Plasma phospholipidomic profile differs between children with phenylketonuria and healthy children

Inês M. S. Guerra[a], Luísa Diogo[b], Marisa Pinho[c], Tânia Melo[a,d], Pedro Domingues[a], M. Rosário Domingues[a,d], Ana S. P.Moreira[a,e,*]

[a] Mass Spectrometry Center, LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[b] Reference Center of Inherited Metabolic Diseases, Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra, 3000-075 Coimbra, Portugal

[c] ECOMARE, CESAM - Center for Environmental and Marine Studies, Department of Biology, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

[d] CESAM - Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[e] CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

*Corresponding author. *E-mail address:* ana.moreira@ua.pt

Abstract

Phenylketonuria (PKU) is a disease of the catabolism of phenylalanine (Phe), caused by an impaired function of the enzyme phenylalanine hydroxylase. Therapeutics is based on the restriction of Phe intake, which mostly requires a modification of the diet. Dietary restrictions can lead to imbalances in specific nutrients, including lipids. In the present study, the plasma phospholipidome of PKU and healthy children (CT) was 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 the lipid species dataset showed a distinction between PKU and CT groups. Univariate analysis revealed that 146 species of phospholipids were significantly different between both groups. Lipid species showing significant variation included phosphatidylcholines, containing polyunsaturated fatty acids (PUFA), which were more abundant in PKU. The high level of PUFA-containing lipid species in children with PKU may be related to a diet supplemented with PUFA. 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 compared to CT. However, further studies with larger cohorts are needed to clarify whether these changes are specific to phenylketonuric children.

Keywords: Inborn errors of metabolism; phenylketonuria; plasma; phospholipids; lipidomics; mass spectrometry.

1. Introduction

Phenylketonuria (PKU) is the most prevalent inborn error in amino acid metabolism [1–3]. PKU is characterized by an impaired activity of the enzyme phenylalanine hydroxylase (PAH), resulting in a complete or partial inability of the enzyme to convert L-phenylalanine (Phe) to L-tyrosine. Deficiency of the PAH enzyme results in increased levels of P he in the blood (hyperphenylalaninemia) [4–6]. The accumulation of Phe can lead to intellectual impairment, microcephaly, seizures, and motor deficits [2,7,8]. Early diagnosis through neonatal screening and rapid therapeutic implementation are essential to prevent these possible complications [9]. The therapeutic approach for PKU is based on a lifelong Phe-restricted diet, with a low intake of natural proteins [1].

In the low-Phe diet, protein-rich foods like eggs, fish, meat and dairy products, which are the main natural dietary sources of long-chain polyunsaturated fatty acids (PUFA), are excluded [10]. Thus, to provide the required amount of daily protein intake, PKU patients consume commercial formulas with Phe-free essential amino acids, as well containing vitamins, minerals, and other nutrients [11]. To ensure that the essential PUFA dietary requirements are met, amino acid formulas containing PUFA and PUFA supplements are used together with PKU diet [12]. Despite the use of amino acid formulas and PUFA supplements, as well as some special low-protein foods available to PKU patients, high dietary restrictions can lead to nutritional imbalances, notably in lipids.

Alterations in blood lipids, especially in serum and plasma lipoprotein components, have already been described in PKU patients on a Phe-restricted diet [3]. Statistically significant lower levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were observed in PKU patients compared to healthy subjects [5,13–16]. However, no statistical difference in the levels of TC, LDL-C and HDL-C has been reported in other studies [14,17], highlighting the existence of dissimilar results in the plasma lipid profile of PKU patients.

Moreover, previous studies have revealed changes in the fatty acid (FA) profile of plasma and red blood cell (RBC) samples from PKU patients [3]. The existing literature is ambiguous about the prevalence of long-chain PUFA

deficiency in patients with PKU. However, some studies have shown a significant decrease in important PUFA, such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6), in patients with PKU who maintained a Phe-restricted diet (with no information available on PUFA supplementation) [18–22]. These reduced levels have been associated with dietary restrictions [22], as PKU patients do not consume foods of animal origin rich in PUFA [2,3,23]. The low dietary intake of long-chain PUFA is thought to be the main cause of the decrease in the levels of DHA, EPA and AA, as their endogenous synthesis is very low [24,25].

Given the important role of long-chain PUFA for the normal cognitive and visual development, controlled trials were also performed to elucidate the effect of PUFA supplementation on the FA status of patients with PKU, using plasma or RBC samples [4,23,26–31]. Although some conflicting results between studies were reported, in most cases an increase in the content of n-3 PUFA was observed [3].

Since FA are mainly esterified in other classes of lipids, namely in phospholipids (PL) which contain about 50% of the total amount of plasma FA [32], variations in the plasma FA profile of PKU patients may impact these classes. Some of the studies that have shown variations in the FA profile of PKU individuals have been performed using the plasma PL fraction [4,20,21,23,29,33]. However, changes at the level of individual PL species in PKU patients have never been explored.

Since PL have an important role in the signalling and regulatory functions in health and disease [3], further characterization of plasma PL in PKU patients is needed to identify the plasticity of the PL profile due to dietary restrictions.

The present study represents the first report comparing the plasma phospholipidome of children with PKU and healthy children (control group, CT), highlighting the differences in the lipid profile of children with PKU at the level of PL species. For that, the PL plasma extracts obtained from children with PKU and healthy children (CT) were characterized by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) for the identification of polar lipid species. Complementarily, gas chromatography-mass spectrometry (GC-MS) analysis was carried out for the determination of the composition of FA.

2. Material and Methods

2.1. 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 of age, 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. At the time of the study, all patients, except one, had genetic studies completed. p.R261Q in five alleles, p.R270K in four and p.R158Q in three, were the most common pathogenic variations found. No correlation between phenotype and genotype was established. Summary information on patients is shown in Supplementary Table S1. 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 the CT children varied between 7 and 16 years. With an average of 12 years of age, the CT group included 4 females and 8 males. The collection of the blood samples was taken in the fasted state. Blood (2 mL) was collected into lithium-heparin tubes and centrifuged (2000 ×g, 10 min) to recover the plasma fraction. The plasma samples, after collection, were stored at -80ºC until the lipid extraction.

The collection of plasma samples required for this study was accepted by the Hospital Ethics Committee (vote number: CHUC-094-16) and all parents (or legal representatives) signed informed consent, in agreement with the ethical standards of the Declaration of Helsinki.

2.2. Plasma Phospholipids Extraction

The PL were extracted from plasma samples by solid-phase extraction (SPE) with a Visiprep SPE vacuum Manifold (Supelco, Sigma-Aldrich, Bellefonte, PA, USA) [34,35]. Briefly, protein precipitation was performed by mixing 100 µL of plasma sample with 900 µL of acetonitrile (ACN) with 1% formic acid, followed by vortexing (30 s) and centrifugation (626 ×g, 5 min). The resulting supernatant was transferred to a SPE column (HybridSPE-Phospholipid 30 mg, SUPELCO, Sigma-Aldrich, Bellefonte, PA, USA), after conditioning step (1 mL of ACN). As most supernatant was eluted, the column was washed with 1 mL of ACN with 1% formic acid and 1 mL of ACN. The PL were then eluted with ACN with 5% aqueous ammonia (2 x 1 mL). The PL were recovered and dried under a nitrogen stream. The extracts were then 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 Corporation, Billerica, MA, USA). The filtered samples were collected in amber vials, dried and stored at -80 ºC until further lipid analysis.

2.3. Phospholipids Quantification

After SPE extraction, the total amount of PL was quantified by phosphorus (P) measurement [36], as previously done [34,37]. PL-enriched extracts were dissolved in 100 µL of dichloromethane, and a volume of 10 µL was transferred, to a glass tube previously washed with 5% nitric acid, in duplicate. After drying the solvent under a stream of nitrogen, 125 µL of 70% perchloric acid were added to each tube. Samples were incubated in a heat block (Stuart, Staffordshire, U.K.) for 1 h at 180 °C, followed by cooling to room temperature. After that, 825 µL of Milli-Q water, 125 µL of ammonium molybdate (20 mM prepared in Milli-Q water), and 125 µL of ascorbic acid (567 mM prepared in Milli-Q water) were added to each sample, being vortexed between each addition. Samples were then placed during 10 min at 100°C in a water bath. Afterwards, using a cold-water bath, the samples were cooled down. 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 calculated from a calibration curve prepared by carrying out the same procedure (without the heating block step) with standards containing up to 2 µg of P obtainedfrom 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 [38].

2.4. Characterization of Fatty Acids Profile by Gas Chromatography Coupled to Mass Spectrometry (GC-MS)

FA analysis was performed by GC-MS after transmethylation of PL-enriched extracts [39], as routinely used in authors' laboratory [35,37]. Briefly, an amount of lipid extract equivalent to 15 µg of total PL was dissolved in a volume of 1 mL of internal standard methyl nonadecanoate (Sigma, St. Louis, MO, USA) prepared in *n*-hexane (3.63 µM). 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. Thereafter, 2 mL of NaCl solution (171 mM) were added, being centrifuged at 626 ×g for 5 min. A volume of 600 µL of the organic phase was then 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 to 160 °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

achieved by MS spectrum comparison with the chemical database NIST library and "The Lipid Web" [40], and take into account the retention times and MS spectra of FAME standards (Supelco 37 Component FAME Mix, Sigma-Aldrich, Darmstadt, Germany). FA quantification was conducted using calibration curves obtained from FAME standards under the same instrumental conditions.

2.5. Characterization of the Phospholipid Profile by Hydrophilic Interaction Liquid Chromatography Coupled to High-Resolution Tandem Mass Spectrometry (HILIC-MS/MS)

PL-enriched extracts were analysed by 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) [34,35]. The mobile phases were as follow: 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 during 30 min, returning to the initial conditions in 5 min, followed by a 5 min re-equilibration period 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 mixed with an aliquot of 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 temperature 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 sheath gas flow of 15 U and a capillary temperature of 250 ºC. A top-10 data-dependent method was used. Acquisition cycles consisted of one full scan mass spectrum, with a resolution of 70,000, automatic gain control (AGC) target of 1×10^6, and ten data-dependent MS/MS scans with a resolution of 17,500 and AGC target of 1×10^5, that were repeated continuously through the experiments with a dynamic exclusion of 60 s and 2×10^4 of threshold intensity. MS/MS were acquired using a stepped normalized collisional energy of 25, 30, and 35 eV. Data acquisition was performed using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA). The MZmine 2.32 software [41] 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 (RT) 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 of each species by the peak area of the lipid standard with the closest RT.

2.6. Statistical Analysis

Multivariate and univariate statistical analyses were performed using R version 3.6.2 [42] in Rstudio version 1.2.5 [43]. The R package Metaboanalyst was used for imputation and log transformation of the HILIC-MS data (lipid species) and GC-MS data (fatty acids) [44]. EigenMS was also used for normalization of LC-MS data [45]. The R built-in function and the R package pcaMethods were used to perform principal component analysis (PCA) [46]. 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) [47]. Heatmaps were created from autoscaled data using the R package pheatmap [48], and using "Euclidean" as clustering distance, and "ward.D" as the clustering method. All graphics and boxplots were created using the R packages ggplot2 [49], plyr[50], dplyr[51], tidyr[52] and ggrepel[53].

3. Results

3.1. Phospholipids quantification

The total amount of PL recovered after SPE (expressed in µg PL per 100 µL of plasma; mean ± standard deviation) was 41.27 ± 7.30 and 53.78 ± 21.73 for PKU and CT samples, respectively. No statistically significant differences were found by univariate analysis between the two groups (q value > 0.05).

3.2. Fatty Acid Composition analysis by GC-MS

For an overview of the composition of esterified FA, GC-MS analysis was performed. A total of 9 FA were identified in the PKU and CT groups (Table 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 (LA, 18:2 n-6) (17.74 ± 4.62 in PKU and 12.21 ± 6.73 in CT).

Table 1. Fatty acid (FA) profile of extracts enriched in phospholipids (PL) obtained from plasma samples of phenylketonuric (PKU) and healthy children (control, CT), determined by GC–MS. The data represents mean values of FA in µg per 100 µg PL ± 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
18:1n-9	13.04 ± 2.81	8.09 ± 2.24
18:1	4.24 ± 0.47	3.55 ± 0.25
18:2n-6	17.74 ± 4.62	12.21 ± 6.73
20:4n-6	10.40 ± 3.31	7.76 ± 3.12
20:5n-3	2.81 ± 0.35	2.58 ± 0.11
22:6n-3	5.37 ± 1.14	4.17 ± 1.14

Multivariate principal component analysis (PCA) analysis was achieved 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 1).
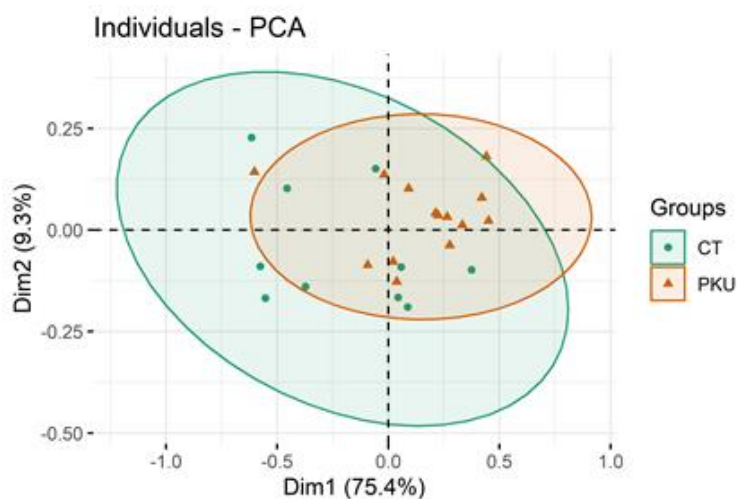

Figure 1. Principal component analysis (PCA) score plot of the fatty acids data set obtained by GC–MS analysis of phospholipid-enriched extracts obtained from plasma samples of phenylketonuric (PKU) and healthy children (control, CT).

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 2, Supplementary Table S2). 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), EPA and DHA (*q*-value < 0.05). No statistical difference was observed for palmitic acid (16:0), LA, and AA.
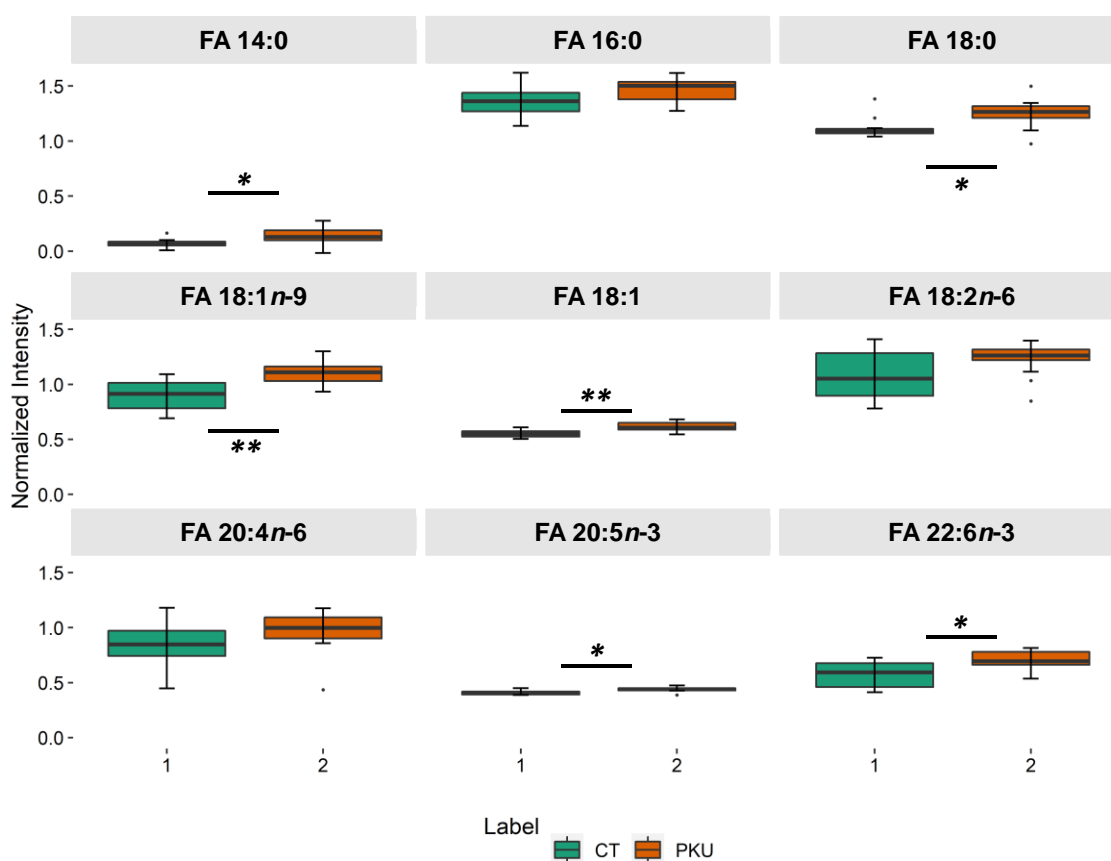

Figure 2. Boxplots of the fatty acids (FA) identified by GC-MS from control (CT) and phenylketonuric (PKU) children organized (left to right, top to bottom) by increasing number of carbon atoms and double bonds. FA are labelled as follows: FA xx:i (xx=number of carbon atoms; i= number of double bonds), followed by *n*-y for unsaturated FA (y=double-bond position related to the methyl end). Data from FA quantification were glog transformed using Metaboanalyst [44]. 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 (Supplementary Table S2).

3.3. 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 PL (*m/z* values), belonging to 9 different classes: phosphatidylcholine (PC; including diacyl, alkyl/alkenyl-acyl and oxidized species), lyso PC (LPC; including monoacyl, alkyl/alkenyl and oxidized species), sphingomyelin (SM), phosphatidylethanolamine (PE; including diacyl, alkyl/alkenyl-acyl and oxidized species), lyso PE (LPE), phosphatidylglycerol (PG), lyso PG (LPG), phosphatidylinositol (PI), and phosphatidylserine (PS). In addition to the PL species, 3 species of sphingolipids belonging to the class of ceramides (Cer) have also been identified (Supplementary Table S3). The PC, LPC, SM and Cer species were identified and semi-quantified in the positive mode as $[M+H]^+$ ions.. Confirmation of the PC, LPC and SM classes were obtained by analysing the MS/MS data of $[M+H]^+$ ions with the identification of the product ion of exact mass 184.0739 , corresponding to the phosphocholine polar head (Figure S1a and S2a). 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 product ions corresponding to the fatty acyl chains such as $[RCOO]^-$ (Figure S1b). 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. Confirmation of the phospholipid class for

the PE and LPE species was obtained, from MS/MS data in the positive mode, of [M+H]+ ions, by 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 the [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 only based on the mass accuracy (error < 5 ppm) and RT. As an example, MS/MS spectra of isomeric plasmanyl and plasmenyl species, as well as an oxidized PL species are also shown in the Supplementary (Figures S7 and S8).

To visualize sample grouping, the multivariate analysis by PCA was performed on the lipid species data set. Two identified outliers, from the PKU and CT group, were removed from further analysis. The PCA score plot of the lipid species demonstrated 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 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 3).
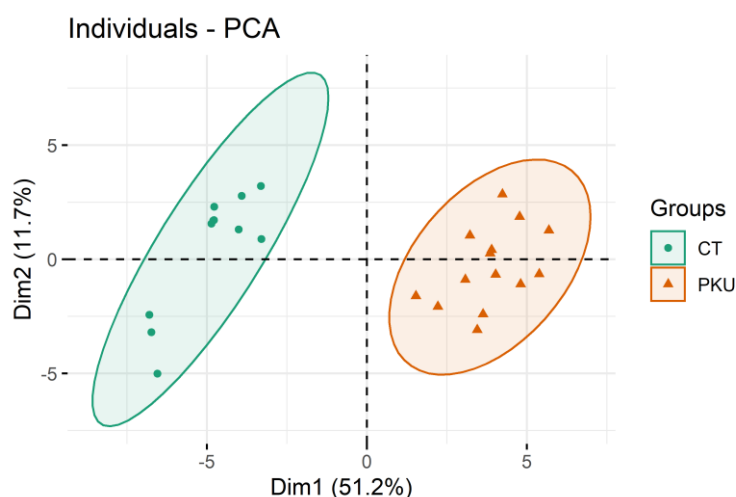
Figure 3. Principal component analysis (PCA) score plot of the lipid species dataset obtained by HILIC-MS/MS analysis of phospholipid-enriched extracts obtained from plasma samples of phenylketonuric (PKU) and healthy (control, CT) children.

Univariate analysis (Shapiro–Wilk test followed by Welch *t*-test or Mann-Whitney test) was also performed to evaluate lipid species with significant variation between the two groups (CT *vs* PKU). The results revealed that 146 of 190 lipid species were statistically different between groups (q-value < 0.05) (Supplementary Table S4). Boxplots of the 16 main species with the lowest q-values (q-value < 0.001) are shown in Figure 4. These 16 species that exhibited 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 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).
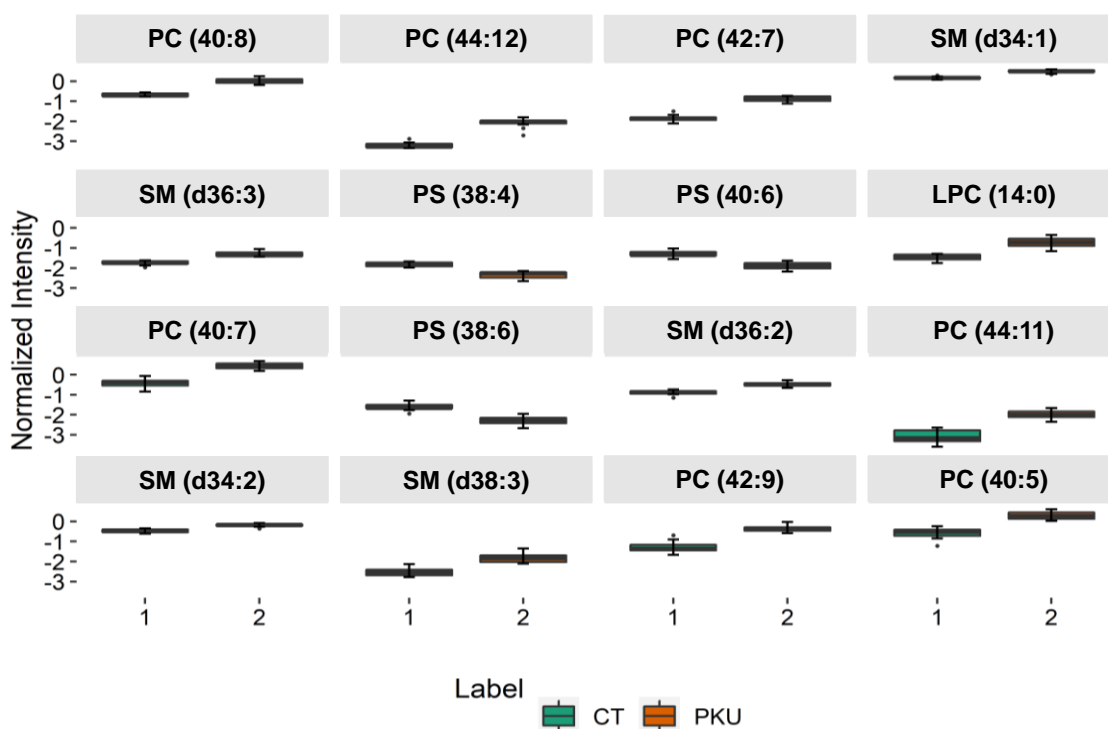
Figure 4. Boxplots of the 16 main lipid species showing the major variation between control (CT) and phenylketonuric (PKU) children sorted (left to right, top to bottom) by the lower *q*-values of the Welch t-test or Mann-Whitney test (Supplementary Table S4). Data were glog transformed after missing value imputation using Metaboanalyst [44] and normalized with EigenMS [45]. 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.

A heatmap (double dendrograms) with two-dimensional hierarchical clustering was created using the top 25 *q*-values (*q*-value < 0.001) from Welch *t*-test or Mann-Whitney test (Figure 5). In the first level of the upper hierarchical dendrogram, it is possible to observe that the samples were 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 coherent with what was shown in the PCA score plot (Figure 3). In respect to the clustering of individual lipid species by similarity in changes in lipid expression, the first group at the first level of the dendrogram (on the left of the heatmap in Figure 5) 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 included 13 PC, 5 SM, 1 LPC, and 2 PI species, which were more abundant in the PKU group. Concerning the

composition of these top 25 species, PC species with high total carbon chain length (C38-44) and level of unsaturation (4-12), bearing PUFA, were more abundant in the PKU individuals.
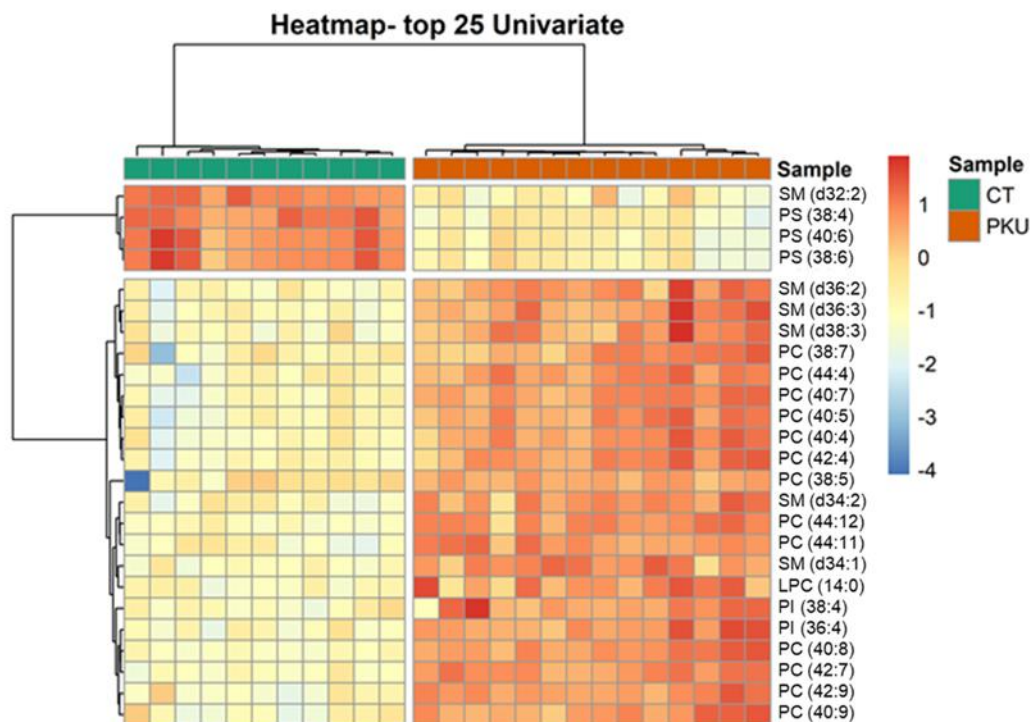

Figure 5. Two-dimensional hierarchical clustering heatmap of the 25 main lipid species with the lowest *q*-values of the Welch t-test or Mann-Whitney test. The dendrogram at the top shows the clustering of the samples, and on the left shows the clustering of the lipid species. Levels of relative abundance are shown on the colour scale, with numbers indicating the fold difference from the mean. Phospholipid species are indicated 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.

4. Discussion

Previous studies have revealed alterations in lipoprotein levels of serum/plasma and the FA profile of plasma and red blood cells of PKU individuals [3,5,16,19,26,29,33,54,55]. Such changes can affect the composition of PL as they are the main components of human plasma lipoproteins and incorporate FA into their composition [56]. Indeed, some of the changes reported in the FA profile of PKU individuals have been found in the plasma PL fraction [4,20,21,23,29,33]. To our knowledge, this study represents the first report comparing plasma

phospholipidome of PKU children and healthy children, highlighting the changes in the lipid profiles of PKU children at the level of individual lipid species.

Initially, 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 16:0 and FA with 18 carbon chains as highly abundant FA [35,57]. In accordance to the multivariate analysis of the FA dataset by PCA (Figure 1), the PKU and CT groups were not separated.

Regarding to the univariate analysis of the FA dataset, the PKU group showed a significant increase in the level of PUFA DHA and EPA, compared to the CT group (Figure 2). Consistent with these results, in seven studies [4,23,26–29,58], higher levels of DHA in the total serum/plasma PL fraction were observed in PKU individuals supplemented with PUFA. Only two of these seven studies showed an increase in the level of EPA in plasma [23,28], as observed in this study. It is known that in healthy individuals only 2-5% and 5-10% of α-linolenic acid are converted to DHA and EPA, respectively[59]. Consequently, the DHA and EPA status is determined by food intake [60]. As PKU individuals do not consume the typical food sources of DHA, such as fish, the increase in DHA level in PKU patients taking supplements/amino acid mixtures with DHA could be a consequence of such DHA supplementation. However, it is known that dietary DHA causes 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 [61]. Such a reaction may also have contributed to the increased level of EPA in PKU patients with DHA supplementation. The levels of AA were not significantly different between PKU and CT groups, which is consistent with other studies [4,28,29].

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 [3], especially DHA and EPA, as observed in this study. Such an increase can have positive effects on the health of PKU patients. It is well known that PUFAs are important structural and functional constituents of all cell membranes and are crucial for the normal visual and cognitive development [24,62]. Also, DHA and EPA have been associated with anti-inflammatory properties [63,64]. 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 [63]. Therefore, it is possible to speculate that 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 [24,65]. Furthermore, the higher levels of *n*-3 PUFA are associated with a reduction in the risk of developing cardiovascular diseases and atherosclerosis [62,63,66,67]. 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.

The PCA of the lipid species showed a separation between the PKU and CT groups (Figure 3), highlighting the importance of the study at the level of the lipid species. The results of the univariate analysis (Figure 4 and 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 [56,68], showed a significant increase in PKU children compared to CT. Thirteen PC species were in the top 25 lipid species with a major variation. Although the composition of FA was only identified for 6 of them, it was possible to deduce that the remaining 7 PC species also contain PUFA in their composition. 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, also an up-regulation of some ether PC as well as ether PE has been observed in PKU children, both representing 15.06% of all statistically significant lipid species (Supporting Information, Table S4). The physiological functions of these unique alkyl-acyl PLs are not fully understood. However, it has been reported that ether-linked PL, especially plasmalogens, have antioxidant properties because of their ability to scavenge oxygen radicals, as they are preferentially oxidized [69–71], and seem to be transformed into inoffensive products that can be reutilized by the organism [71,72]. The increase of

some ether-like PC and PE may be beneficial for PKU children because they can decrease oxidative stress and lipid peroxidation [3].

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 [73]. Alterations in PI content are probably associated with their signalling roles, as they are important precursors of signalling molecules, such as PIPs that regulate metabolic processes [74]. 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 [75].

PS is the major class of anionic phospholipids in eukaryotic membranes [76]. 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 [76]. 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 [77]. 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 [78]. 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 the variable diet and supplementation in the PKU

group. Additionally, the quality of PUFA supplementation taken by the patients might influence the PKU lipidomic profile. 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). In further studies, it should be interesting to evaluate the alterations in the plasma lipid profiles with the type of diet and supplementation practiced by the PKU patients. These constraints should be considered in order to better understand how the lipid profile of phenylketonuric is modified, as well as to analyse alterations in possible metabolic pathways. For example, the amino acid restriction, necessary in PKU, could influence the function of mTOR resulting in alterations in lipolysis/lipogenesis [79,80]. 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.

5. Conclusions

The present study reports, for the first time, the application of mass spectrometry-based lipidomics to identify the plasma phospholipidome of children with phenylketonuria and healthy children. The changes observed in the profile of esterified FA in the PL of PKU children, in particular the increase in the levels of *n*-3 PUFA, namely DHA and EPA, may be associated with PUFA supplementation. In terms of individual PL species, the most significant changes were revealed by increased plasma levels of PC, PI and SM and decreased PS species. In particular, an increase in several PC with PUFA was observed. The increased content of PL species containing PUFA could have a positive impact on reducing the inflammatory state of patients, as well as reducing the risk of developing cardiovascular disease and atherosclerosis. Also, the PKU group showed an increase in ether-like PC and PE, which are associated with antioxidant activity. Regarding the higher levels of SM, it is necessary to clarify the variation and the significance of this class of lipids in children with PKU, as

they have previously been associated with atherosclerosis and risk factors for coronary heart disease in human adults.

Associated Content

Supporting Information

(Table S1) Data collected for each of PKU patients. (Table S2) Significant variations between the FA identified by GC-MS from PKU and CT groups. (Table S3) 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). (Table S4) The 146 lipid species showing significant variation between PKU and control (CT) groups. (Figure S1) Representative MS/MS spectra of phosphatidylcholine (PC) lipid species. (Figure S2) Representative MS/MS spectra of sphingomyelin (SM) lipid species. (Figure S3) Representative MS/MS spectrum of ceramide (Cer) lipid species. (Figure S4) Representative MS/MS spectra of phosphatidylethanolamine (PE) lipid species. (Figure S5) Representative MS/MS spectrum of phosphatidylglycerol (PG) lipid species. (Figure S6) Representative MS/MS spectrum of phosphatidylglycerol (PI) lipid species. (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. (Figure S8) Representative MS/MS spectra of oxidized phosphatidylcholine (PC) lipid species.

Acknowledgments

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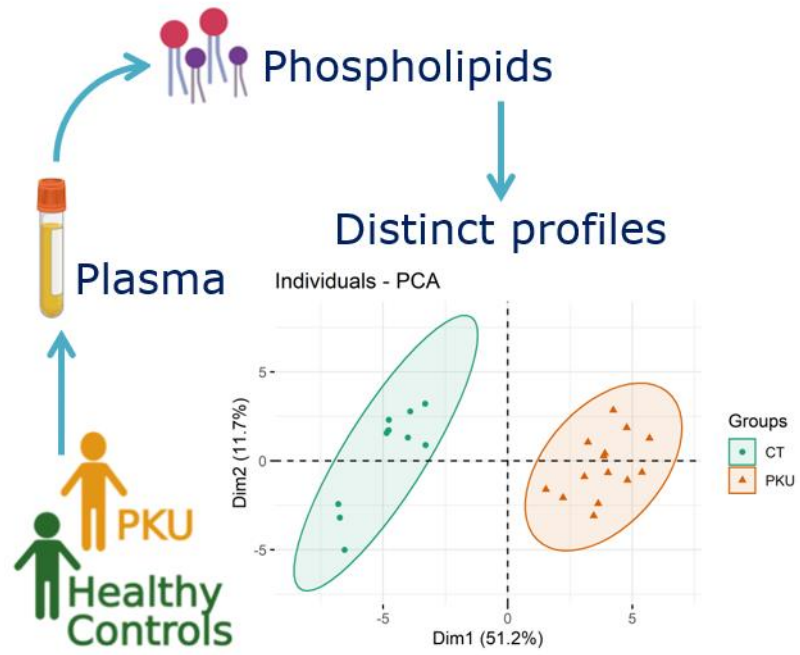
Supplementary Information

Plasma phospholipidomic profile differs between children with phenylketonuria and healthy children

Inês M. S. Guerra[a], Luísa Diogo[b], Marisa Pinho[c], Tânia Melo[a,d], Pedro Domingues[a], M. Rosário Domingues[a,d], Ana S. P.Moreira[a,e,*]

[a] Mass Spectrometry Center, LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] Reference Center of Inherited Metabolic Diseases, Hospital Pediátrico, Centro Hospitalar e Universitário de Coimbra, 3000-075 Coimbra, Portugal
[c] ECOMARE, CESAM - Center for Environmental and Marine Studies, Department of Biology, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal
[d] CESAM - Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[e] CICECO - Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

*Corresponding author. *E-mail address:* ana.moreira@ua.pt

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Supplementary Table S1. Data collected for each of phenylketonuric (PKU) patients.

Age	Sex	Forms of the disease	Phe (mg/dl) screening	AA mixture supplement	PUFA supplement	PAH gene	
17	M	Moderate	13.9	Yes	No	p.R261Q	p.L308F
14	F	Mild	9.1	Yes	Keyomega®	p.I65T	p.R261Q
14	F	Moderate	15	Yes	Keyomega®	p.R243X	p.R243X
13	M	Mild	6.8	Yes	No	p.R176L	p.R243Q
12	F	Moderate	16.5	Yes	Keyomega®	p.R252W	p.R270K
12	M	Classic	20	Yes	DHA richoil®	p.R158Q	p.R158Q
12	F	Moderate	11	Yes	DHA richoil®	p.R158Q	p.L249F
11	F	Moderate	14.7	Yes	DHA richoil®	p.R261Q	p.R270K
8	F	Mild*	6.5	No	No	p.G352Vfsx12	p.I421T
7	M	Classic	27.2	Yes	No	IVS10-11G>A	IVS12+1G>A
6	F	Moderate	13.8	Yes	No	p.I65T	p.P281L
4	M	Mild#	7.9	Yes	No	p.R261Q	p.R261Q
3	M	Moderate#	15.6	Yes	No	na	na
3	M	Mild#	8	Yes	No	p.R270K	p.A403V
4	M	Classic#	26.3	Yes	No	p.R270K	IVS10-11G>A

The cohort included moderate, mild and classical PKU patients based on Phe levels at newborn screening. Dietary treatment was started up to 10 days of age, in average.
#PKU patients were submitted to an acute BH_4 test.
*PKU patient was on Kuvan® treatment.
na-Genetic test result not available.

Supplementary Table S2. Significant variations between the FA identified by GC-MS from PKU and CT groups, sorted 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 (*q*-values <0.05) between the groups revealed by Welch t-test or Mann-Whitney test are marked with * if *q* < 0.05 and ** if *q*< 0.01. ns – not significant.

Fatty acids	FDR, *q*-value	Statistical significance level
14:0	0.03179	*
16:0	0.065880	ns
18:0	0.027164	*
18:1*n*-9	0.005159	**
18:1	0.003238	**
18:2*n*-6	0.090307	ns
20:4*n*-6	0.152209	ns
20:5*n*-3	0.033109	*
22:6*n*-3	0.027164	*

Supplementary Table S3. 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, considering that in animals the *sn*-1 position generally contains smaller and saturated fatty acids, while fatty acids with a higher unsaturation degree are located at *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$_3$COO]$^-$)					
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$_3$COO]$^-$)

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 S4. The 146 lipid species showing significant variation between PKU and control (CT) groups, 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 in PKU group
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	*	↑
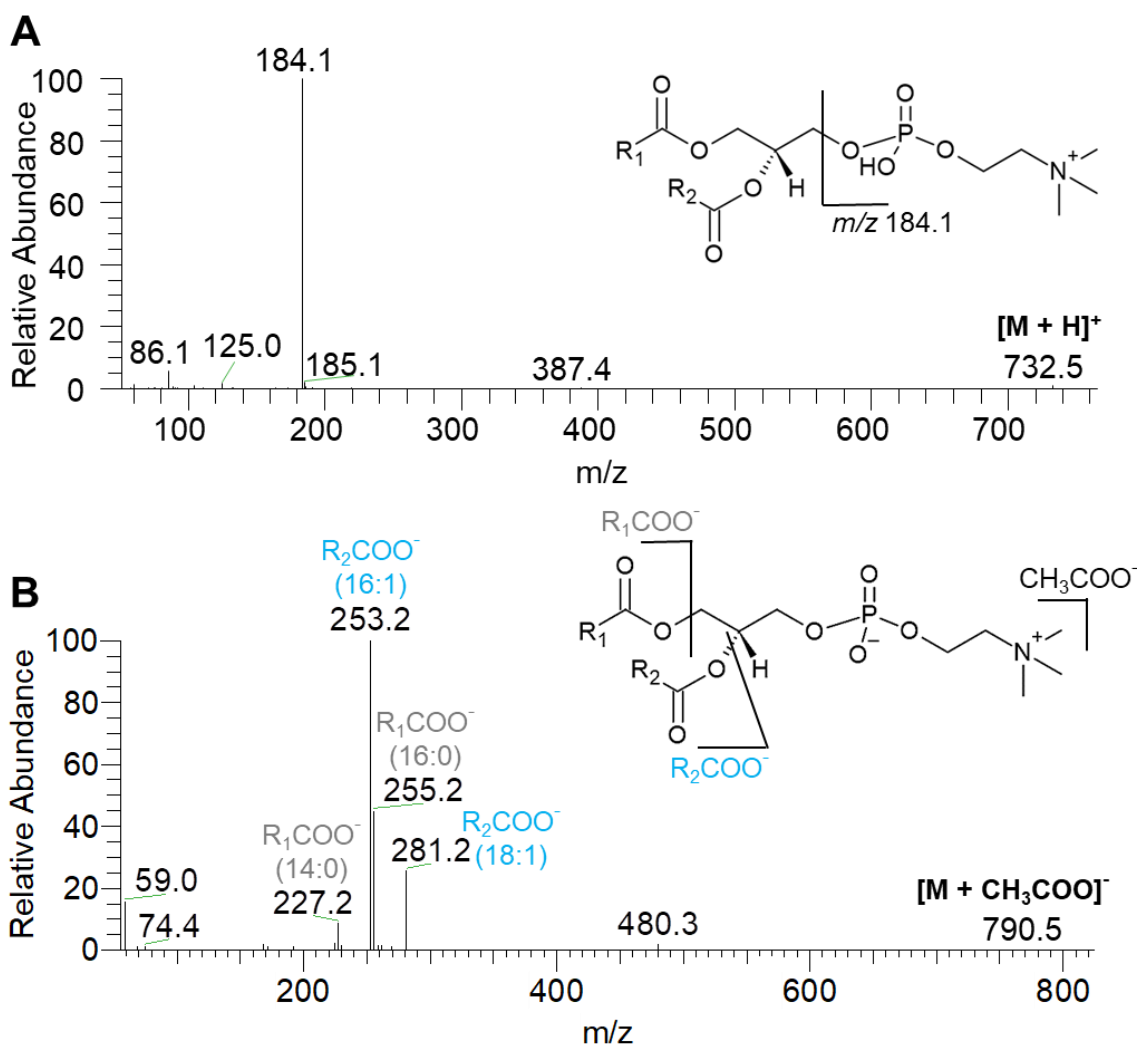
Supplementary Figure S1. Representative MS/MS spectra of phosphatidylcholine (PC) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species PC (32:1) observed in positive mode as [M + H]$^+$ ion at m/z 732.5. Confirmation of phospholipid class was achieved by the identification of the product ion 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$_3$COO]$^-$ 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$_1$COO$^-$) and 18:1 (R$_2$COO$^-$) 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$_1$COO$^-$) and 16:1 (R$_2$COO$^-$) 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.
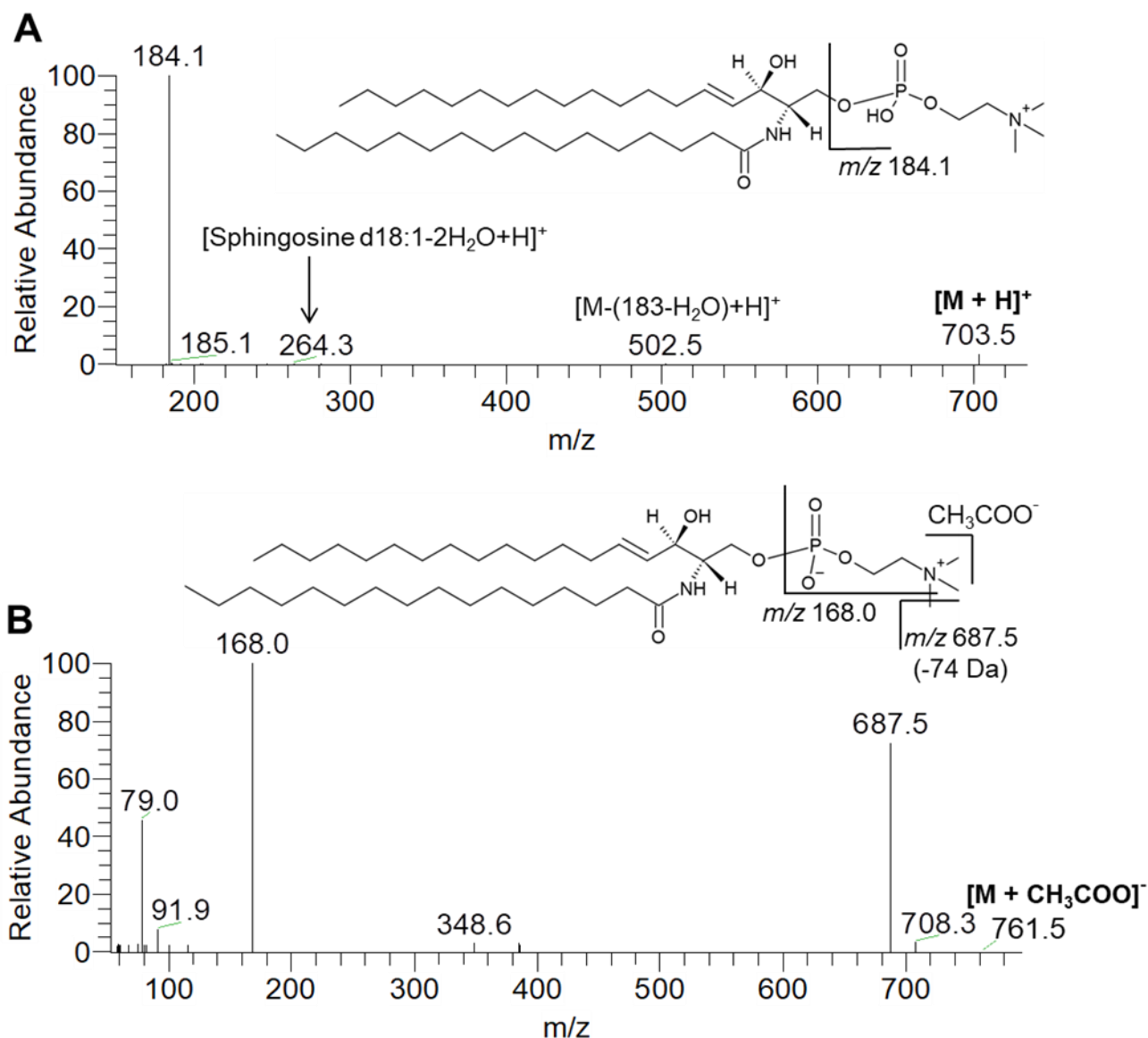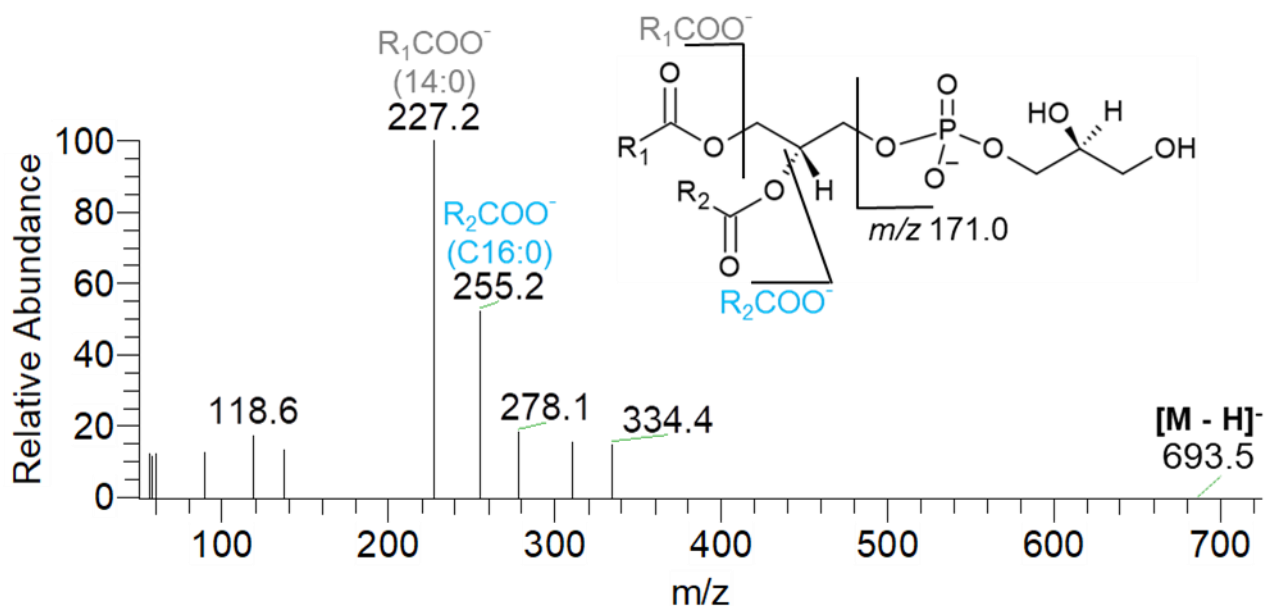
Supplementary Figure S2. Representative MS/MS spectra of sphingomyelin (SM) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species SM (d34:1) observed in positive mode as $[M + H]^+$ ion at m/z 703.5. Confirmation of phospholipid class was achieved by the identification of the product ion at 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$_2$O+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.
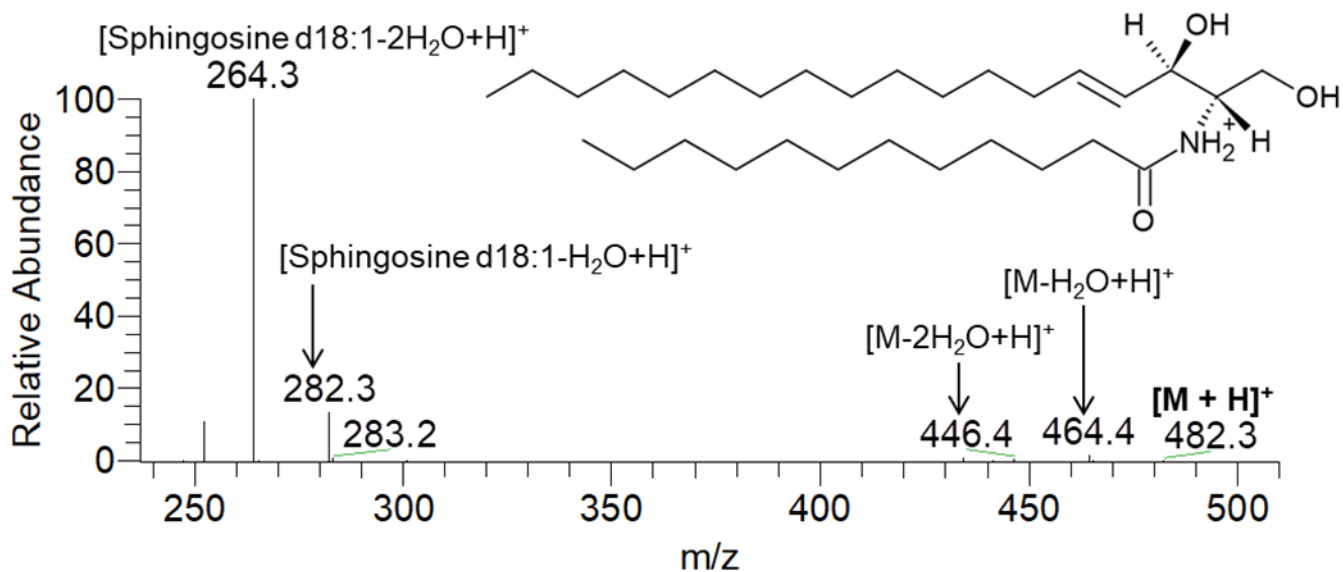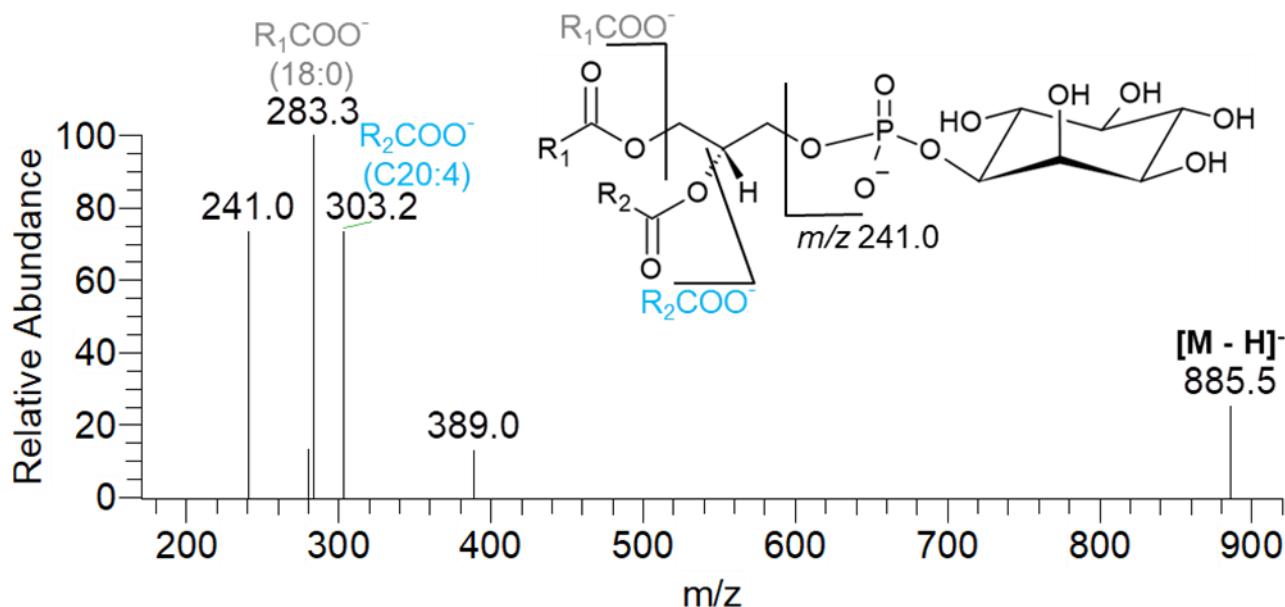
Supplementary Figure S3. Representative MS/MS spectrum of ceramide (Cer) lipid species. HILIC-MS/MS spectrum of the lipid species Cer (d30:1) observed in positive mode as $[M + H]^+$ ion at m/z 482.3. Confirmation of phospholipid class was achieved by the identification of the abundant product ions of 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.
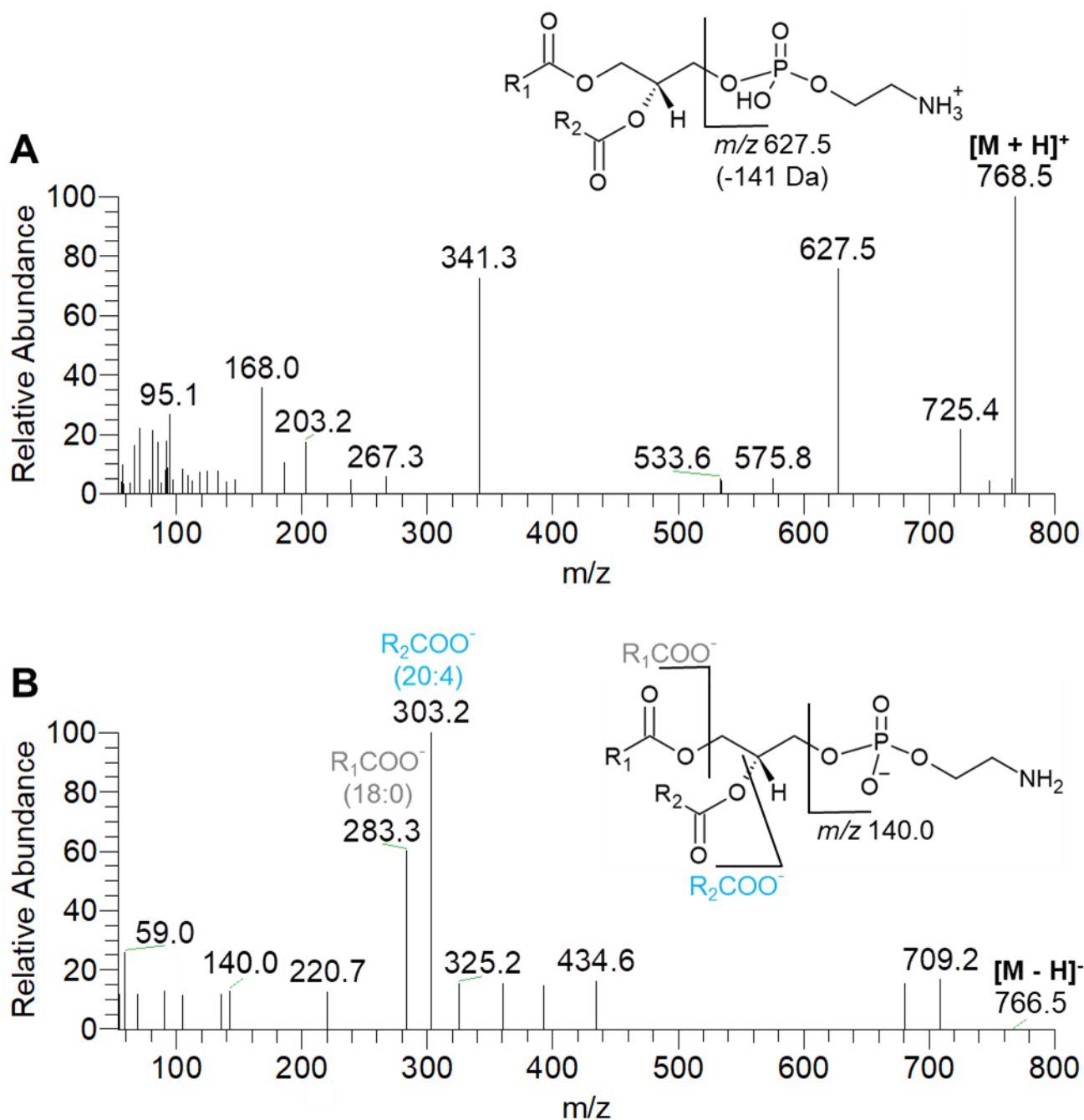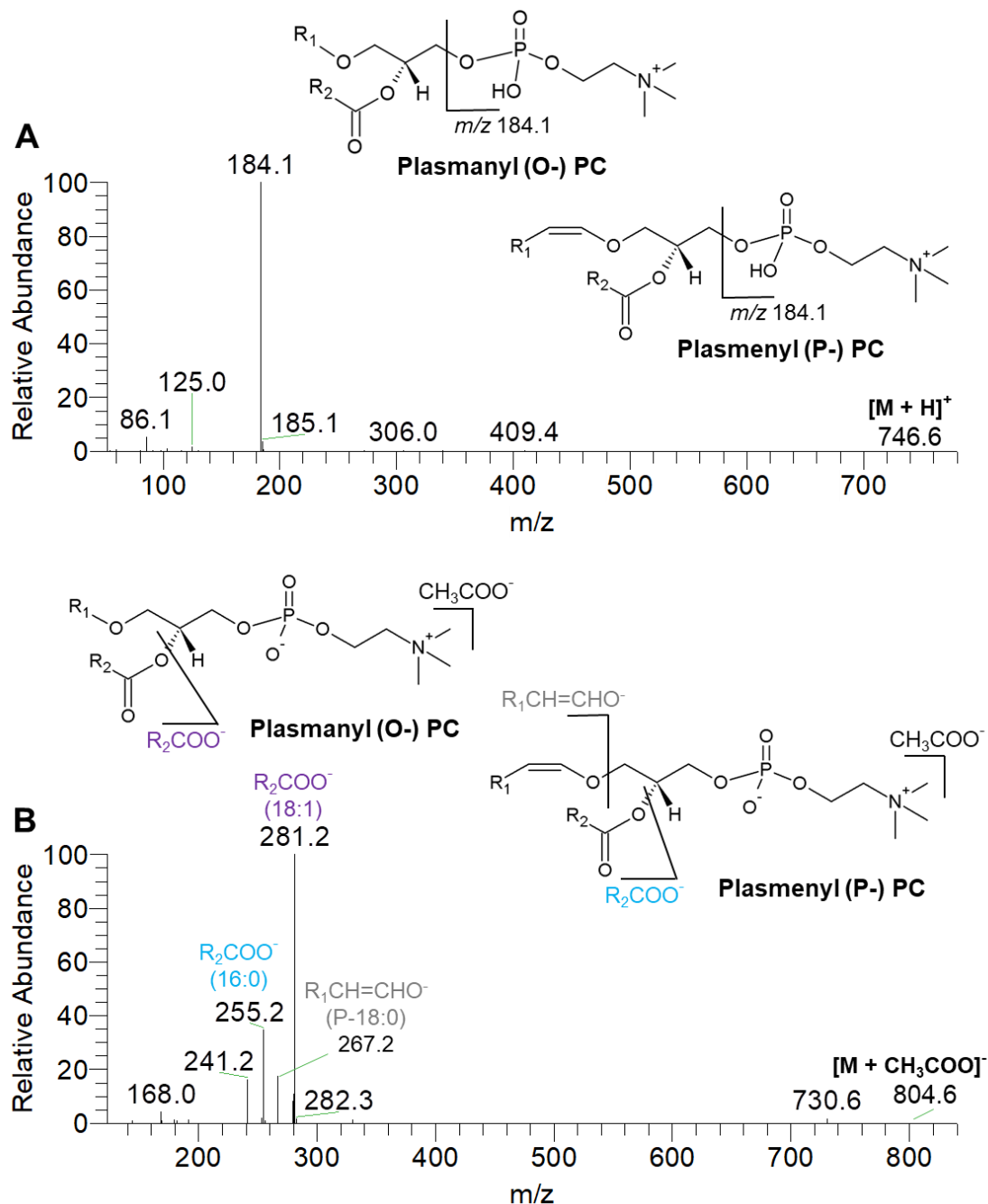
Supplementary Figure S4. Representative MS/MS spectra of phosphatidylethanolamine (PE) lipid species. **A)** HILIC-MS/MS spectrum of the lipid species PE (38:4) observed in positive mode as [M + H]$^+$ ion at *m/z* 768.5. Confirmation of phospholipid class was achieved by the identification of the neutral 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$_1$COO$^-$) and 20:4 (R$_2$COO$^-$), 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.
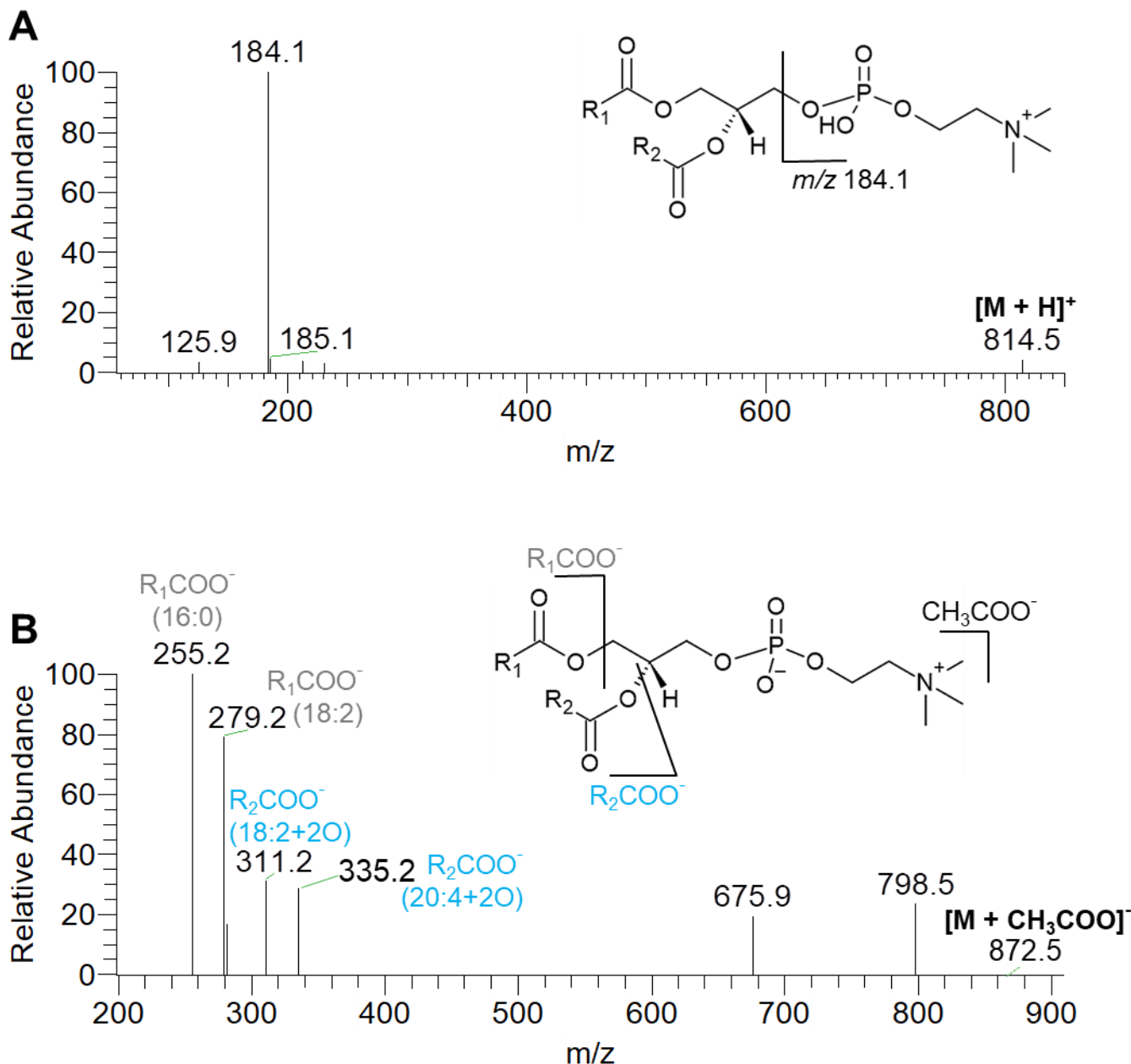
Supplementary Figure S5. Representative MS/MS spectrum of phosphatidylglycerol (PG) lipid species. HILIC-MS/MS spectrum of the lipid species PG (30:0) observed in negative mode as [M - H]$^-$ ion at m/z 693.5. Confirmation of phospholipid class may be achieved by the identification of the product ion 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$_1$COO$^-$) and 16:0 (R$_2$COO$^-$) 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 phosphoinositol 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$_1$COO$^-$) and 20:4 (R$_2$COO$^-$) 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 + CH3COO]- 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 (R1CH=CHO-) and 16:0 (R2COO-), 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 (R2COO-), 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 (R2COO-).

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: C5H15NO4P; 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 (R$_1$COO$^-$) and 20:4+2O (R$_2$COO$^-$) 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$_1$COO$^-$) and 18:2+2O (R$_2$COO$^-$) 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.