anti-inflammatory properties and reduction of the risk of development cardiovascular disease, while *n*-6 PUFA are associated with the promotion of inflammatory processes [79,80]. The changes observed in PUFA levels after supplementation may be due to the high percentage of *n*-3 PUFA in the supplements. However, the detailed composition of the supplements was not reported

in all studies, and that is important because their composition may influence the results.

The conflicting results reported on the changes in FA profile in PKU, as well as in lipoprotein components (discussed in section I.2.3.1), when comparing the same type of sample, may be driven by small numbers of patients involved in the studies, as well as by other variables, such as age, sex, body mass index and disease severity, that are not taken into account. Data from adults are particularly limited and the oldest PKU patients are in the age of the 50s. Further studies in older populations of PKU patients are required to confirm the risk for the development of dyslipidaemia, as well as of other comorbidities associated to PKU. Also, methodological aspects, such as the procedure used for lipid extraction from biological samples, the approach used for sample analysis and the way how results are expressed, may contribute to the conflicting results. The results obtained also depend on the type of sample analysed. For example, plasma FA profile is an indicator of recent fat intake, while the RBC FA profile reflects longer-term intake [81]. Regarding the FA analysis, the different conditions, such as reagents, temperature and time, used to prepare derivatives from FA for GC, also influence the FA profile obtained and lead to different results. In future studies, standardization is needed to make results comparable.

Table I.2. Changes in FA observed in plasma and RBC of PKU patients, subjected to a Phe-restricted diet and after an additional long-chain PUFA supplementation, reported in published lipidomics studies with statistical analysis.

Reference	Analytical method	Lipid extraction method	Derivatives for GC	Type of sample (results expressed as)	Number of PKU patients	Age range	Results	
			FAME or PFBE (reagent, temperature, and time)				↓Reduction	↑Increase
Phe-restricted diet								
Galli *et al* [62]	GC-MS	Bligh and Dyer Folch *et al*	FAME (methanolic hydrochloride)	Plasma (%weight total FA) Red blood cells (%weight total FA)	15 (8F+7M)	3-12 yr	20:4*n*-6, 20:5*n*-3, 22:6*n*-3 16:0, 16:1, 18:0	18:1*n*-9 22:4*n*-6
Moseley *et al* [63]	GC-FID	No extraction step	FAME (conditions not clarified)	Plasma (% total FA) Red blood cells (% total FA)	27 (18F+9M)	7-39 yr	20:4*n*-6, 20:5*n*-3, 22:6*n*-3, total *n*-3, *n*-3/*n*-6 ratio 18:1*n*-9, 20:5*n*-3, 22:6*n*-3, total *n*-3, *n*-3/*n*-6 ratio	18:3*n*-3, 22:5*n*-6 22:5*n*-6, total *n*-6
Van Gool *et al* [64]	GC-FID	Bligh and Dyer	FAME (14% boron trifluoride in methanol, 100 ºC, 1h)	Plasma PL fraction (% total FA by weight)[a] Red blood cells PL fraction (% total FA by weight)[a]	9 (3F+6M)	6m-25 yr	18:3*n*-3, 20:4*n*-3, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3, total *n*-3 18:3*n*-3, 20:4*n*-6, 20:4*n*-3, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3, total *n*-3	20:3n-6, 22:4*n*-6, 22:5*n*-6 total *n*-6 18:2*n*-6, 18:3*n*-6, 22:4*n*-6, 22:5*n*-6, total *n*-6
Sanjurjo *et al* [65]	GC-FID	No extraction step	FAME (methanol-benzene 4:l (v/v) and acetyl chloride, 100°C, 1h)	Plasma (% total FA) Red blood cells PL fraction (% total FA)[b]	40 (15F+25M)	2m-20 yr	16:0, 16:1, 20:4*n*-6, 22:6*n*-3 14:0, 16:1, 18:1*n*-9, 18:3*n*-3, 20:5*n*-3, 22:6*n*-3	18:2*n*-6, 20:3*n*-6 20:4*n*-6, 22:4*n*-6, 22:5*n*-6
Giovannini *et al* [66]	GC-FID	Folch *et al*	FAME (methanol/ hydrochloric acid, 90 ºC, 1h)	Plasma PL fraction (%weight)[b]	45	9-14 yr	SFA, 22:6*n*-3, total *n*-3	MUFA, 18:3*n*-3

Aldámiz-Echevarría *et al* [67]	GC-FID	No extraction step	FAME (methanol-benzene 4: l (v/v) and acetyl chloride, 100°C, 1h)	Plasma total FA (g/100g total FA)	47	INF	20:5 *n*-3, 22:6 *n*-3, SFA, total *n*-3	18:1*n*-9, MUFA, *n*-6/*n*-3 ratio
				Plasma PL fraction (g/100g PL)			20:5 *n*-3, 22:6 *n*-3, total *n*-3	16:0, 18:1*n*-9, total *n*-6, *n*-6/*n*-3 ratio
Stroup *et al* [70]	GC-MS	No extraction step	PFBE (triethylamine and 10% pentafluorobenzyl bromide in acetonitrile, 15 min, room temperature)	Red blood cells (% total FA)	25 (15F+10M)	18-49 yr	18:0, 18:1*n*-9, 20:5*n*-6, 22:6*n*-6, total *n*-3, *n*-3/*n*-6 ratio, SFA	18:3*n*-3, 18:3*n*-6, 20:3*n*-6, 22:4*n*-6, total n-6, *n*-6/*n*-3 ratio
Effect of additional long-chain PUFA supplementation								
Agostoni *et al* [57]	GC-FID	Folch et al	FAME (methanolic hydrochloric acid)	Plasma PL fraction (%weight)	20	3-17 yr	-	22:6 *n*-3, total *n*-3
Beblo *et al* [77]	GC-FID	Folch et al	FAME (methanolic hydrochloric acid)	Plasma PL fraction (%weight/weight)	36	1-11 yr	18:2*n*-6, 20:4*n*-6, 22:5*n*-6, total *n*-6	20:5*n*-3, 22:5*n*-3, 22:6*n*-3, total *n*-3, *n*-3/*n*-6 ratio
Agostoni *et al* [56]	GC-FID	Folch *et al*	FAME (methanolic hydrochloric acid)	Plasma total FA (%weight)	21	5-10 yr	20:3*n*-6, MUFA	20:5*n*-3, 22:5*n*-3, 22:6*n*-3, PUFA
Demmelmair *et al* [58]	GC-FID	Folch *et al*	FAME (sodium methoxide, room temperature)	Plasma PL fraction (mg/L)	109	5-13 yr	-	22:6 *n*-3
Koletzko *et al* [78]	GC-FID	*n*-hexane/isopropanol (3:2 vol/vol)	FAME (methanolic hydrochloric acid, 85°C, 45 min)	Plasma PL fraction (%weight)	10 (3F+7M)	1-3 w	-	20:4*n*-6, 22:6*n*-3
Agostoni *et al* [76]	GC-FID	Folch *et al*	FAME (methanolic hydrochloric acid, 90°C,1h)	Red blood cells PL fraction (%)	42 (22F+20M)	1-5w	-	22:6*n*-3, total *n*-3
Cleary *et al* [75]	GC-MS	No extraction step	FAME (sulfuric acid in methanol, 70 ºC, 3h)	Red blood cells PL fraction (%)	53	1-10 yr	-	22:6 *n*-3

Methods to recover phospholipid fraction: [a]SPE, solid phase extraction; [b]TLC, thin layer chromatography; FA, fatty acid(s); FAME, fatty acid methyl ester; F, female; GC-FID, gas chromatography-flame ionization detector; INF, information not found; GC-MS, gas chromatography-mass spectrometry; M, male; m, months; MUFA, monosaturated fatty acid(s); PFBE, pentafluorobenzyl ester; PL, phospholipid(s); PUFA, polyunsaturated fatty acid(s); SFA, saturated fatty acid(s); w: weeks. yr: years.

I.2.4. OXIDATIVE STRESS IN PKU

Over the last years, the role of oxidative stress in PKU pathogenesis has been investigated in PKU animal model and biological samples from PKU patients under treatment [82,83]. The results indicate that oxidative stress may represent an important element in the pathophysiology of PKU. Although the cause of increased oxidative stress in this disease is poorly understood, it is assumed to result from the accumulation of toxic metabolites which induce the production of free radicals and/or from the reduction of antioxidant defences, possibly due to the dietary treatment that lead to a deficient intake of micro or macronutrients with antioxidant properties [84]. The altered redox status in PKU patients with a low-Phe diet has been associated, in particular, with selenium (Se), ubiquinone-10 (Q_{10}) and L-carnitine deficiencies (Figure I.3).

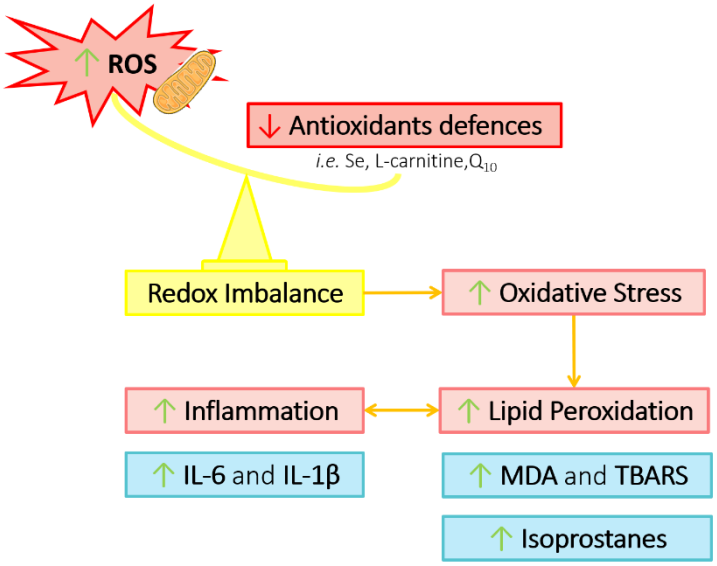

Figure I.3. Systematic representation of oxidative stress in PKU.

Se deficiency has been observed in plasma of PKU patients with Phe-restricted diet [82,83,85,86]. The reduced levels of Se may impair normal plasma/RBC glutathione peroxidase (GSHPx). GSHPx is a Se-containing enzyme that removes hydrogen peroxide by coupling its reduction to water with oxidation of reduced glutathione [82]. Consequently, Se deficiency reduces the ability to cope with the usual production of reactive species, which may result in increased ROS levels and oxidative stress. In fact, it was demonstrated that RBC GSHPx activity in PKU patients is significantly lower than in healthy controls [85,87] due to a poor Se intake. Furthermore, Se supplementation restored the activity of GSHPx [86], and the

concentration of plasma Se was strongly correlated with the GSHPx activity in RCB [85,86].

Q_{10} is a lipophilic antioxidant, important for the prevention of peroxidation of lipids in blood and tissues [82,87]. Low Q_{10} plasma/serum levels have been found in PKU patients when compared with healthy controls [83,87]. The low Q_{10} levels in PKU patients were mainly associated with high plasma Phe concentrations and, to a lesser extent, to the natural protein restriction, Tyr deficiency and a down regulation of the mevalonate pathway. Moreover, high levels of Phe seem to inhibit the activity of key enzymes, 3-hydroxy-3-methylglutaryl-CoA reductase (cholesterol synthesis) and mevalonate-5-pyrophosphate decarboxylase (mevalonate pathway), leading to decreased Q_{10} biosynthesis [82,83,87]. It has been reported that the low Q_{10} values in PKU patients are associated with higher levels of plasma malondialdehyde (MDA), a product derived from lipid peroxidation. This suggest an important role of Q_{10} in the prevention of lipid peroxidation [82]. However, the high levels of MDA may also report an increase in oxidative stress [88].

L-carnitine protects the cells from the effect of ROS. This molecule can reduce MDA levels by facilitating FA transport and thereby lowering its availability for lipid peroxidation. Decreased plasma total L-carnitine levels were found in PKU patients who strictly adhered to the diet. Also, a significant negative correlation between thiobarbituric acid-reactive substances, a parameter of lipid peroxidation, and L-carnitine plasma levels was observed [82,83,86]. Furthermore, it was demonstrated a significant inverse correlation between blood levels of L-carnitine and MDA, indicating that lipid peroxidation in PKU patients occurs mainly due to shortage of L-carnitine [86].

Oxidative stress and inflammation have been reported in PKU and seem to be related. In fact, increased levels of plasma cytokines, namely interleukin-6 and interleukin-1β, were found in treated PKU patients (age: 10-22 years), indicating a pro-inflammatory state in PKU. Also, it was found an increase in the anti-inflammatory cytokine interleukin-10. Besides that, there is a negative correlation between interleukin-6 and interleukin-10, suggesting an attempt to repair the response to inflammation processes [89]. On the other side, the increase of interleukin-1β was positively correlated with the increase of isoprostanes (lipid peroxidation biomarkers formed by non-enzymatic peroxidation of AA), excreted in the urine of PKU patients.

These results suggest that the inflammatory process is enhanced in PKU patients and is associated with lipid oxidative damage [89].

To the best of our knowledge, the few studies relating oxidative stress and lipids in PKU reported higher levels of oxidative stress and lipid peroxidation markers (MDA), but no studies identified oxidized lipid species. It is well known that oxidized lipids, not only enzymatically produced eicosanoids, but also lipid oxidation products formed by radical induced oxidation, have key roles in the onset of inflammation [90,91], namely in chronic diseases, such as cardiovascular [92] and neurodegenerative disorders [93]. Lipidomics studies at molecular level (*i.e.* with identification of individual lipid molecular species, including those oxidatively modified) are needed in order to identify oxidized lipid species, understand their role in PKU pathogenesis and stablish the possible correlation with the risk of developing other chronic complications.

I.3. THE NEED FOR LIPIDOMICS IN PKU

Lipidomics is the systematic and large-scale study of structure, function and interactions of lipids with other lipids, proteins and other molecules in biological samples (blood, tissues, cells, among others), as well as the study of lipid changes that occur during pathophysiological disturbances [94,95].

Lipidomics analysis uses mass spectrometry (MS) approaches, most often combined with liquid chromatography [96]. These approaches allow the identification and quantification of a large range of molecular species from distinct lipid classes [97]. The main steps of a typical lipidomics workflow (Figure I.4) are: extraction of lipids from biological samples, data acquisition by MS methodologies (either by direct infusion or coupled to liquid chromatography) and data analysis. The extraction is commonly performed by Bligh and Dyer [98] or Folch [99], both methods based on the use of chloroform and methanol, as well as using solid-phase extraction (SPE). In what concern to the data acquisition, two different strategies can be considered: untargeted MS, which aims the identification and quantification of as many lipid species as possible, or targeted MS, which usually aims at the detection and quantification of a panel of specific lipids. Untargeted lipidomics is usually used to prospect disease biomarkers. In validation studies, targeted methods are further designed for identified biomarkers, envisioning their implementation in clinical

laboratories [100–102]. This type of methodology is also applied in the analysis of oxidatively modified lipids, which is called oxidative lipidomics [103]. The big amount of data obtained by MS are analysed using bioinformatic tools.

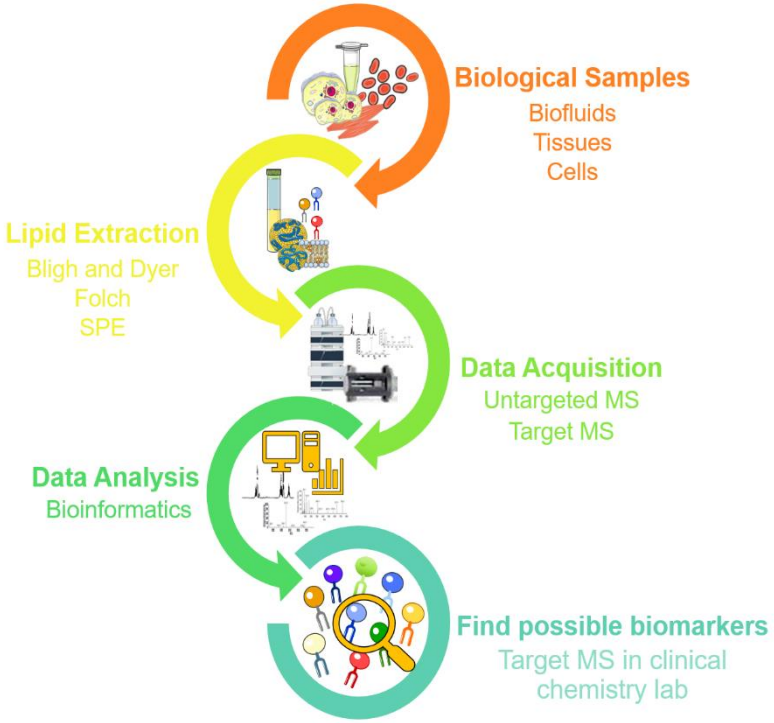

Figure I.4. Basic lipidomics workflow. Adapted from Domingues *et al* [102] and from Lydic and Goo [45].

Lipidomics is particularly useful to study changes in lipids at molecular level that can occur as consequence of the metabolic adaptation in a disease environment [104–111]. Thus, lipidomics has been applied in the study of several diseases [104–111], and, in particular, of some IEM, such as FA oxidation defects [112–114] and peroxisomal disorders [115]. To date, there are no lipidomics studies at molecular level, using MS-based strategies, in PKU.

Despite the important current knowledge, further studies are needed to understand in detail the changes in the lipid profile of PKU patients. As highlighted in this review, previous studies reported that lipoprotein lipids and FA composition of plasma/serum and RBC can be changed in PKU patients. Such changes can affect molecular species of different lipid classes, and, in particular, changes in PL can be expected.

PL, the major building blocks of biological membranes, incorporate about 50% of the total amount of FA in the plasma. PL are important players in the regulation and

control of cellular functions in health and in disease [116]. It is widely recognized that disturbances in PL homeostasis are associated with several diseases and their study can give new insights in the knowledge of disease pathophysiology, new prognosis biomarkers, or risk of other comorbidities [116,117]. However, changes in the PL profile of PKU patients have never been explored.

Lipidomics studies are needed to face the lack of knowledge regarding changes in molecular species of PL, but also of other lipid classes, in PKU. Such knowledge would contribute to understand the lipid metabolism adaptation in PKU patients, monitor their outcome, namely concerning the risk for other chronic diseases, and find possible prognosis biomarkers.

I.4. AIM OF THE WORK

Despite the changes already reported at the level of lipoproteins components and fatty acid (FA) profile, as well as the evidences of increased oxidative stress and lipid peroxidation in PKU patients, further studies are needed to understand in detail the changes in the lipid profile of PKU patients, in particular at the level of phospholipids (PL), which incorporate about 50% of the total amount of FA in plasma and have important signalling and regulatory functions.

Therefore, the main aim of this work was to study the changes in the plasma phospholipidome of children with PKU in order to identify the occurrence of alterations in the lipid metabolism. For this, plasma phospholipid-enriched extracts obtained from children with PKU and healthy children (control group, CT) were analysed by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) for identification of polar lipid species. Complementarily, gas chromatography-mass spectrometry (GC-MS) analysis was performed for determination of FA composition.

II. Material and Methods

The text of this chapter was submitted for publication in the following article:

I.M.S. Guerra, H L. Diogo, M. Pinho, T. Melo, P. Domingues, M.R. Domingues, A.S.P. Moreira, Plasma phospholipidomic profile differs between children with phenylketonuria and healthy children, *Submitted* (30 December 2020).

II.1. STUDY DESIGN OVERVIEW

Plasma samples were obtained from PKU children (*n* = 15) between 3 and 17 years old, followed at the Centro de Referência de Doenças Hereditárias do Metabolismo - Centro Hospitalar Universitário de Coimbra (Coimbra, Portugal). With an average of 9 years old, the studied group included 7 females and 8 males. Patients were on a lifelong Phe-restricted diet, prescribed with Phe-free essential amino acid formulas (13 patients) and one of two types of PUFA supplements: KeyOmega® (Vitaflo) powdered blend of 200 mg AA and 100 mg DHA per sachet (3 patients), or DHA Richoil™ (D.M.F. Dietetic Metabolic Food SRL) containing 250 mg of DHA per capsule (3 patients), according to Phe tolerance and PUFA plasma levels. For the controls (CT, *n* = 12), recruited among those submitted to blood collection for other causes (*e.g.*, minor surgery procedures) and in good general health and normal diet, the age of individuals varied from 7 to 16 years, with an average of 12 years old and included 4 females and 8 males. The blood samples were collected in the fasted state. Blood (2 mL) was collected into lithium-heparin tubes and centrifuged (2000 g, 10 min) to recover the plasma fraction. After collection, the plasma samples of the children were stored at −80°C until further study of the lipid profile. The study was approved by the Hospital Ethics Committee and all parents (or legal representatives) signed informed consent.

II.2. EXTRACTION OF PLASMA PHOSPHOLIPIDS

The phospholipids (PL) from each plasma sample were obtained by solid phase extraction (SPE) using a Visiprep SPE vacuum Manifold (Supelco, Sigma-Aldrich, Bellefonte, PA) [118,119]. Briefly, proteins precipitation was performed by mixing 100 µL of plasma sample with 900 µL of acetonitrile (ACN) with 1% formic acid, followed by vortex (30 s) and centrifugation (2000 rpm, 5 min). The resulting supernatant was transferred to the SPE column (HybridSPE-Phospholipid 30 mg, SUPELCO, Sigma-Aldrich, Bellefonte, PA), previously conditioned with 1 mL of ACN. After elution of almost all the supernatant, the column was then washed with 1 mL of ACN with 1% formic acid and 1 mL of ACN. The PL retained on the Hybrid SPE-PL column were eluted with two consecutive 1 mL aliquots of ACN with 5% aqueous ammonia. This fraction was collected, dried under a nitrogen stream, dissolved in dichloromethane and filtered using a HAMILTON glass syringe and

syringe filter (0.22 µm pore size, 4 mm diameter, Millex-GV Durapore® (PVDF) membrane, hydrophilic, Millipore). The filtered samples were collected in amber vials, dried again and stored at -80 ºC until further analysis.

II.3. PHOSPHOLIPIDS QUANTIFICATION BY PHOSPHORUS MEASUREMENT

The quantification of the total amount of phospholipids (PL) recovered after extraction was performed by phosphorus (P) measurement according to the method of Bartlett and Lewis [120], as previously described [118,121]. PL-enriched extracts were dissolved in 100 µL of dichloromethane, and a volume of 10 µL was transferred, in duplicate, to a glass tube, previously washed with 5% nitric acid. The solvent was dried under a nitrogen stream, and a volume of 125 µL of 70% perchloric acid was added to each tube. Samples were incubated in a heating block (Stuart, U.K.) for 1 h at 180 °C. After cooling to room temperature, a volume of 825 µL of Milli-Q water, 125 µL of ammonium molybdate (25 g L^{-1} prepared in Milli-Q water), and 125 µL of ascorbic acid (100 g L^{-1} prepared in Milli-Q water) were added to each sample, with vortex mixing between each addition. Samples were then incubated in a water bath at 100 °C for 10 min. Afterwards, samples were immediately cooled down in a cold-water bath. The absorbance was measured at 797 nm in a Multiskan GO1.00.38 Microplate Spectrophotometer (Thermo Scientific,Hudson, NH, USA) controlled by SkanIT software, version 3.2 (Thermo Scientific).The P content of each extract was determined from a calibration curve prepared by performing the same procedure (without the heating block step) with standards containing 0.1 to 2 µg of P prepared from a sodium dihydrogen phosphate dihydrate solution (100 µg mL^{-1} of P). The total amount of PL was then estimated by multiplying the amount of P by 25 [122].

II.4. CHARACTERIZATION OF FATTY ACIDS PROFILE BY GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (GC-MS)

Fatty acids (FA) were analysed by gas chromatography-mass spectrometry (GC-MS) after transmethylation of phospholipid (PL)-enriched extracts according to the method described by Aued-Pimentel *et al* [123], routinely used in authors' laboratory [119,121]. Briefly, an amount of lipid extract containing 15 µg of total PL was dissolved in 1 mL of internal standard methyl nonadecanoate (Sigma, St. Louis, MO, USA) prepared in *n*-hexane (1.135 µg mL^{-1}). FA were converted to fatty acid methyl

esters (FAME) by adding 200 µL of methanolic KOH solution (2 M) and intense vortex-mixing for 1-2 min. A volume of 2 mL of saturated NaCl solution (10 g L^{-1}) was then added. After centrifugation at 2000 rpm for 5 min, a volume of 600 µL of the organic phase was collected and dried under a nitrogen stream. The resulting FAME derivatives were dissolved in 100 µL of n-hexane and 2 µL were then used for GC-MS analysis (Agilent Technologies 8860 GC System, santa Clara, CA, USA) The GC equipment was connected to an Agilent 5977B Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 58 °C for 2 min, a linear increase to160 °C at 25 °C min^{-1}, followed by a linear increase at 2 °C min^{-1} to 210 °C, then at 20 °C min^{-1} to 225 °C, standing at 225 °C for 15 min. The injector and detector temperatures were 220 and 230°C, respectively. Helium was used as the carrier gas at a flow rate of 1.4 mL min^{-1}. The data acquisition software used was GCMS5977B/Enhanced MassHunter. The acquired data were analysed using the software Agilent MassHunter Qualitative Analysis 10.0. FA identification was performed by MS spectrum comparison with the chemical database NIST library and "The Lipid Web" [124], and considering the retention times and MS spectra of FAME standards(Supelco 37 Component FAME Mix, Sigma-Aldrich, Darmstadt, Germany). FA quantification was performed using calibration curves obtained from FAME standards under the same instrumental conditions.

II.5. CHARACTERIZATION OF THE PHOSPHOLIPID PROFILE BY HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION TANDEM MASS SPECTROMETRY (HILIC-MS/MS)

Phospholipid (PL)-enriched extracts were analysed by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) using a high-performance liquid chromatography (HPLC) system (Ultimate 3000 Dionex, Thermo Fisher Scientific, Bremen, Germany) with an autosampler coupled online to the Q-Exactive® hybrid quadrupole Orbitrap® mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) [118,119]. The solvent system consisted of two mobile phases: mobile phase A [ACN/MeOH/water 50:25:25 (v/v/v) with 5 mM ammonium acetate] and mobile phase B [ACN/MeOH 60:40 (v/v) with 5 mM

ammonium acetate]. Initially, 5% of mobile phase A was held isocratically for 2 min, followed by successive linear increases to 70% of A within 11 min, and then to 90% of A in 7 min. The solvent (90% of A) was held isocratically for 30 min, returning to the initial conditions in 5 min, followed by a re-equilibration period of 5 min prior next injection. In a glass vial with a micro-insert, a volume of 5 µL of each PL-enriched extract, previously resuspended in dichloromethane (1 µg μL^{-1} PL), was added to 4 µL of a mixture of PL standards (Avanti Polar Lipids, Inc., Alabaster, AL; 0.02 µg of dimyristoyl phosphatidylcholine, 0.02 µg of dimyristoyl phosphatidylethanolamine, 0.012 µg of dimyristoyl phosphatidylglycerol, 0.04 µg of dimyristoyl phosphatidylserine) and 91 µL of starting eluent system (95% of eluent B and 5% of eluent A). A volume of 5 µL of this mixture was then introduced into the ACE®HILIC-N column (100 mm × 1 mm, 3 µm) with a flow rate of 50 µL min^{-1}. The column oven was maintained at 35 ºC. The mass spectrometer with Orbitrap technology was operated in a positive/negative switching toggles between positive (electrospray voltage of 3.0 kV) and negative (electrospray voltage of −2.7 kV) ion modes with a capillary temperature of 250 ºC and sheath gas flow of 15 U.A top-10 data-dependent method was used. Acquisition cycles consisted of one full scan mass spectrum at high resolution (resolution of 70,000 and automatic gain control (AGC) target of 1×10^6) and ten data-dependent MS/MS scans (resolution of 17,500 and AGC target of 1×10^5) that were repeated continuously throughout the experiments with the dynamic exclusion of 60 s and intensity threshold of 2×10^4. A stepped normalized collisional energy of 25, 30, and 35 eV was used. Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA). MZmine 2.32 software [125] was used for filtering LC-MS raw data, peak detection, peak processing, and assignment against an in-house database. Only peaks with an intensity higher than 1×10^4 were considered. The validated peaks were within the typical retention time of the respective lipid class and exact mass accuracy with an error of less than 5 ppm. The identification of the most lipid species was further validated by analysis of the MS/MS data, allowing to confirm polar head group and find the fatty acyl chain(s) for most of the molecular species. The normalization of the LC-MS data was performed by exporting the integrated peak areas of the extracted ion chromatograms and dividing the peak area value of each species by the peak area value of the lipid standard with the closest retention time.

II.6. STATISTICAL ANALYSIS

Multivariate and univariate analyses were performed using R version 3.6.2 [126] in Rstudio version 1.2.5 [127]. The R package Metaboanalyst was used for imputation and log transformation of the HILIC-MS data (lipid species) and GC-MS data (fatty acids) [128]. EigenMS was also used for normalization of LC-MS data [129]. Principal component analysis (PCA) was performed with the R built-in function and the R package pcaMethods [130]. Shapiro–Wilk test followed by Welch t-test (for variables with normal distribution) or Mann-Whitney (for variables with non-normal distribution) were performed with R built-in functions. P-values were corrected for multiple testing using Benjamin-Hochberg method (with R built-in function) for the false discovery rate (FDR, *q*-values) [131]. Heatmaps were created from autoscaled data using the R package pheatmap [132], "Euclidean" as clustering distance, and "ward.D" as the clustering method. All graphics and boxplots were created using the R packages ggplot2 [133], plyr [134], dplyr [135], tidyr [136] and ggrepel [137].

III. RESULTS

The text and figures/tables of this chapter was submitted for publication in the following article:

I.M.S. Guerra, H L. Diogo, M. Pinho, T. Melo, P. Domingues, M.R. Domingues, A.S.P. Moreira, Plasma phospholipidomic profile differs between children with phenylketonuria and healthy children, *Submitted* (30 December 2020).

III.1. PHOSPHOLIPIDS QUANTIFICATION

The total amount of phospholipids (PL) (expressed in µg PL 100 μL^{-1} of plasma; mean ± standard deviations) obtained from PKU samples was 41.27 ± 7.30 and from the CT group was 53.78 ± 21.73. No significant differences were found by univariate analysis between the two groups (*q*-value > 0.05).

III.2. FATTY ACID COMPOSITION ANALYSIS BY GC-MS

For an overview of the composition of esterified fatty acids (FA), GC-MS analysis was performed. A total of 9 FA were identified in the PKU and CT groups (Table III.1). Palmitic acid (16:0) was the most abundant lipid in both groups (30.75 ± 6.94 in PKU and 22.91 ± 7.48 in CT), followed by stearic acid (18:0) (18.80 ± 4.92 in PKU and 13.09 ± 3.77 in CT) and linoleic acid (18:2*n*-6) (17.74 ± 4.62 in PKU and 12.21 ± 6.73 in CT).

Table III.1. Fatty acid (FA) profile of enriched in phospholipid (PL) obtained from plasma samples of phenylketonuric (PKU) and healthy children (control, CT), determined by GC-MS analysis of fatty acid methyl ester derivatives. The values show are means (µg FA 100 µg PL^{-1}) ± standard deviations.

Fatty acids	PKU	CT
14:0	1.45 ± 0.32	1.08 ± 0.34
16:0	30.75 ± 6.94	22.91 ± 7.48
18:0	18.80 ± 4.92	13.09 ± 3.77
18:1*n*-9	13.04 ± 2.81	8.09 ± 2.24
18:1	4.24 ± 0.47	3.55 ± 0.25
18:2*n*-6	17.74 ± 4.62	12.21 ± 6.73
20:4*n*-6	10.40 ± 3.31	7.76 ± 3.12
20:5*n*-3	2.81 ± 0.35	2.58 ± 0.11
22:6*n*-3	5.37 ± 1.14	4.17 ± 1.14

Multivariate analysis by principal component analysis (PCA) analysis was performed using the FA data set. The PCA score plot of the two first dimensions (Dim) showed that the two groups were not differentiated, with the model capturing 84.7% of the total variance in the data set (Dim 1: 75.4%; and Dim 2: 9.3%) (Figure III.1).

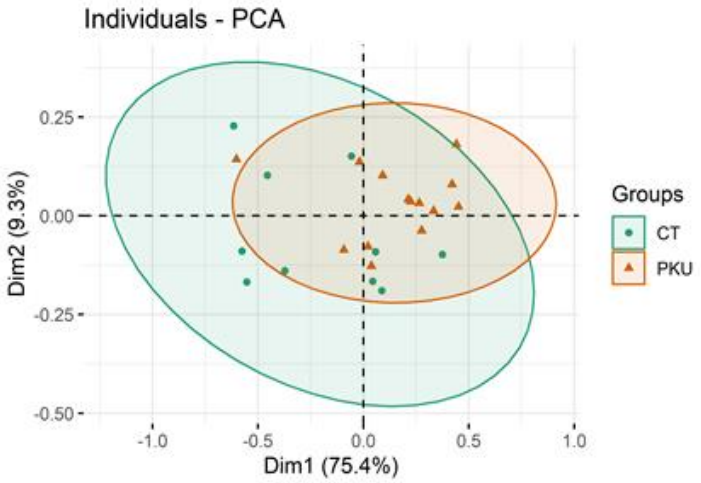

Figure III.1. Principal component analysis (PCA) score plot of the fatty acids data set obtained by GC-MS analysis of phospholipid-enriched extracts obtained from plasma samples of phenylketonuric (PKU) and healthy children (control, CT).

Univariate analysis (Shapiro–Wilk test followed by Welch *t*-test or Mann-Whitney test) was used to assess the existence of significant differences in the FA composition by comparing the two groups (CT *vs* PKU). Of the 9 FA identified, 6 showed significant differences between groups, with higher content in children with PKU when compared to the CT group (Figure III.2). The most significant increases were observed in oleic acid (18:1*n*-9) (*q*-value <0.01), followed by myristic acid (14:0), stearic acid (18:0), eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3), (*q*-value < 0.05). No statistical difference was observed for palmitic acid (16:0), linoleic acid (LA, 18:2*n*-6), and arachidonic acid (AA, 20:4*n*-6).

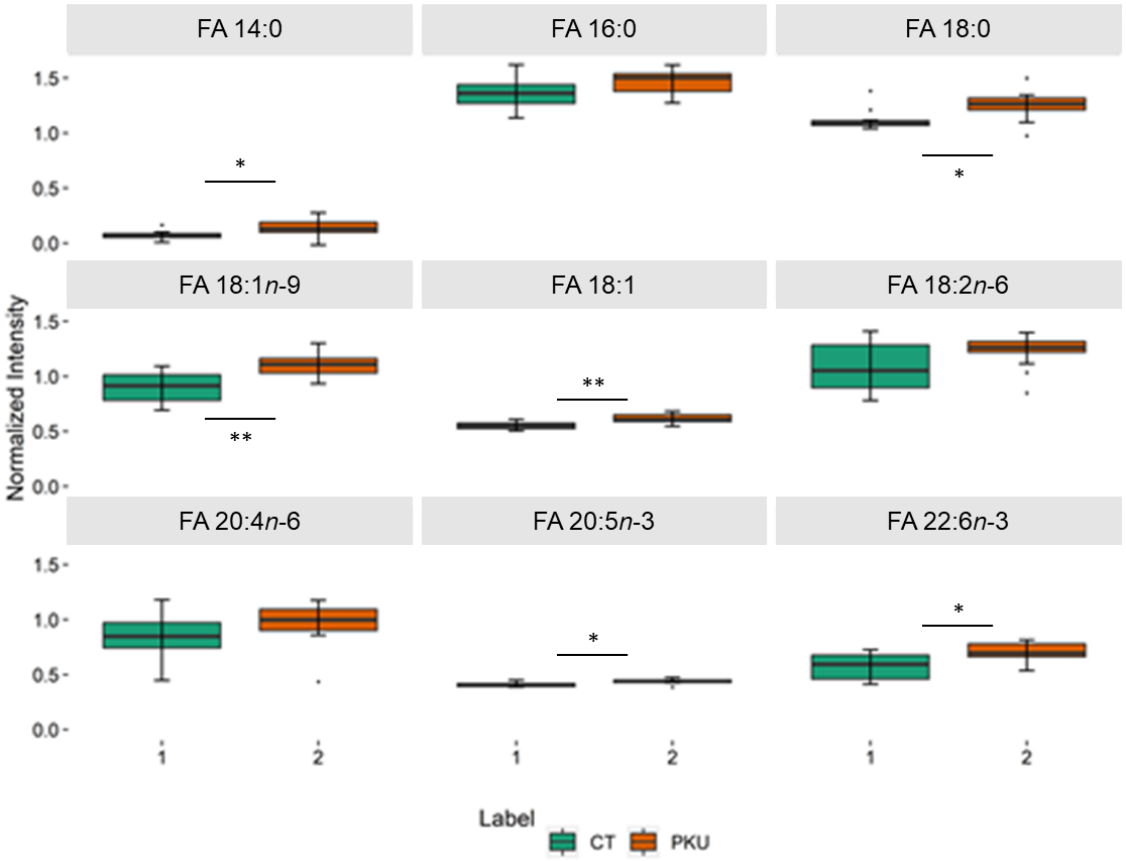

Figure III.2. Boxplots of the fatty acids (FA) identified by GC-MS from control (CT) and phenylketonuric (PKU) children sorted (left to right, top to bottom) by increasing number of carbon atoms and double bonds. FA are labelled as follows: FA xx:i (xx=number of carbon atoms; i= number of double bonds), followed by *n*-y for unsaturated FA (y=double-bond position related to the methyl end). Significant differences between the groups revealed by Welch t-test or Mann-Whitney test are identified by horizontal lines and marked with * if $q < 0.05$ and ** if $q < 0.01$.

III.3. IDENTIFICATION OF THE PHOSPHOLIPID PROFILE BY HILIC-MS/MS

The plasma phospholipid profile of the two experimental groups (PKU and CT) was characterized using high-resolution HILIC-MS/MS. In total, we identified 187 species of phospholipids (PL) (*m/z* values), belonging to 9 different classes: phosphatidylcholine (PC; including diacyl, alkyl/alkenyl-acyl and oxidized species), lysophosphatidylcholine (LPC; including monoacyl, alkyl/alkenyl and oxidized species), sphingomyelin (SM), phosphatidylethanolamine (PE; including diacyl, alkyl/alkenyl-acyl and oxidized species), lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG), lysophosphatidylglycerol (LPG), phosphatidylinositol (PI), and phosphatidylserine (PS). In addition to the PL species, 3 species of

shingolipids belonging to the class of ceramides (Cer) have also been identified (Supplementary Table S1).

The PC, LPC, SM and Cer species were identified and semi-quantified in the positive mode as $[M+H]^+$ ions. A total of 77 PC, 33 LPC, 21 SM and 3 Cer were identified. Confirmation of the PC and LPC phospholipid classes was obtained by analysing the MS/MS data of $[M+H]^+$ ions with the identification of the product ion of exact mass 184.0739 (error < 5ppm), corresponding to the phosphocholine polar head (Figure S1a). The FA composition of the PC and LPC species was determined, from the negative mode MS/MS data of the $[M+CH_3COO]^-$ ions, by identifying the of product ions corresponding to the fatty acyl chains such as $[RCOO]^-$ (Figure S1b). As observed for PC and LPC, the MS/MS spectra of the SM species, observed in the positive mode as $[M+H]^+$ ions, showed an abundant product ion with an exact mass of 184.0739, corresponding to the polar head group of phosphocholine (Figure S2a). Distinctly from PC and LPC which have even *m/z* values, SM have odd *m/z* values. Also, the MS/MS spectra of $[M+H]^+$ ions of SM showed product ions of the sphingoid base chain, allowing the sphingoid base and FA amide substituent to be deduced (Figure S2a).Confirmation of SM species was also obtained by the identification in the MS/MS spectra of $[M+CH_3COO]^-$ ions of the product ion at *m/z* 168.0425, corresponding to the phosphocholine polar head without a methyl group, and observing the characteristic neutral loss of 74 Da, corresponding to the loss of methyl acetate (Figure S2b). The MS/MS fragmentations of the sphingoid base of Cer, in the positive mode as $[M+H]^+$ ions, allowed to confirm the identification of the respective sphingoid base and the FA amide substituent (Figure S3).

PE, LPE, PG, LPG, PI and PS species were identified and semi-quantified in the negative mode as $[M-H]^-$ ions. Overall, 27 PE, 1 LPE, 11 PG, 5 LPG, 8 PI and 4 PS species were identified. Confirmation of the phospholipid class for the PE and LPE species was obtained, from MS/MS data in the positive, of $[M+H]^+$ ions, the identifying the typical neutral loss of 141 Da, corresponding to the polar head of phosphoethanolamine (Figure S4a). The product ion at *m/z* 171.0058, corresponding to the glycerol phosphate anion, in the MS/MS spectra of $[M-H]^-$ ions of PG and LPG species that allow these classes of phospholipids to be confirmed, was not visible due to the low intensity of the respective molecular ions (Figure S5). These species were identified based on the retention time (RT) and exact mass measurements

Confirmation of the phospholipid class for PI species, in the negative mode as $[M-H]^-$ ions, was obtained by identifying the product ion at *m/z* 241.0113, corresponding to the inositol head group (Figure S6). The FA composition of PE, LPE, PG, LPG and PI was confirmed, in the MS/MS spectra of the $[M-H]^-$ ions, by identification of the product ions corresponding to the fatty acyl chains such as $[RCOO]^-$. The identification of the PS species was based on the mass accuracy (error < 5 ppm) and retention time. As an example, MS/MS spectra of isomeric plasmanyl and plasmenyl species, as well as of oxidized PL species are also shown in Supplementary figures (Figures S7 and S8).

To visualize sample grouping, the multivariate analysis by PCA was performed on the lipid species data set. The PCA score plot of the lipid species showed that the two groups were separated in the first two dimensions, and the model captured 62.9% of the total variance (Dim1: 51.2%; and Dim2: 11.7%) (Figure III.3). The two groups (CT and PKU) were separated along Dim1, the main discriminating component, with the CT group located at negative values of Dim1 and the PKU group located at positive values of Dim1 (Figure III.3).

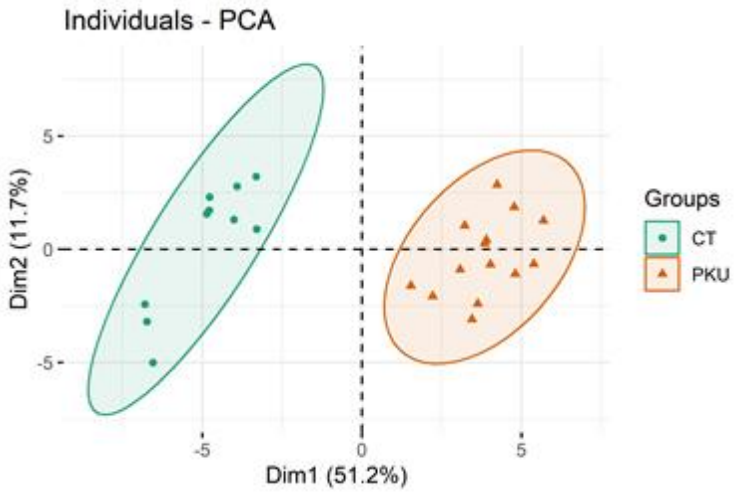

Figure III.3. Principal Component Analysis (PCA) score plot of the lipid species dataset obtained by HILIC-MS/MS analysis of phospholipid-enriched extracts obtained from plasma samples of phenylketonuric (PKU) and healthy (control, CT) children.

Univariate analysis (Shapiro–Wilk test followed by Welch *t*-test or Mann-Whitney test) was also performed to evaluate the lipid species with significant variation between the two groups (CT *vs* PKU). The results showed that 146 of 190

lipid species were significantly different between groups (q-value < 0.05) (Supplementary Table S2). Boxplots of the 16 main species with the lowest q-values (q-value < 0.001) are shown in Figure III.4. These 16 species that showed major variation included 7 PC, 5 SM, 3 PS, and 1 LPC. All of these PC species (40:8, 44:12, 42:7, 40:7, 44:11, 42:9 and 40:5) were significantly increased in PKU children compared to CT individuals. Also, LPC (14:0) and SM (d34:1, d36:3, d36:2, d34:2, and d38:3) showed the same trend as that observed in PC species. In contrast, PS species (38:4, 40:6, and 38:6) were significantly reduced in PKU individuals. It was possible to identify the FA composition of 4 of the 16 species, namely of PC (40:8) identified as PC (20:4/20:4), PC (42:7) identified as PC (20:1/22:6), PC (40:7) identified as PC (18:1/22.6), and SM (d34:1) identified as SM (d18:1/16:0).

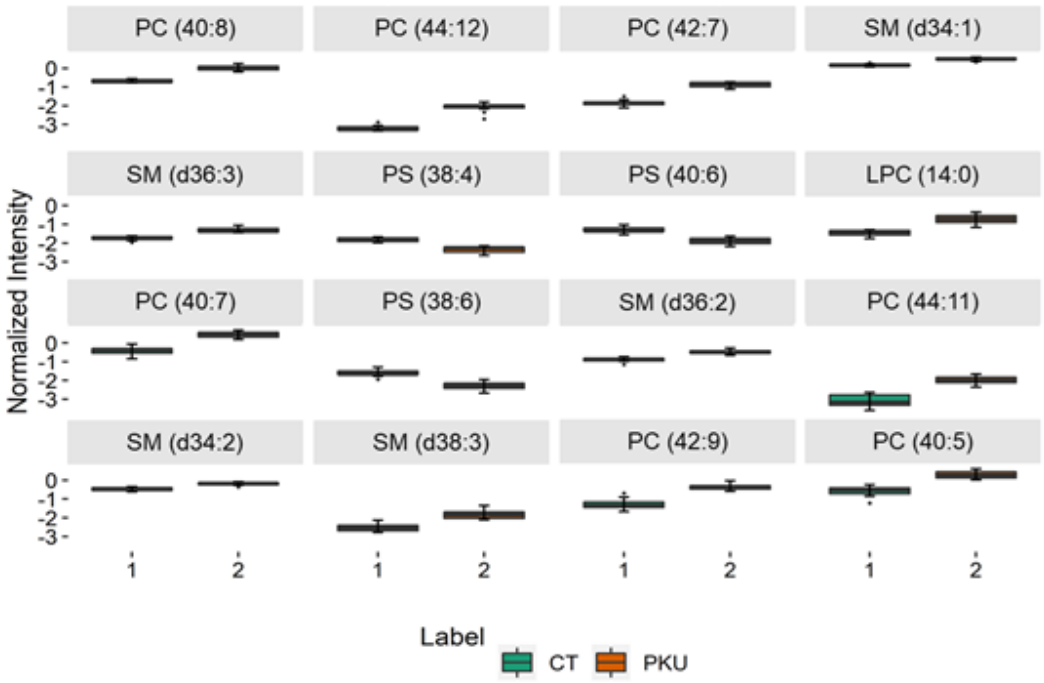

Figure III. 4. Boxplots of the 16 main lipid species showing the major variation between control (CT) and phenylketonuric (PKU) children sorted (left to right, top to bottom) by the lower q-values of the Welch t-test or Mann-Whitney test. All phospholipid species displayed in the figure showed a q-value < 0.001 after Benjamin-Hochberg correction for the false discovery rate (FDR). The phospholipid species are labelled as follows: AAAA (xx:i) (AAAA=lipid class abbreviation; xx=number of carbon atoms in fatty acid(s); i=number of double bonds). Abbreviations of the lipid classes: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin.

The results of the univariate analysis were used to create a heatmap (double dendrograms) with two-dimensional hierarchical clustering, using the top 25 q-values (q-value < 0.001) the Welch t-test or Mann-Whitney test (Figure III.5.). The primary

split in the upper hierarchical dendrogram showed the samples clustered independently into two groups: one cluster for the CT group, and another for the PKU group. The separation of the CT group from the PKU group was consistent with what was observed in the PCA score plot (Figure III.3). The clustering of individual lipid species with respect to their similarity in changes in lipid expression showed two groups at the first level of the dendrogram (on the left of the heatmap in Figure III.5). From top to bottom, the first group included 1 SM (d32:2) and 3 PS (38:4, 40:6 and 38:6), which were more abundant in the CT group. The second group contained 13 PC, 5 SM, 1 LPC, and 2 PI species, which were more abundant in the PKU group. Regarding the composition of these top 25 species, PC species with high total carbon chain length (C38-44) and level of unsaturation (4-12), bearing polyunsaturated fatty acids (PUFA), were more abundant in the PKU individuals.

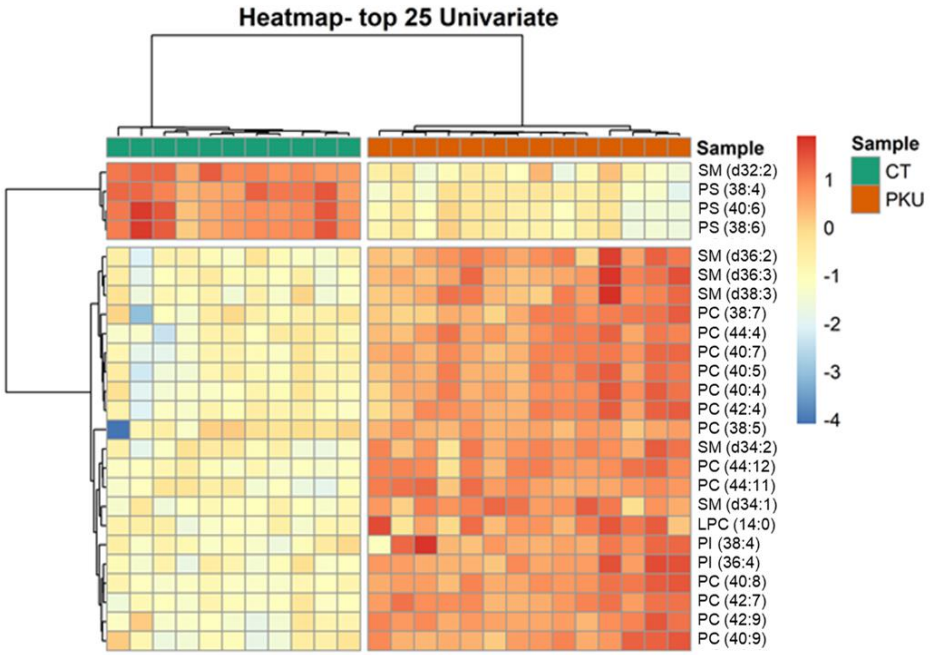

Figure III. 5. Two-dimensional hierarchical clustering heatmap of the 25 main lipid species with the lowest *q*-values of the Welch t-test or Mann-Whitney test. The dendrogram at the top represents the clustering of the samples, and the dendrogram on the left represents the clustering of the lipid species. Levels of relative abundance are indicated on the colour scale, with numbers indicating the fold difference from the mean. Phospholipid species are labelled as follows: AAAA (xx:i) (AAAA=lipid class; xx=number of carbon atoms in fatty acid(s); i=number of double bonds). Abbreviations of the lipid classes: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

IV. DISCUSSION

The text of this chapter was submitted for publication in the following article:

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Previous studies have revealed changes in lipoprotein levels of serum/plasma and the fatty acid profile of plasma and red blood cells of PKU individuals [138]. Such changes can affect the composition phospholipids (PL), as they are the main components of human plasma lipoproteins and incorporate FA into their composition [139]. Indeed, some of the changes reported in the FA profile of PKU individuals have been found in the plasma PL fraction [57,58,64,66,67,77]. To our knowledge, this study represents the first report comparing plasma phospholipidome of PKU children and healthy children, highlighting the changes in the lipid profile of PKU children at the level of individual lipid species.

Firstly, by a complete analysis of the FA composition of the plasma PL fraction, a total of 9 FA was identified. The profile of FA identified is consistent with the profile previously reported in the literature for the plasma/serum PL fraction, including as the most abundant FA, the SFA 16:0 (palmitic acid) and FA of 18 carbon chains [119,140]. According to the multivariate analysis of the FA dataset by PCA (Figure III.1), the PKU and CT groups were not separated.

In terms of univariate analysis of the FA dataset, the PKU group showed a significant increase in the level of PUFA docosahexaenoic acid (DHA, 22:6*n*-3) and eicosapentaenoic acid (EPA, 20:5*n*-3), compared to the CT group (Figure III.2). Consistent with these results, in seven studies [56–58,77,78,141,142], higher levels of DHA in the total plasma/serum PL fraction were observed in PKU individuals supplemented with PUFA. Only two of these seven studies showed an increased level of EPA in plasma [56,77], as observed in this study. It is known that, in healthy individuals, the conversion of ALA to DHA is only 2% to 5% and to EPA is less than 5% to 10% [143]. Consequently, DHA and EPA status is determined by food intake [144]. As PKU individuals do not consume the typical food sources in DHA, such as fish, the increase of DHA level observed in this study could be a consequence of the PUFA supplements in the case of patients with PUFA supplementation, which include DHA in their composition. However, it is known that dietary DHA causes in a dose-dependent, and saturable increase in plasma DHA level and modest increases in EPA. DHA itself can serve as a substrate for metabolic retroconversion to EPA through a β-oxidation reaction [145]. Such a reaction may also have contributed to the increased level of EPA observed for the PKU group in the present study. On the other hand, the

levels of arachidonic acid (AA, 20:4*n*-6) did not have statistical differences between the PKU and CT groups, which is consistent with other studies [56–58].

Overall, the effect of long-chain PUFA supplements on the FA profile of PKU patients reveals an increase in the content of *n*-3 PUFA [138], especially DHA and EPA, as observed in this study. Such an increase can have positive effects the health of PKU patients. It is well known that PUFA are important structural and functional constituents of all cell membranes and are essential for the normal visual and cognitive development [146,147]. Also, DHA and EPA have been associated with anti-inflammatory properties [148,149]. The increase in DHA and EPA promotes the increased generation of anti-inflammatory cytokines and the generation of special pro-resolving lipid mediators, such as resolvins, protectins and maresins [148].Therefore, it is possible to speculate that the DHA supplementation would result in an increased capability to produce special pro-resolving lipid mediators and a decrease in the inflammatory state. Such an anti-inflammatory action may be relevant, considering that a pro-inflammatory state has already been described in patients with PKU [89,147]. Furthermore, the higher levels of *n*-3 PUFA are associated with a reduction in the risk of developing cardiovascular diseases and atherosclerosis [146,148,150,151]. Despite the health benefits associated with consuming PUFA, particularly *n*-3 PUFA, it should be noted that the question about the dosage of PUFA needed to achieve optimal effects has remained unanswered.

In contrast to the PCA of the FA profile, the PCA of the lipid species showed a separation between the PKU and CT groups (Figure III.3), highlighting the importance of the study at the level of the lipid species. The results of the univariate analysis (Figure III.4 and III.5) revealed a significant increase in the PC, SM and PI species and a decrease of PS species in the PKU group.

Lipid species of the PC class, the PL class most abundant in biological membranes and in plasma lipoproteins [139,152], showed a significant increase in PKU children compared to CT. Thirteen PC species were in the top 25 lipid species with major variation. Among these, we were able to identify the composition of FA for 6 of them: PC (38:5) – (18:1/20:4); PC (38:7) – (16:1/22:6); PC (40:4) – (18:0/22:4) and (20:0/20:4); PC (40:7) – (18:1/22:6); PC (40:8) – (20:4/20:4); and PC (42:7) – (20:1/22:6). Although the composition of FA was not found for the remaining 7 PC species, it was possible to deduce that they also contain PUFA in their composition,

since they are PC with a high total carbon chain length (C40-44) and a high level of unsaturation (4-12). The increased number of plasmatic PC bearing long-chain PUFA in PKU children may be a consequence of their daily PUFA supplementation.

Beyond the common diacyl PC, it has also been observed up-regulation of some ether PC (3 species with $q < 0.001$, 3 with $q < 0.01$ and 5 with $q < 0.05$), as well as ether PE (6 species with $q < 0.001$, 4 with $q < 0.01$ and 1 with $q<0.05$) in PKU children, both representing 15.06% of all statistically significant lipid species (Supporting Information, Table S2). The physiological functions of these unique alkyl-acyl PL are not fully understood. However, it has been reported that ether-linked PL, especially plasmalogens, have antioxidant properties due to their ability to scavenge oxygen radicals, as they are preferentially oxidized [153–155]. The increase of some ether-like PC and PE may be beneficial for PKU children, because they can decrease oxidative stress and lipid peroxidation [138].

Regarding the increase of PI species, namely PI (36:4) and PI (38:4), observed in PKU children, it is known that PI species influence the structure and function of plasma lipoproteins, but their role in plasma is unclear and requires further investigation [156]. Alterations in PI content are probably associated with their signalling roles, as they are important precursors of signalling molecules, such as phosphatidylinositol phosphates (PIPs) that regulate metabolic processes [157]. Therefore, we speculate that the observed increase in PI species could be associated with a decrease in its phosphorylation to phosphoinositides, by phosphatidylinositol kinases (*e.g.* phosphatidylinositol 3-kinase), due to the reduction in oxidative stress [158].

PS is the major class of anionic phospholipids in eukaryotic membranes [159]. The decrease in their levels in the PKU group, namely of PS (38:4), PS (38:6) and PS (40:6), may occur due to the increased biosynthesis of PC, because mammalian cells produce PS by exchanging the headgroup for serine [159]. It should be noted that the full function of plasma PS is not yet known, and their downregulation is not clearly understood. However, the PS class, at the cellular level, has been associated with anti-inflammatory roles [160]. In the future, it is important to study the roles of this class in the plasma.

SM is the most abundant class of sphingolipids in lipoproteins, constituting about 20% of total plasma PL. This class of lipids can be hydrolysed by the action of

sphingomyelinase, giving rise to Cer, which by the action of sphingomyelin synthase are again converted to SM. Our study reports a significant increase of 6 SM in the plasma of PKU children, compared to the CT group. Previous studies have reported that higher plasma levels of SM are associated with increased atherosclerosis and risk factors for coronary heart disease in adults [161]. Consequently, in the future, it is necessary to clarify the variation and the significance of elevated levels of this class of lipids in children with PKU.

The lipidomic approaches used here revealed alterations in the PL profile of PKU patients. Such alterations could have clinical application for monitoring the disease and possible comorbidities, as well as therapeutic response. Nonetheless, we recognize that the strength of our results is limited, namely by the small sample size and variable PUFA supplementation in the PKU group. However, this first study is important to give new clues for subsequent studies taking into account the prevalence of PKU (a rare disease) and the specific study population, children (among whom it might be quite difficult to obtain samples for research analysis). Also, a longitudinal analysis is needed to assess the overall progression of changes in the PL profile over time in PKU patients, compared to controls.

V. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

PKU is a disease of phenylalanine (Phe) catabolism, caused by deficiency of the enzyme phenylalanine hydroxylase. Therapeutic is based on Phe restriction in the diet, which requires a reduction of normal food stuff. Dietary restrictions can lead to imbalances in specific nutrients, notably lipids. Previous studies showed changes in the level of lipoproteins components and FA profile (including FA profile of PL fraction), as well as evidences of increased oxidative stress and lipid peroxidation in PKU patients. However, changes at level of individual PL species in PKU patients have never been explored.

According to the proposed objectives, the present study reports the GC-MS analysis of FA composition, as well as the application of HILIC-MS/MS to identify the plasma phospholipidome of phenylketonuric children and healthy children. Thus, it was possible to detect that PKU children showed significant differences in the FA profile and in the identified PL species, when compared with healthy children. The changes in the profile of the esterified FA in PL of PKU children, pointed by the increased in the *n*-3 PUFA, namely DHA and EPA, may be associated with the PUFA supplementation in the case of the patients with such supplementation. In terms of individual PL species, the most significant changes were revealed by the increase of the plasmatic levels of PC, PI and SM and the decrease of PS species. In particular, it was observed an increase in several PC with PUFA. The increase content of PUFA-containing PL species could have a positive impact in the reduction of the inflammatory state of patients, as well as in the reduction of the risk of development cardiovascular diseases and atherosclerosis. Also, PKU group showed an increase of ether-like PC and PE, which are associated to antioxidant activity. Regarding to the higher levels of SM, it is necessary to clarify the variation and the meaning of this lipid class in children with PKU, since they have already been associated with atherosclerosis and with the risk factors for coronary heart disease in human adults.

Further studies using MS-based lipidomics are needed to understand in detail the changes in the FA and PL profile of PKU patients. Also, a more representative phenylketonuric population are needed in order to validate the obtained results. It should be interesting to make a comparative study between phenylketonuric patients, since not everyone has the same degree of illness.

Overall, these results obtained may open new research avenues towards a greater understanding of the PL role in this disease.

VI. REFERENCES

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VII. SUPPLEMENTARY INFORMATION

Supplementary Table S1. Phospholipids and sphingolipids identified by HILIC-MS and MS/MS of phospholipid (PL)-enriched extracts obtained from plasma samples collected, in fasting, from PKU patients and healthy controls (mass error < 5 ppm). Observed *m/z* values and respective errors were checked for all samples. Lipid species are labelled as follows: AAAA (xx:i) (AAAA=lipid class abbreviation; xx=number of carbon atoms in fatty acid(s); i=number of double bonds). The 'O-' prefix is used for plasmanyl species to indicate the presence of an alkyl ether substituent, whereas the 'P-' prefix is used for plasmenyl species to indicate the alk-1-enyl ether substituent. Molecular species with known fatty acyl constituents are labelled using (*sn*-1/*sn*-2) nomenclature, assuming that in animals the *sn*-1 position generally contains smaller and saturated fatty acids, while fatty acids with a higher unsaturation degree are located in *sn*-2. *, Lipid species identified only by retention time and mass accuracy. **, Lipid species identified by retention time, mass accuracy and confirmation of polar head group by MS/MS of $[M+H]^+$ ions. Lipid class abbreviations: Cer, ceramide; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Lipid species	Theoretical *m/z*	Observed *m/z*	Error (ppm)	Fatty acyl chain(s)	Formula
Cer identified as $[M+H]^+$					
Cer(d30:1)	482.4573	482.4563	-2.1121	(d18:1/12:0)	C30H60NO3
Cer(d34:1)	538.5199	538.5186	-2.4493	*	C34H68NO3
HexCer(d38:0)	758.6510	758.6522	1.5897	*	C44H88NO8
PC identified as $[M+H]^+$ (determination of fatty acyl chains by MS/MS of $[M + CH_3COO]^-$)					
PC(O-28:0)	664.5281	664.5280	-0.1761	**	C36H75NO7P
PC(O-30:1)/PC(P-30:0)	690.5438	690.5426	-1.6900	**	C38H77NO7P
PC(O-30:0)	692.5594	692.5584	-1.4670	**	C38H79NO7P
PC(30:1)	704.5230	704.5250	2.7934	(14:0/16:1)	C38H75NO8P
PC(30:0)	706.5387	706.5404	2.4316	(14:0/16:0)	C38H77NO8P
PC(O-32:2)/PC(P-32:1)	716.5594	716.5576	-2.5343	(O-16:1/16:1) and/or (P-16:0/16:1)	C40H79NO7P
PC(O-32:1)/PC(P-32:0)	718.5751	718.5756	0.7417	(O-16:0/16:1) and/or (O-16:1/16:0) and (P-16:0/16:0)	C40H81NO7P
PC(O-32:0)	720.5907	720.591	0.3927	(O-16:0/16:0)	C40H83NO7P
PC(32:2)	730.5387	730.5403	2.2148	(16:1/16:1) and (14:0/18:2)	C40H77NO8P
PC(32:1)	732.5543	732.5527	-2.2265	(16:0/16:1) and (14:0/18:1)	C40H79NO8P
PC(32:0)	734.5700	734.5701	0.1606	(16:0/16:0)	C40H81NO8P
PC(O-32:0)+O	736.5856	736.5843	-1.8083	**	C40H83NO8P
PC(O-34:4)/PC(P-34:3)	740.5599	740.5572	-3.7021	**	C42H79NO7P
PC(O-34:3)/PC(P-34:2)	742.5751	742.5763	1.6604	(O-16:1/18:2) and/or (P-16:0/18:2)	C42H81NO7P
PC(O-34:2)/PC(P-34:1)	744.5907	744.5914	0.9173	(O-16:0/18:2) and (P-18:1/16:0)	C42H83NO7P
PC(O-34:1)/PC(P-34:0)	746.6064	746.6059	-0.6255	P-18:0/16:0), (O-16:0/18:1) and possible (O-18:1/16:0)	C42H85NO7P
PC(32:1)+O	748.5492	748.5491	-0.1950	(16:0/16:1+O)	C40H79NO9P
PC(34:5)	752.5230	752.5198	-3.6305	**	C42H75NO8P
PC(34:4)	754.5387	754.5391	0.5540	(14:0/20:4)	C42H77NO8P
PC(34:3)	756.5543	756.5530	-1.7593	**	C42H79NO8P
PC(34:2)	758.5700	758.5710	1.3183	(16:0/18:2)	C42H81NO8P
PC(34:1)	760.5856	760.5847	-1.2254	(16:0/18:1)	C42H83NO8P
PC(P-36:5)	764.5594	764.5579	-1.9841	(P-16:0/20:5)	C44H79NO7P
PC(O-36:5)/PC(P-36:4)	766.5751	766.5757	0.8258	(O-16:1/20:4) and/or (P-16:0/20:4)	C44H81NO7P

PC(O-36:4)/PC(P-36:3)	768.5907	768.5912	0.6284	(P-18:1/18:2) and (O-16:0/20:4)	C44H83NO7P
PC(O-36:3)/PC(P-36:2)	770.6064	770.6035	-3.7204	(O-18:1/18:2) and/or (P-18:0/18:2)	C44H85NO7P
PC(O-36:2)/PC(P-36:1)	772.6220	772.6194	-3.3859	(O-18:0/18:2), (P-18:1/18:0) and (O-18:1/18:1) and/or (P-18:0/18:1)	C44H87NO7P
PC(34:2)+O	774.5649	774.5661	1.5531	(16:0/18:2+O)	C42H81NO9P
PC(34:1)+O	776.5805	776.5773	-3.7948	**	C42H83NO9P
PC(36:6)	778.5387	778.5379	-1.0044	(14:0/22:6)	C44H77NO8P
PC(36:5)	780.5543	780.5535	-1.0646	(16:0/20:5), (16:1/20:4) and (18:2/18:3)	C44H79NO8P
PC(36:4)	782.5700	782.5707	0.9175	(16:0/20:4) and (18:2/18:2)	C44H81NO8P
PC(36:3)	784.5856	784.5857	0.0867	(16:0/20:3) and (18:1/18:2)	C44H83NO8P
PC(36:2)	786.6013	786.5999	-1.7569	(18:0/18:2) and (18:1/18:1)	C44H85NO8P
PC(P-38:6)	790.5751	790.5715	-2.6146	(P-16:0/22:6)	C46H81NO7P
PC(O-38:6)/PC(P-38:5)	792.5907	792.5889	-2.2925	(P-18:1/20:4) and (O-16:0/22:6)	C46H83NO7P
PC(O-38:5)/PC(P-38:4)	794.6064	794.6041	-2.8530	(O-18:1/20:4) and/or (P-18:0/20:4)	C46H85NO7P
PC(O-38:4)/PC(P-38:3)	796.6220	796.6184	-3.7860	**	C46H87NO7P
PC(O-38:3)/PC(P-38:2)	798.6377	798.6342	-1.5865	**	C46H89NO7P
PC(38:8)	802.5387	802.5380	-0.8498	**	C46H77NO8P
PC(38:7)	804.5543	804.5510	-2.4001	(16:1/22:6)	C46H79NO8P
PC(38:6)	806.5700	806.5707	0.8902	(16:0/22:6)	C46H81NO8P
PC(38:5)	808.5856	808.5854	-0.2869	(18:1/20:4)	C46H83NO8P
PC(36:6)+2O	810.5285	810.5310	3.0696	**	C44H77NO10P
PC(38:4)	810.6013	810.6022	1.1325	(18:0/20:4)	C46H85NO8P
PC(38:3)	812.6169	812.6161	-1.0239	(18:0/20:3) and (18:2/20:1)	C46H87NO8P
PC(36:4)+2O	814.5598	814.5603	0.5991	(18:2/18:2+2O) and (16:0/20:4+2O)	C44H81NO10P
PC(38:2)	814.6326	814.6301	-3.0468	**	C46H89NO8P
PC(38:1)	816.6482	816.6462	-2.4882	**	C46H91NO8P
PC(P-40:6)	818.6064	818.6042	-2.6472	(P-18:0/22:6)	C48H85NO7P
PC(O-40:6)/PC(P-40:5)	820.6220	820.6194	-3.1878	**	C48H87NO7P
PC(O-40:5)/PC(P-40:4)	822.6377	822.6345	-3.8498	**	C48H89NO7P
PC(O-40:4)/PC(P-40:3)	824.6533	824.6518	-1.8396	(O-20:0/20:4)	C48H91NO7P
PC(40:10)	826.5387	826.5368	-2.2770	**	C48H77NO8P
PC(40:9)	828.5543	828.5508	-3.6582	**	C48H79NO8P
PC(40:8)	830.5699	830.5682	-2.0853	(20:4/20:4)	C48H81NO8P
PC(40:7)	832.5856	832.5849	-0.8792	(18:1/22:6)	C48H83NO8P
PC(40:6)	834.6013	834.5990	-2.7342	(18:0/22:6)	C48H85NO8P
PC(40:5)	836.6169	836.6146	-2.7874	**	C48H87NO8P
PC(40:4)	838.6326	838.6338	1.4524	(18:0/22:4) and (20:0/20:4)	C48H89NO8P
PC(42:11)	852.5543	852.5503	-1.5612	**	C50H79NO8P
PC(42:10)	854.5700	854.5672	-3.2554	**	C50H81NO8P
PC(40:4)+O	854.6275	854.6303	3.2798	**	C48H89NO9P
PC(42:9)	856.5856	856.5832	-2.8392	**	C50H83NO8P
PC(42:8)	858.6013	858.5978	-1.6096	(20:4/22:4) and (20:2/22:6)	C50H85NO8P
PC(42:7)	860.6169	860.6138	-3.6392	(20:1/22:6)	C50H87NO8P
PC(42:6)	862.6326	862.6328	0.2527	(20:0/22:6)	C50H89NO8P
PC(42:5)	864.6482	864.6464	-2.1188	**	C50H91NO8P
PC(42:4)	866.6639	866.6623	-1.8254	**	C50H93NO8P

PC(40:5)+2O	868.6068	868.6044	-2.7193	**	C48H87NO10P
PC(44:12)	878.5700	878.5672	-3.1665	**	C52H81NO8P
PC(44:11)	880.5856	880.5821	-1.5126	**	C52H83NO8P
PC(44:10)	882.6013	882.5981	-3.6053	**	C52H85NO8P
PC(44:9)	884.6169	884.6129	-3.7666	**	C52H87NO8P
PC(44:8)	886.6326	886.6327	0.1331	**	C52H89NO8P
PC(44:5)	892.6795	892.6802	0.7483	**	C52H95NO8P
PC(44:4)	894.6952	894.6927	-2.7730	**	C52H97NO8P
LPC identified as $[M+H]^+$ (determination of fatty acyl chain by MS/MS of $[M + CH_3COO]^-$)					
LPC(14:0)	468.3090	468.3082	-1.7446	(14:0)	C22H47NO7P
LPC(16:0)	496.3403	496.3414	2.1840	(16:0)	C24H51NO7P
LPC(16:1)	494.3247	494.3255	1.6851	(16:1)	C24H49NO7P
LPC(16:1)+O	510.3196	510.3189	-1.3345	(16:1+O)	C24H49NO8P
LPC(18:0)	524.3716	524.3726	1.8746	(18:0)	C26H55NO7P
LPC(18:1)	522.3560	522.3573	2.5519	(18:1)	C26H53NO7P
LPC(18:1)+O	538.3509	538.3488	-3.8674	**	C26H53NO8P
LPC(18:2)	520.3403	520.3397	-1.1838	(18:2)	C26H51NO7P
LPC(18:2)+2O	552.3301	552.3318	2.9928	**	C26H51NO9P
LPC(18:2)+O	536.3352	536.3367	2.7371	(18:2+O)	C26H51NO8P
LPC(18:3)	518.3247	518.3223	3.9092	(18:3)	C26H49NO7P
LPC(18:3)+O	534.3196	534.3174	-2.5865	(18:3+O)	C26H49NO8P
LPC(20:0)	552.4029	552.4037	1.4174	(20:0)	C28H59NO7P
LPC(20:1)	550.3873	550.3887	2.6036	(20:1)	C28H57NO7P
LPC(20:2)	548.3716	548.3692	-2.7664	(20:2)	C28H55NO7P
LPC(20:3)	546.3560	546.3563	0.6095	(20:3)	C28H53NO7P
LPC(20:4)	544.3403	544.3411	1.4403	(20:4)	C28H51NO7P
LPC(20:4)+O	560.3352	560.3337	-2.7341	*	C28H51NO8P
LPC(20:5)	542.3247	542.3219	-3.2582	(20:5)	C28H49NO7P
LPC(22:0)	580.4342	580.4328	-2.4413	**	C30H63NO7P
LPC(22:1)	578.4186	578.4179	-1.1514	**	C30H61NO7P
LPC(22:4)	572.3716	572.3714	-0.3791	(22:4)	C30H55NO7P
LPC(22:5)	570.3560	570.3561	0.2332	**	C30H53NO7P
LPC(22:6)	568.3403	568.3395	-1.4358	(22:6)	C30H51NO7P
LPC(24:0)	608.4655	608.4648	-1.1784	**	C32H67NO7P
LPC(O-16:0)	482.3611	482.3602	-1.7663	(O-16:0)	C24H53NO6P
LPC(O-16:1)/LPC(P-16:0)	480.3454	480.3461	1.4531	(P-16:0)	C24H51NO6P
LPC(O-18:0)	510.3924	510.3927	0.6818	(O-18:0)	C26H57NO6P
LPC(O-18:1)/LPC(P-18:0)	508.3767	508.376	-1.3809	**	C26H55NO6P
LPC(O-18:1)/LPC(P-18:0)+2O	540.3665	540.3662	-0.6144	**	C26H55NO8P
LPC(O-20:0)	538.4237	538.4238	0.2749	**	C28H61NO6P
LPC(P-18:1)	506.3611	506.3606	-0.8926	**	C26H53NO6P
LPC(P-20:0)	536.4080	536.4075	-0.9359	**	C28H59NO6P
SM identified as $[M+H]^+$					
SM(d30:0)	649.5285	649.5258	-2.5418	*	C35H74N2O6P
SM(d30:1)	647.5128	647.5124	-0.6193	**	C35H72N2O6P

SM(d32:0)	677.5598	677.5587	-1.5512	**	C37H78N2O6P
SM(d32:1)	675.5441	675.5435	-0.8897	**	C37H76N2O6P
SM(d32:2)	673.5285	673.5281	-0.5211	**	C37H74N2O6P
SM(d34:0)	705.5911	705.5894	-2.3399	**	C39H82N2O6P
SM(d34:1)	703.5754	703.5742	-1.7070	(d18:1/16:0)	C39H80N2O6P
SM(d34:2)	701.5598	701.5581	-2.3533	(d18:2/16:0)	C39H78N2O6P
SM(d36:0)	733.6224	733.6206	-2.3868	**	C41H86N2O6P
SM(d36:1)	731.6067	731.6055	-1.6416	**	C41H84N2O6P
SM(d36:2)	729.5911	729.5902	-1.1664	**	C41H82N2O6P
SM(d36:3)	727.5754	727.5734	-2.7502	**	C41H80N2O6P
SM(d38:1)	759.6380	759.6367	-1.7127	(d18:1/20:0)	C43H88N2O6P
SM(d38:2)	757.6224	757.6209	-1.9152	**	C43H86N2O6P
SM(d38:3)	755.6067	755.6056	-1.4571	**	C43H84N2O6P
SM(d40:1)	787.6693	787.6672	-2.6674	**	C45H92N2O6P
SM(d40:2)	785.6537	785.6526	-1.3377	**	C45H90N2O6P
SM(d40:3)	783.6380	783.6365	-1.9154	**	C45H88N2O6P
SM(d42:2)	813.6850	813.6835	-1.7832	**	C47H94N2O6P
SM(d42:3)	811.6693	811.6682	-1.3565	**	C47H92N2O6P
SM(d44:2)	841.7163	841.7141	-2.5555	*	C49H98N2O6P
PE identified as $[M-H]^-$ (confirmation of polar head group by MS/MS of $[M+H]^+$)					
PE(30:0)	662.4760	662.4763	0.4528	*	C35H69NO8P
PE(32:1)	688.4917	688.4929	1.6965	*	C37H71NO8P
PE(34:1)	716.5230	716.5223	-0.9769	(16:0/18:1)	C39H75NO8P
PE(34:2)	714.5070	714.5081	1.5395	(16:0/18:2)	C39H73NO8P
PE(34:3)	712.4920	712.4924	0.5614	**	C39H71O8NP
PE(36:2)	742.5387	742.5398	1.4814	(18:0/18:2) and (18:1/18:1)	C41H77NO8P
PE(36:3)	740.5230	740.5255	3.3760	(18:1/18:2)	C41H75NO8P
PE(36:4)+O	754.5023	754.5035	1.5944	*	C41H73NO9P
PE(38:4)	766.5390	766.5390	0.0000	(18:0/20:4)	C43H77O8NP
PE(38:5)	764.5230	764.5219	-1.4388	(18:1/20:4)	C43H75NO8P
PE(38:6)	762.507	762.5082	1.5738	(16:0/22:6)	C43H73O8NP
PE(40:4)+O	810.5649	810.5625	-2.9572	**	C45H81NO9P
PE(40:6)	790.5300	790.5263	-0.1265	(18:0/22:6)	C45H77O8NP
PE(40:7)	788.5230	788.5235	0.6341	(18:1/22:6)	C45H75NO8P
PE(40:8)	786.5070	786.5078	1.0172	**	C45H73O8NP
PE(P-34:0)/PE(O-34:1)	702.5440	702.5447	0.9964	*	C39H77NO7P
PE(P-34:1)/PE(O-34:2)	700.5280	700.5289	1.2847	(O-16:0/18:2), (O-16:1/18:1) and/or (P-16:0/18:1) and/or (P-18:1/16:0)	C39H75NO7P
PE(P-34:2)/PE(O-34:3)	698.5130	698.5143	1.8611	(P-16:0/18:2) and/or (O-16:1/18:2)	C39H73NO7P
PE(P-36:1)/PE(O-36:2)	728.5590	728.5585	-0.6863	(O-18:0/18:2), (P-18:0/18:1) and/or (O-18:1/18:1)	C41H79NO7P
PE(P-36:2)/PE(O-36:3)	726.5440	726.5455	2.0646	(P-18:1/18:1), (P-18:0/18:2) and/or (O-18:1/18:2)	C41H77NO7P
PE(P-36:3)/PE(O-36:4)	724.5280	724.529	1.3802	(P-18:1/18:2) and (O-16:0/20:4)	C41H75NO7P
PE(P-36:4)/PE(O-36:5)	722.5130	722.5142	1.6609	(P-16:0/20:4) and/or (O-16:1/20:4)	C41H73NO7P
PE(P-38:3)/PE(O-38:4)	752.5590	752.5576	-1.8603	(O-18:0/20:4), (P-18:0/20:3) and/or (O-18:1/20:3)	C43H79NO7P
PE(P-38:4)/PE(O-38:5)	750.5440	750.544	0.0000	(P-18:0/20:4) and/or (O-18:1/20:4)	C43H77NO7P

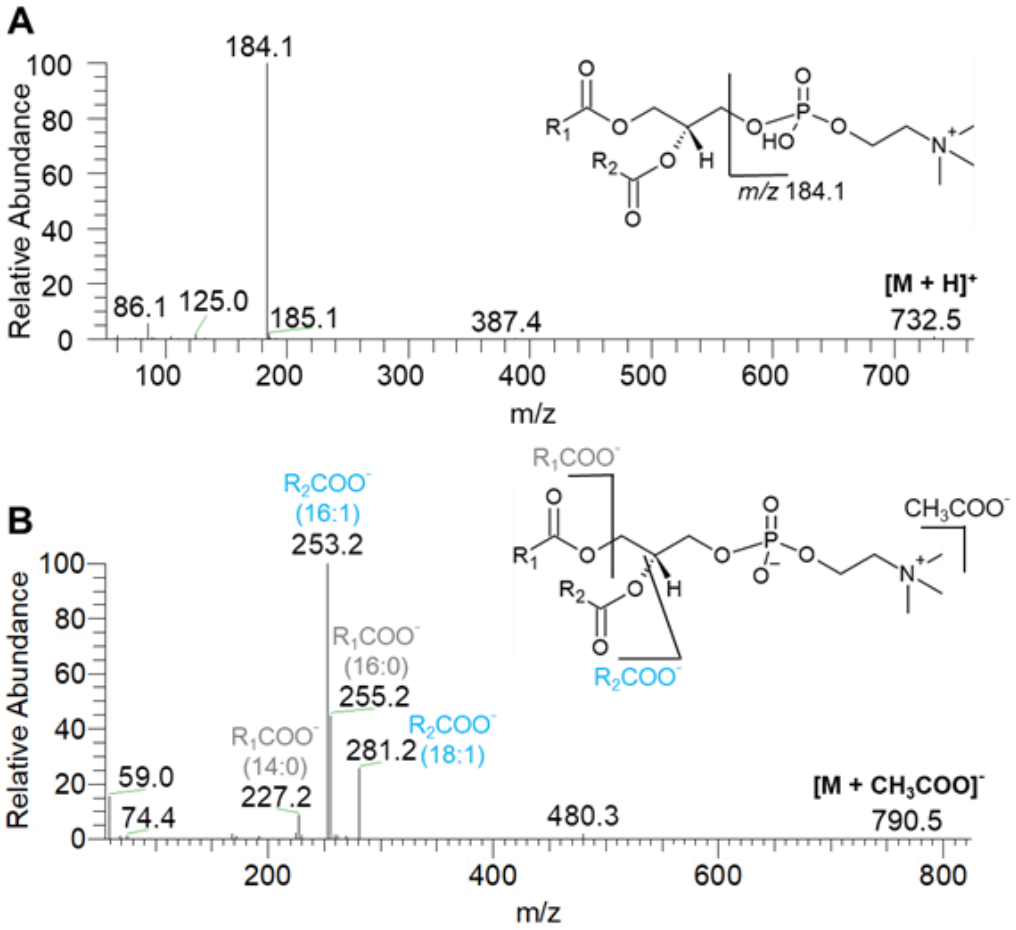
PE(P-38:5)/PE(O-38:6)	748.5280	748.5281	0.1336	(P-18:1/20:4)	C43H75NO7P
PE(P-40:4)/PE(O-40:5)	778.5750	778.5729	-2.6972	*	C45H81NO7P
PE(P-36:6)	718.4810	718.4829	2.6445	**	C41H69O7NP
LPE identified as $[M-H]^-$ (confirmation of polar head group by MS/MS of $[M+H]^+$)					
LPE(18:4)	472.2464	472.2464	-0.0339	*	C23H39NO7P
PG identified as $[M-H]^-$					
PG(18:0/C5aldehyde)	609.3404	609.3419	2.4978	*	C29H54O11P
PG(18:0/C5carboxylic)	625.3353	625.3364	1.7703	*	C29H54O12P
PG(26:0)	637.4081	637.4092	1.7257	(12:0/14:0)	C32H62O10P
PG(30:0)	693.4707	693.4701	-0.8652	(14:0/16:0)	C36H70O10P
PG(34:1)	747.5176	747.5183	0.9364	(16:0/18:1)	C40H76O10P
PG(36:7)	763.4550	763.4588	1.9648	*	C42H68O10P
PG(36:6)	765.4707	765.4713	0.7838	*	C42H70O10P
PG(36:3)	771.5176	771.5192	2.0738	*	C42H76O10P
PG(36:2)	773.5333	773.5332	-0.1293	(18:0/18:2)	C42H80O10P
PG(36:1)	775.5489	775.5471	-2.3209	(18:0/18:1)	C42H80O10P
PG(40:6)	821.5333	821.5322	-1.3390	*	C46H78O10P
LPG identified as $[M-H]^-$					
LPG(14:0)	455.2410	455.2424	3.0797	(14:0)	C20H40O9P
LPG(16:1)	481.2566	481.258	2.8093	*	C22H42O9P
LPG(16:0)	483.2723	483.2734	2.2803	*	C22H44O9P
LPG(18:2)	507.2723	507.2730	1.3839	*	C24H44O9P
LPG(18:1)	509.2879	509.2891	2.2620	(18:1)	C24H46O9P
PI identified as $[M-H]^-$					
PI(34:2)	833.5180	833.5184	0.4799	*	C43H78O13P
PI(34:1)	835.5337	835.5337	0.0000	*	C43H80O13P
PI(36:4)	857.5180	857.5173	-0.8163	(16:0/20:4)	C45H78O13P
PI(36:3)	859.5337	859.5322	-1.7451	*	C45H80O13P
PI(36:2)	861.5493	861.5494	0.1161	(18:0/18:2) and (18:1/18:1)	C45H82O13P
PI(36:1)	863.5650	863.5623	-3.1266	*	C45H84O13P
PI(38:4)	885.5493	885.5499	0.6775	(18:0/20:4)	C47H82O13P
PI(40:6)	909.5493	909.5508	1.6492	(18:0/22:6)	C49H82O13P
PS identified as $[M-H]^-$					
PS(30:3)	700.4190	700.4182	-1.1422	*	C36H63NO10P
PS(38:6)	806.4972	806.5000	3.4718	*	C44H73NO10P
PS(38:4)	810.5285	810.5304	2.3441	*	C44H77NO10P
PS(40:6)	834.5285	834.5308	2.7560	*	C46H77NO10P

Supplementary Table S2. The 146 lipid species showing significant variation between PKU and control group, ordered by increasing values of *q*-value (* if $q < 0.05$, ** if $q < 0.01$ and *** if $q < 0.001$**; ↑ if increased and ↓ if decreased in PKU group).** Lipid species are labelled as follows: AAAA (xx:i) (AAAA=lipid class abbreviation; xx=number of carbon atoms in fatty acid(s); i=number of double bonds). The 'O-' prefix is used for plasmanyl species to indicate the presence of an alkyl ether substituent, whereas the 'P-' prefix is used for plasmenyl species to indicate the alk-1-enyl ether substituent. Lipid class abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

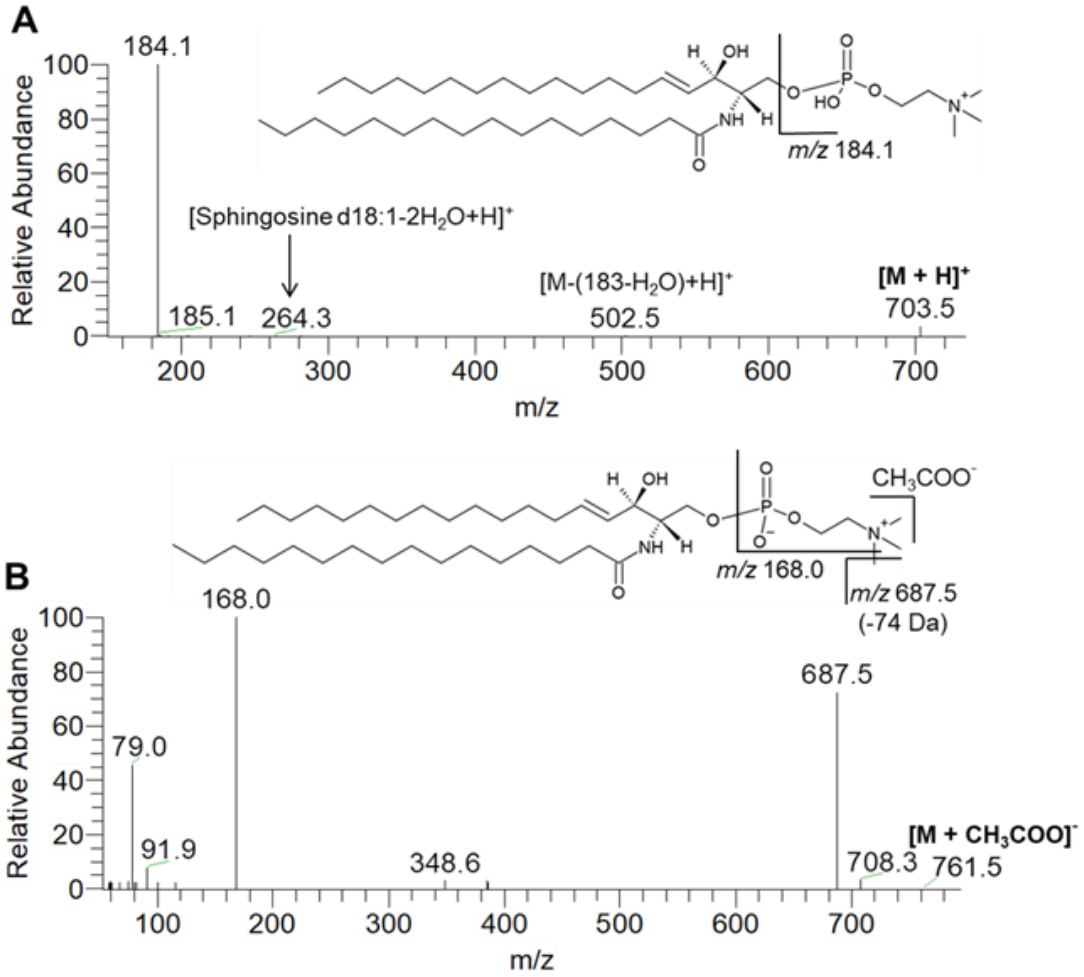
Lipid species (C:N)	FDR, *q*-value	Statistical significance level	Change
PC (40:8)	9.21E-12	***	↑
PC (44:12)	2.23E-11	***	↑
PC (42:7)	6.64E-11	***	↑
SM (d34:1)	8.77E-10	***	↑
PS (38:4)	4.13E-08	***	↓
SM (d36:3)	4.13E-08	***	↑
PS (40:6)	1.16E-07	***	↓
LPC (14:0)	1.21E-07	***	↑
PC (40:7)	1.48E-07	***	↑
PS (38:6)	1.48E-07	***	↓
SM (d36:2)	1.50E-07	***	↑
PC (44:11)	6.06E-07	***	↑
SM (d34:2)	6.06E-07	***	↑
SM (d38:3)	6.19E-07	***	↑
PC (42:9)	1.01E-06	***	↑
PC (40:5)	1.01E-06	***	↑
PC (44:4)	1.54E-06	***	↑
PI (38:4)	2.27E-06	***	↑
PC (40:9)	2.87E-06	***	↑
PC (38:5)	2.94E-06	***	↑
PC (38:7)	2.94E-06	***	↑
PC (40:4)	2.94E-06	***	↑
PC (42:4)	2.94E-06	***	↑
PI (36:4)	2.94E-06	***	↑
SM (d32:2)	2.94E-06	***	↓
SM (d42:3)	3.40E-06	***	↑
SM (d36:1)	3.59E-06	***	↑
SM (d32:1)	3.68E-06	***	↑
PC (38:8)	4.11E-06	***	↑
LPC (22:0)	4.49E-06	***	↑
PC (34:3)	4.49E-06	***	↑
SM (d36:0)	4.49E-06	***	↑
PG (34:1)	4.83E-06	***	↑
PC (32:2)	5.00E-06	***	↑
LPC (20:0)	5.52E-06	***	↑
LPC (P-18:1)	5.52E-06	***	↑
PC (34:4)	5.52E-06	***	↑
PC (42:5)	5.52E-06	***	↑
PC (42:6)	6.85E-06	***	↑
LPC (18:0)	7.25E-06	***	↑
PE (P-34:1)/PE (O-34:2)	1.17E-05	***	↑
PG (36:1)	1.17E-05	***	↑
PC (36:6)	1.79E-05	***	↑

PC (40:4)+O	1.79E-05	***	↑
PC (36:3)	1.83E-05	***	↑
LPC (16:1)+O	1.96E-05	***	↓
LPC (16:1)	2.51E-05	***	↑
LPC (24:0)	2.53E-05	***	↑
PI (40:6)	2.53E-05	***	↑
SM (d40:3)	2.97E-05	***	↑
PC (44:9)	3.16E-05	***	↑
PC (32:1)	3.26E-05	***	↑
PC (30:1)	3.82E-05	***	↑
PE (P-38:5)/PE (O-38:6)	3.82E-05	***	↑
LPC (16:0)	4.17E-05	***	↑
PC (44:8)	4.17E-05	***	↑
SM (d38:2)	4.17E-05	***	↑
PC (44:5)	4.24E-05	***	↑
SM (d44:2)	4.29E-05	***	↑
PC (42:10)	4.44E-05	***	↑
SM (d34:0)	5.00E-05	***	↑
SM (d40:1)	5.00E-05	***	↑
LPC (18:1)	5.56E-05	***	↑
LPC (O-18:0)	5.56E-05	***	↑
PC (42:8)	6.94E-05	***	↑
LPC (22:1)	7.52E-05	***	↑
PC (P-40:6)	7.53E-05	***	↑
PE (P-36:1)/PE (O-36:2)	7.53E-05	***	↑
SM (d30:1)	7.53E-05	***	↑
LPC (20:3)	9.80E-05	***	↑
SM (d38:1)	1.08E-04	***	↑
PC (44:10)	1.11E-04	***	↑
LPC (18:3)	1.42E-04	***	↑
PG (36:2)	1.42E-04	***	↑
PE (32:1)	1.46E-04	***	↑
PE (36:2)	1.46E-04	***	↑
LPC (O-16:0)	1.51E-04	***	↑
LPC (20:2)	1.73E-04	***	↑
PE (36:3)	2.66E-04	***	↑
LPC (O-18:1)/LPC (P-18:0)	2.79E-04	***	↓
SM (d40:2)	2.79E-04	***	↑
PC (O-40:4)/PC (P-40:3)	3.21E-04	***	↑
LPC (22:5)	3.91E-04	***	↑
PE (P-34:0)/PE (O-34:1)	5.36E-04	***	↑
PE (P-38:4)/PE (O-38:5)	5.47E-04	***	↑
PC (30:0)	5.50E-04	***	↑
PE (38:5)	5.67E-04	***	↑
PC (34:5)	5.88E-04	***	↑
SM (d42:2)	5.88E-04	***	↑
PC (38:2)	7.77E-04	***	↑
PE (40:4)+O	7.77E-04	***	↑
PC (42:11)	8.08E-04	***	↑
PS (30:3)	8.37E-04	***	↑
PC (O-28:0)	8.63E-04	***	↓
PE (P-36:3)/PE (O-36:4)	8.63E-04	***	↑
LPC (O-16:1)/LPC (P-16:0)	9.45E-04	***	↑
PI (34:1)	9.45E-04	***	↑
LPC (20:4)	1.03E-03	**	↑
LPG (18:2)	1.05E-03	**	↑
LPC (18:3)+O	1.22E-03	**	↓
LPC (22:6)	1.22E-03	**	↑

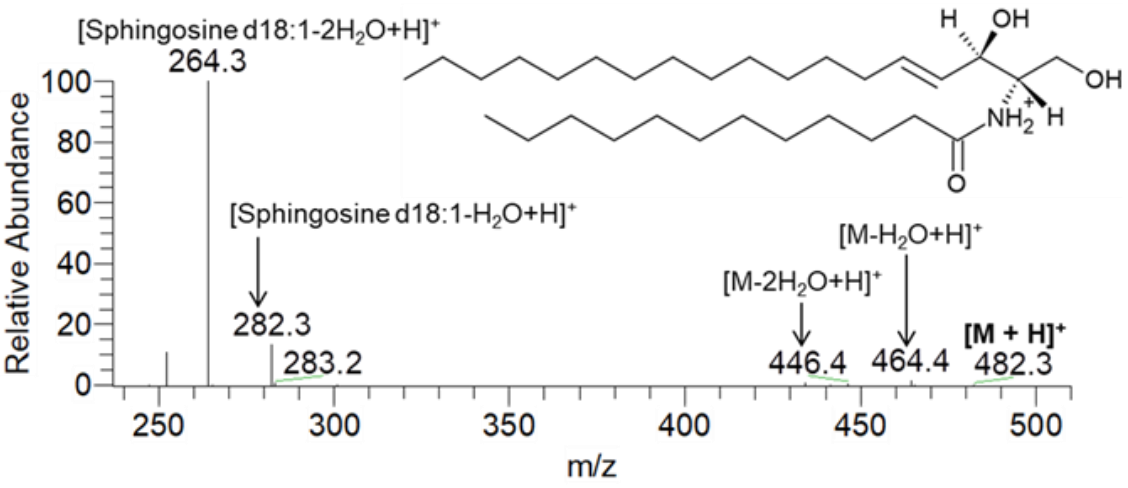
PC (40:10)	1.22E-03	**	↑
PC (36:4)	1.39E-03	**	↑
PC (32:0)	1.53E-03	**	↑
PC (40:5)+2O	1.53E-03	**	↑
PE (34:3)	1.59E-03	**	↑
PC (32:1)+O	1.97E-03	**	↑
PE (34:2)	2.09E-03	**	↑
PE (P-34:2)/PE (O-34:3)	2.14E-03	**	↑
LPC (18:2)+2O	2.17E-03	**	↓
PE (38:4)	2.35E-03	**	↑
PE (P-38:3)/PE (O-38:4)	2.35E-03	**	↑
PE (P-40:4)/PE (O-40:5)	3.01E-03	**	↑
PC (O-34:1)/PC (P-34:0)	3.08E-03	**	↑
PE (40:7)	3.49E-03	**	↑
LPC (20:1)	4.24E-03	**	↑
PC (36:2)	4.41E-03	**	↑
PC (34:2)	5.21E-03	**	↑
LPE (18:4)	5.28E-03	**	↑
PC (O-38:4)/PC (P-38:3)	6.11E-03	**	↑
LPC (22:4)	6.64E-03	**	↑
LPC (18:2)	7.04E-03	**	↑
PI (36:2)	7.33E-03	**	↑
PC (O-40:5)/PC (P-40:4)	7.56E-03	**	↑
PI (36:1)	7.76E-03	**	↑
PC (36:5)	8.64E-03	**	↑
PC (40:6)	8.64E-03	**	↑
PI (36:3)	8.64E-03	**	↑
PE (P-36:6)	8.81E-03	**	↓
LPG (16:1)	9.31E-03	**	↑
PC (38:6)	9.95E-03	**	↑
PG (36:3)	1.13E-02	*	↑
PE (P-36:2)/PE (O-36:3)	1.24E-02	*	↑
LPG (18:1)	1.26E-02	*	↑
LPC (O-18:1)/LPC (P-18:0)+2O	1.31E-02	*	↓
PC (O-38:3)/PC (P-38:2)	1.50E-02	*	↑
LPC (20:5)	1.58E-02	*	↑
PC (34:1)+O	1.69E-02	*	↓
LPC (18:1)+O	1.78E-02	*	↓
PC (O-40:6)/PC (P-40:5)	2.21E-02	*	↑
PE (34:1)	2.47E-02	*	↑
PC (O-38:5)/PC (P-38:4)	2.55E-02	*	↑
PG (30:0)	2.78E-02	*	↑
PC (O-38:6)/PC (P-38:5)	2.95E-02	*	↑
PG (36:6)	3.62E-02	*	↑
PC (O-36:2)/PC (P-36:1)	3.67E-02	*	↑



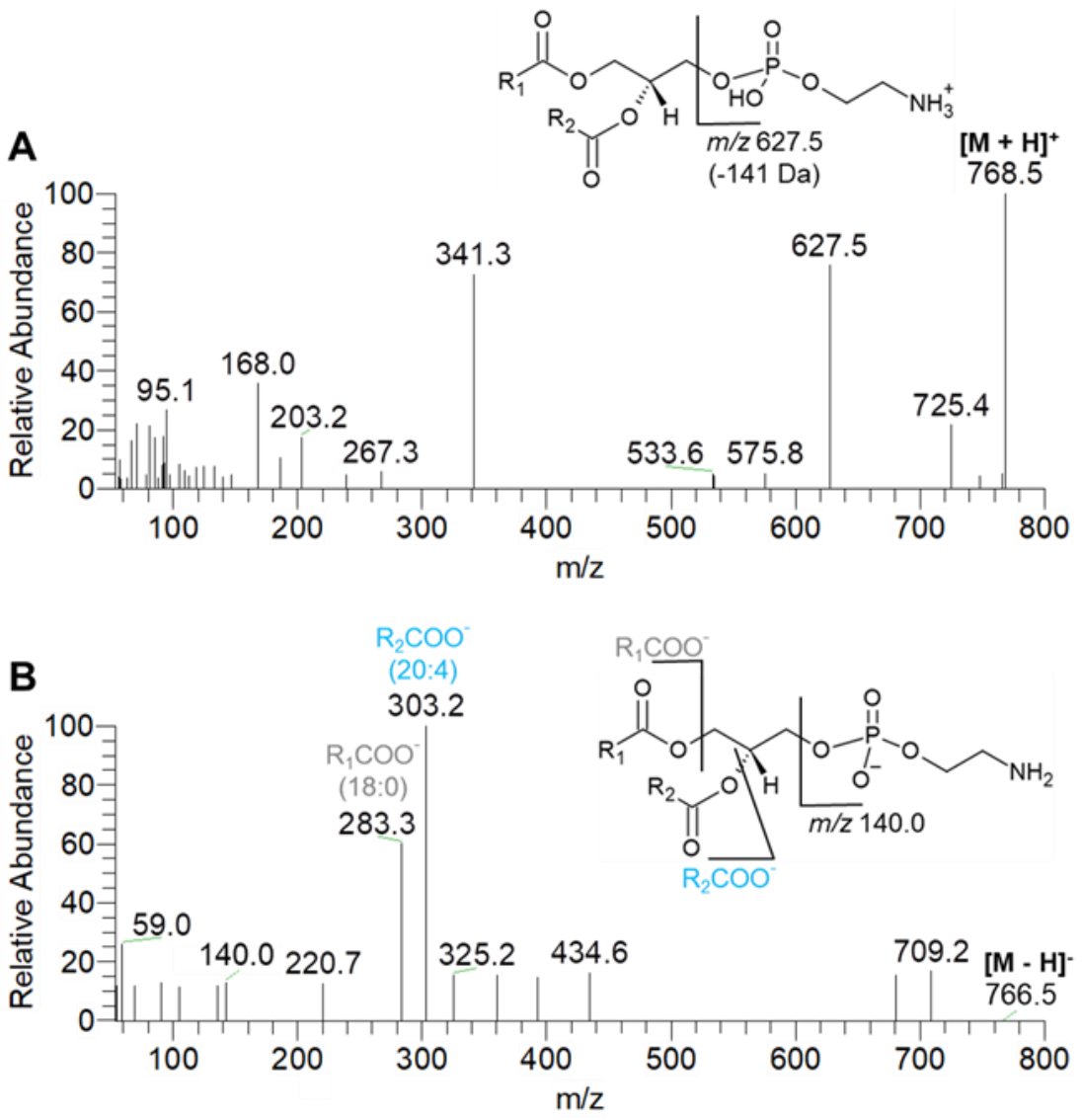
Supplementary Figure S1. Representative MS/MS spectra of phosphatidylcholine (PC) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species PC (32:1) observed in positive mode as $[M+H]^+$ ion at m/z 732.5. Confirmation of phospholipid class was achieved by the identification of the product ion at m/z 184.1 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **B)** HILIC-MS/MS spectrum of the lipid species PC (32:1) observed in negative mode as $[M + CH_3COO]^-$ ion at *m/z* 790.5. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. In this case, two molecular lipid species with distinct fatty acyl composition were identified. The product ions observed at *m/z* 227.2 and 281.2, corresponding to fatty acyl carboxylate anions 14:0 (R_1COO^-) and 18:1 (R_2COO^-) allowed to identify the fatty acyl composition of PC (14:0/18:1). The product ions observed at *m/z* 255.2 and 253.2, corresponding to fatty acyl carboxylate anions 16:0 (R_1COO^-) and 16:1 (R_2COO^-) allowed to identify the fatty acyl composition of PC (16:0/16:1). For LPC, the same fragmentation was observed, with the exception that only one product ion corresponding to a fatty acid was detected.



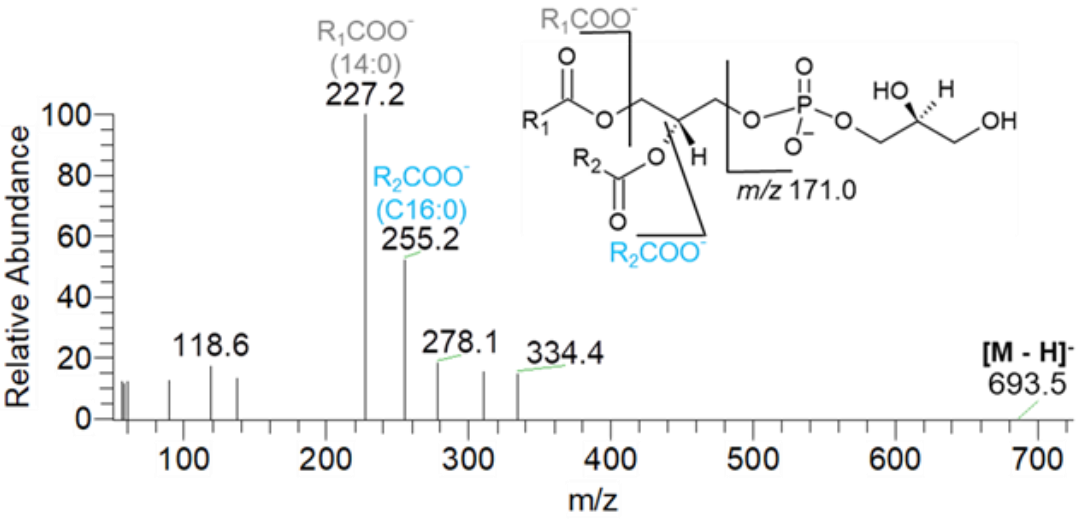
Supplementary Figure S2. Representative MS/MS spectra of sphingomyelin (SM) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species SM(d34:1) observed in positive mode as $[M+H]^+$ ion at *m/z* 703.5. Confirmation of phospholipid class was achieved by the identification of the product ion at *m/z* 184.1 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head; and the product ion of sphingoid base at 264.3, corresponding to [Sphingosine d18:1-$2H_2O$+H]$^+$. The mass difference of 238 Da between the product ions at m/z 502.5 ([M-(183-H_2O)+H]$^+$) and 264.3, plus 18 Da, allowed to infer the presence of 16:0 as fatty acyl amide substituent. **B)** HILIC-MS/MS spectrum of the lipid species SM(d34:1) observed in negative mode as $[M + CH_3COO]^-$ ion at *m/z* 761.5. Confirmation of phospholipid class was achieved by the identification of the product ion at *m/z* 168.0 (formula: $C_4H_{11}NO_4P$; exact mass:168.0425), corresponding to the phosphocholine polar head without a methyl group, and by observing the characteristic neutral loss of 74 Da (formula: $C_3H_6O_2$; exact mass: 74.0368), corresponding to the loss of methyl acetate.



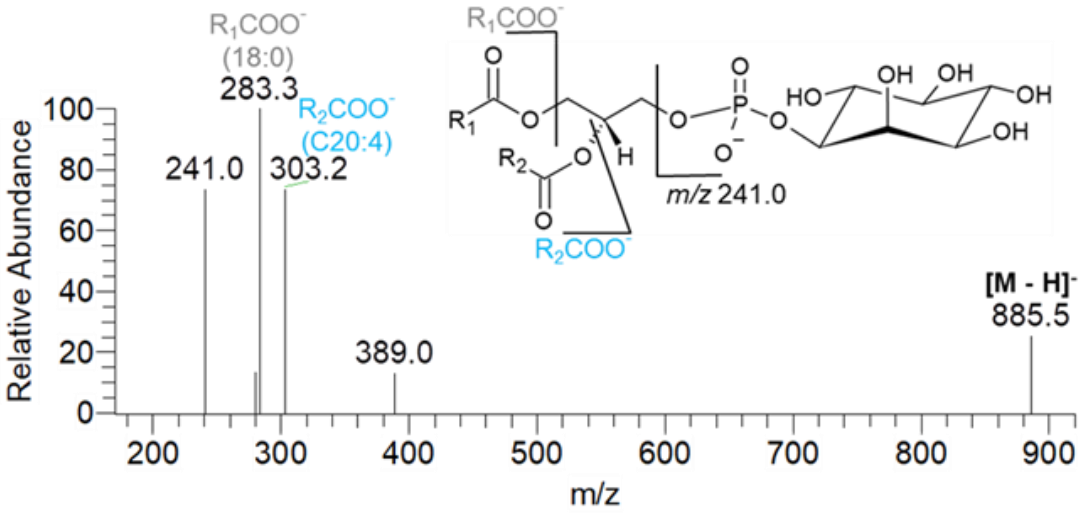
Supplementary Figure S3. Representative MS/MS spectrum of ceramide (Cer) lipid species. HILIC-MS/MS spectrum of the lipid species Cer(d30:1) observed in positive mode as $[M+H]^+$ ion at *m/z* 482.3. Confirmation of phospholipid class was achieved by the identification of the abundant product ions of the sphingoid at *m/z* 264.3 and 282.3, corresponding to [Sphingosine d18:1-$2H_2O$+H]$^+$ and [Sphingosine d18:1-H_2O+H]$^+$. The mass difference of 218 Da between the product ions at *m/z* 446.4 ([M-$2H_2O$+H]$^+$) and 264.3, plus 18 Da, allowed to infer the presence of 12:0 as fatty acyl amide substituent.



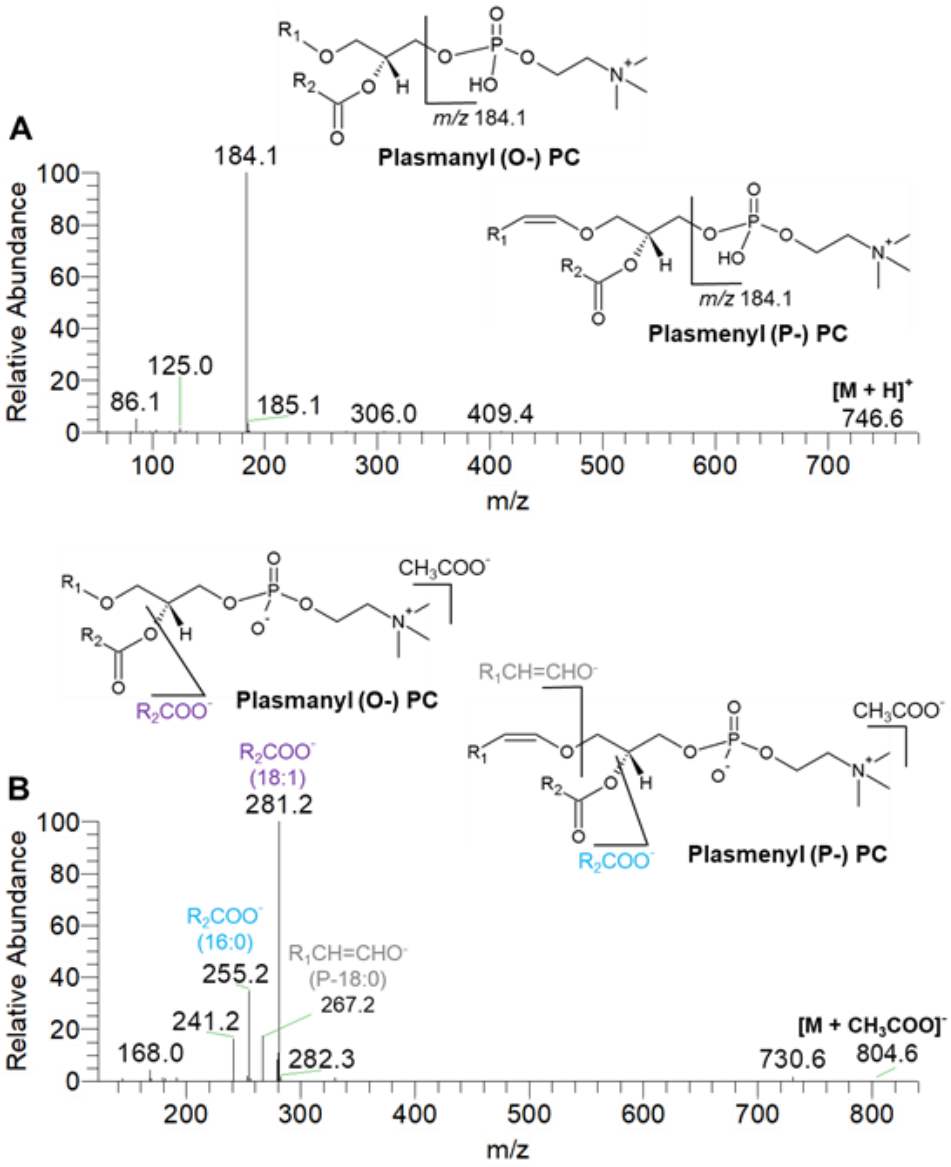
Supplementary Figure S4. Representative MS/MS spectra of phosphatidylethanolamine (PE) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species PE (38:4) observed in positive mode as $[M+H]^+$ ion at *m/z* 768.5. Confirmation of phospholipid class was achieved by the identification of the neutral loss of 141 Da (formula: $C_2H_8NO_4P$; exact mass: 141.0191), corresponding to phosphoethanolamine polar head. **B)** HILIC-MS/MS spectrum of the lipid species PE (38:4) observed in negative mode as $[M-H]^-$ at *m/z* 766.5. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ions observed at *m/z* 283.3 and 303.2, corresponding to fatty acyl carboxylate anions of 18:0 (R_1COO^-) and 20:4 (R_2COO^-), allowed to identify the fatty acyl composition of PE (18:0/20:4). The confirmation as PE species was also achieved by observing the product ion at *m/z* 140.0 (formula: $C_2H_7NO_4P$; exact mass: 140.0113), corresponding to phosphoethanolamine polar head. For LPE, the same fragmentation was observed, with the exception that only one product ion corresponding to a fatty acid was detected.



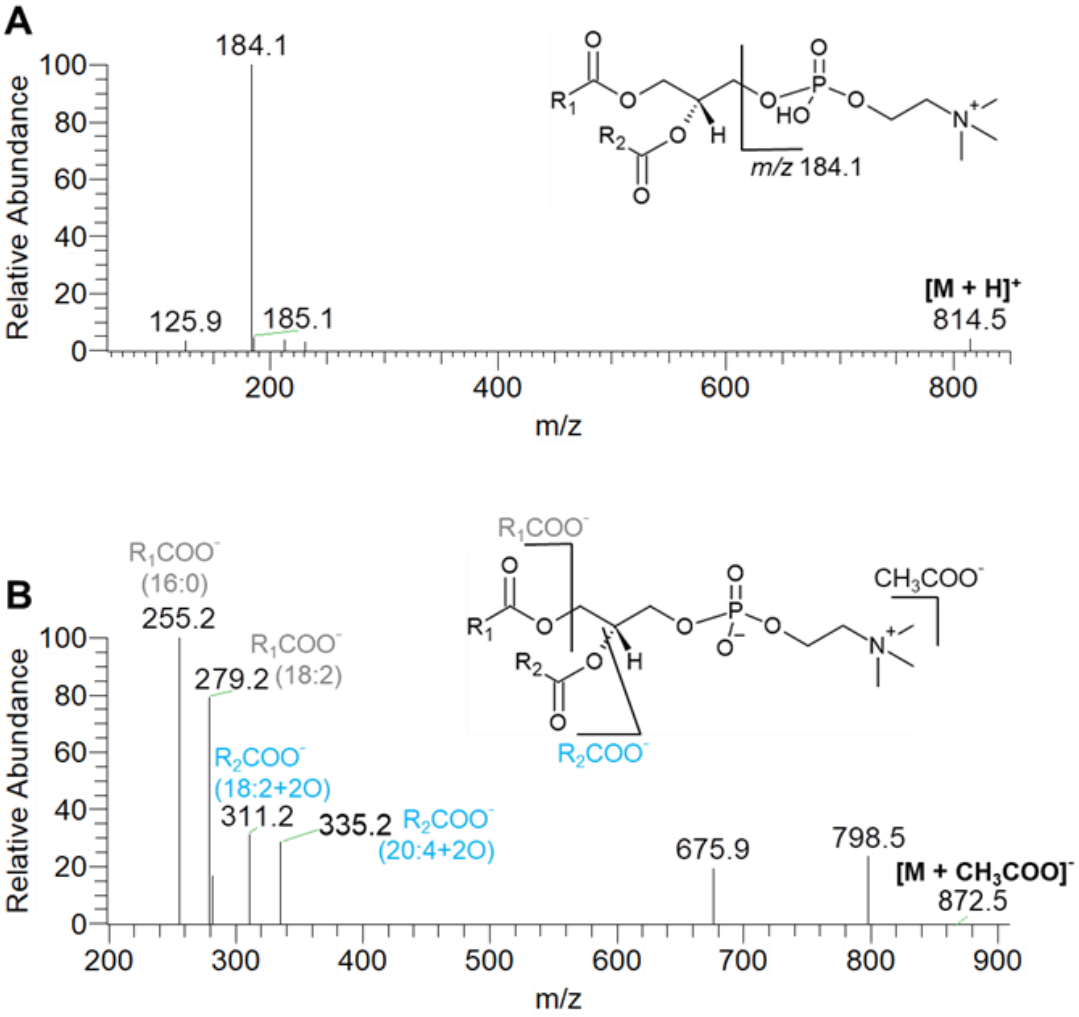
Supplementary Figure S5. Representative MS/MS spectrum of phosphatidylglycerol (PG) lipid species. HILIC-MS/MS spectrum of the lipid species PG (30:0) observed in negative mode as $[M-H]^-$ ion at *m/z* 693.5. Confirmation of phospholipid class may be achieved by the identification of the product ion at *m/z* 171.0 (formula: $C_3H_8O_6P$; exact mass: 171.0058), corresponding to the glycerol phosphate anion (not visible from molecular ions with low intensity, as occurred in this study). Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ions observed at *m/z* 227.2 and 255.2, corresponding to fatty acyl carboxylate anions 14:0 (R_1COO^-) and 16:0 (R_2COO^-) allowed to identify the fatty acyl composition of PG (14:0/16:0). For LPG, the same fragmentation was observed, with the exception that only one product ion corresponding to a fatty acid was detected.



Supplementary Figure S6. Representative MS/MS spectrum of phosphatidylglycerol (PI) lipid species. HILIC-MS/MS spectrum of the lipid species PI (38:4) observed in positive mode as $[M-H]^-$ ion at *m/z* 885.5. Confirmation of phospholipid class was achieved by the identification of the product ion at *m/z* 241.0 (formula: $C_6H_{10}O_8P$; exact mass: 241.0113), corresponding to the inositol head group. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. The product ions observed at *m/z* 283.3 and 303.2, corresponding to fatty acyl carboxylate anions of 18:0 (R_1COO^-) and 20:4 (R_2COO^-) allowed to identify the fatty acyl composition of PI (18:0/20:4).



Supplementary Figure S7. Representative MS/MS spectra of isomeric plasmanyl (O-) and plasmenyl (P-) phosphatidylcholine (PC) lipid species, eluting at the same retention time in LC-MS. **A)** HILIC-MS/MS spectrum of the lipid species PC(O-34:1)/PC(P-34:0) observed in positive mode as $[M+H]^+$ ion at *m/z* 746.6. Confirmation of phospholipid class was achieved by the identification of the product ion at *m/z* 184.1 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **B)** HILIC-MS/MS spectrum of the lipid species PC (O-34:1)/PC(P-34:0) observed in negative mode as $[M+CH_3COO]^-$ at *m/z* 804.6. For plasmenyl (P-) species, fatty acid composition was confirmed by the identification of a product ion corresponding to the ester-linked fatty acid as $[RCOO]^-$ and, in some cases (not visible from molecular ions with low intensity), of another product ion corresponding to the ether-linked fatty acid as $[RCH=CHO]^-$. Plasmanyl (O-) species yielded only a product ion corresponding to the ester-linked fatty acid as $[RCOO]^-$. In the case of the lipid species PC(O-34:1)/PC(P-34:0), three possible molecular lipid species with distinct fatty acyl composition were identified. The product ions observed at *m/z* 267.2 (283-16) and 255.2, corresponding to P-18:0 ($R_1CH=CHO^-$) and 16:0 (R_2COO^-), allowed to identify the fatty acyl composition of PC (P-18:0/16:0). The product ion observed at *m/z* 281.2, corresponding to fatty acyl carboxylate anion of 18:1 (R_2COO^-), allowed to infer the presence of PC (O-16:0/18:1). Also, the presence of PC (O-18:1/16:0) cannot be excluded, as it may contribute for the observation of the product ion at *m/z* 255.2, corresponding to fatty acyl carboxylate anion of 16:0 (R_2COO^-).



Supplementary Figure S8. Representative MS/MS spectra of oxidized phosphatidylcholine (PC) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species PC (36:4)+2O observed in positive mode as $[M+H]^+$ ion at *m/z* 814.5. Confirmation of phospholipid class was achieved by the identification of the product ion at *m/z* 184.1 (formula: $C_5H_{15}NO_4P$; exact mass: 184.0739), corresponding to the phosphocholine polar head. **B)** HILIC-MS/MS spectrum of the lipid species PC (36:4)+2O observed in negative mode as [M+CH3COO]$^-$ at *m/z* 872.5. Fatty acid composition was confirmed by the identification of product ions corresponding to the fatty acyl chains as $[RCOO]^-$. In this case, two molecular lipid species with distinct fatty acyl composition were identified. The product ions observed at *m/z* 255.2 and 335.2, corresponding to fatty acyl carboxylate anions 16:0 ($R1COO^-$) and 20:4+2O (R_2COO^-) allowed to identify the fatty acyl composition of PC (16:0/20:4+2O). The product ions observed at *m/z* 279.2 and 311.2, corresponding to fatty acyl carboxylate anions 18:2 (R_1COO^-) and 18:2+2O (R_2COO^-) allowed to identify the fatty acyl composition of PC (18:2/18:2+2O). For LPC, the same fragmentation was observed, with the exception that only one product ion corresponding to a fatty acid was detected.