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Plasma lipidomics in early APP/PS1 female mouse model and its relationship with brain: Is it affected by the estrous cycle?

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Abstract

Background Alzheimer's disease (AD) is the most prevalent dementia, showing higher incidence in women. Besides, lipids play an essential role in brain, and they could be dysregulated in neurodegeneration. Specifically, impaired plasma lipid levels could predict early AD diagnosis. This work aims to identify the main plasma lipids altered in early AD female mouse model and evaluate their relationship with brain lipidome. Also, the possible involvement of the estrous cycle in lipid metabolism has been evaluated.

Methods Plasma samples of wild-type ($n=10$) and APP/PS1 ($n=10$) female mice of 5 months of age were collected, processed, and analysed using a lipidomic mass spectrometry-based method. A statistical analysis involving univariate and multivariate approaches was performed to identify significant lipid differences related to AD between groups. Also, cytology tests were conducted to confirm estrous cycle phases.

Results Three hundred thirty lipids were detected in plasma, 18 of them showed significant differences between groups; specifically, some triacylglycerols, cholesteryl esters, lysophosphatidylcholines, phosphatidylcholines, and ether-linked phosphatidylcholines, increased in early AD; while other phosphatidylcholines, phosphatidylethanolamines, ceramides, and ether-linked phosphatidylethanolamines decreased in early AD. A multivariate approach was developed from some lipid variables, showing high diagnostic indexes (70% sensitivity, 90% specificity, 80% accuracy). From brain and plasma lipidome, some significant correlations were observed, mainly in the glycerophospholipid family. Also, some differences were found in both plasma and brain lipids, according to the estrous cycle phase.

Conclusions Therefore, lipid alterations can be identified in plasma at early AD stages in mice females, with a relationship with brain lipid metabolism for most of the lipid subfamilies, suggesting some lipids as potential AD biomarkers. In addition, the estrous cycle monitoring could be relevant in female studies.

Keywords Plasma, Brain, Lipid, Early, Alzheimer, Mouse model, Estrous cycle

Background

Nowadays, the main cause of dementia in the ageing population is Alzheimer's disease (AD) [1], and the number of people affected is expected to increase in the coming years [2]. The incidence rate of AD is higher in women [3], and it could be not only because of the greater women's longevity compared to men [4]. In fact, it could be due to sex differences (such as genetic or hormonal differences), gender differences (environmental, social and cultural influences), or a combination of both [5]. Also,

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APOE-ε4 genotype may have a stronger association with AD and neurodegeneration in women than in men, this may be explained by an interaction between the APOE genotype and estrogen [6]. Moreover, amyloid-β (Aβ) deposition has been observed exacerbated in postmenopausal women APOE-ε4 carriers [7]. On the other hand, low educational attainment, which historically is more common in women, is a risk factor for dementia [8]. In addition, women have evidence of more rapid cognitive decline and neurodegeneration than men, despite having similar levels of Aβ and tau [6]. Therefore, female-focused studies identifying potential biomarkers are required to explain the differences and to advance in early detection.

Focusing on animal models, data from studies using female animals are thought to be complex due to the greater variability associated with the estrous cycle [9]. Although female mice do not appear to be more variable than male mice [10], there may be differences in some mechanisms. In fact, recent studies are being conducted in both sexes, but there is few studies using only female mice, or equal number of males and females [11]. Furthermore, there is a lack of mice studies looking for AD biomarkers and considering the estrous cycle.

Regarding AD biomarkers, lipids could play an important role since brain has a high lipid content. They are formed mainly in the liver and adipose tissue and are rapidly taken up by brain transporters through the blood and integrated into brain lipids [12]. Lipids are known to affect the structure and function of the blood–brain barrier (BBB). Some lipids are able to cross the BBB and can regulate the transport of other substrates across the BBB. Under pathological conditions, several vasoactive agents, cytokines and chemical mediators are released from brain due to an increase in the BBB permeability. In addition, BBB dysfunction has been observed in many pathologies of the central nervous system, such as Parkinson's and Alzheimer's diseases [13]. It is believed that BBB disruption may influence the transport of Aβ into the brain, which has implications for AD prognosis [14].

Currently, AD diagnosis is based on expensive and invasive techniques [15], so the need to identify new biomarkers in plasma has gained importance in recent years [16]. In particular, the identification of lipid biomarkers in blood samples [17–19], since an imbalance of lipid metabolism is known to result in abnormal brain functionality, characterizing the disease progression [20]. Despite other risk factors of AD (e.g. increased blood pressure, bodyweight, smoking, decreased physical activity, traumatic brain injury) [21], some studies have reported a link between plasma lipid levels and AD [22, 23]. However, few studies focused on AD plasma lipidome for the identification of a large number of lipids as crucial metabolic factors in the pathophysiology of the disease [22]. Moreover, there is a connection between plasma and brain, since there is evidence of a negative correlation between lysophospholipids and monounsaturated fatty acids in these samples [24]. In general, lipid alterations are correlated with cognitive impairment, showing a relationship with the severity of AD [25, 26], and the development of multiple psychiatric or neurological disorders associated with cognitive decline [27]. In some cases, lipidomics may help to predict the AD progression [28]. Consequently, plasma lipids have potential utility as diagnosis AD biomarkers. On the other hand, a wide variety of mouse models have been used in these studies, with APPswe/PS1dE9 being one of the most studied. It is described as a double transgenic model of Alzheimer's disease (AD) with familial amyloid precursor protein and presenilin-1 mutations.

The aim of this work is to perform a lipidomic study in plasma samples from female wild-type (WT) and APPswe/PS1dE9 transgenic (TG) mice, identifying the main plasma lipids altered in females due to early AD, evaluating the relationship between plasma and brain altered lipids from the same mice and studying differences in lipids due to the estrous cycle.

Materials and methods

Animals

APPswe/PS1dE9 transgenic and wild-type (WT) female mice with C57BL6 x C3H hybrid background (line 85) from the same colony and littermates were used in this study, as both mutations are associated with early AD. This mouse model is useful in the study of amyloid plaque formation and ageing. Specifically, in this model the Aβ aggregation appears at 6 months [29, 30], so an age of 5 months and 10 days (± 4 days) was selected, as this age corresponds to an early stage of AD. Mice were housed in groups (2–6 mice per cage) and maintained under standard housing conditions in a 12:12-h dark–light cycle at 23 ± 1 °C and 60% relative humidity at the Animal Facilities Service from the University of Valencia (Spain). They fed with standard diets and had free access to water ad libitum.

All experimental procedures were approved by the Ethics Committee for Experimentation and Animal Welfare at the University of Valencia (reference: A202203291754, date: 22–08-2022), and performed following relevant animal experimentation guidelines and regulations (RD53/2013 on the Protection of Animals used for experimentation and other scientific purposes Ministry of the Presidency, Spain). Also, this study was carried out following the ARRIVE guidelines (https://arriveguidelines.org.

Genotyping and cytology testing

Following weaning, the QIAamp Fast DNA Tissue Kit (QIAGEN, Germany) was used for PCR genotyping by taking tissue samples from the ear or tail of mice. APPswe/PS1dE9 (TG) mice were identified by the presence of two bands.

The estrous cycle phase was confirmed by vaginal cytology. After the animal was anaesthetised, the technique consisted in a previous washing of the vaginal canal with a saline solution at room temperature with an isotonic concentration of 0.9% sodium chloride (NaCl). A few drops of the same solution were deposited at the entrance of the vaginal canal using a Pasteur pipette, and the contents were released and suctioned three times to collect vaginal cells. A drop of this content was placed on a glass slide and left to dry at room temperature. Following the methodology described in the article by McLean et al. [31] the staining was performed with crystal violet staining (0.1%, w/v). Finally, the smear was examined under the light of the Leica DMD 108 microscope (Wetzlar, Germany) to determine the cell type present. In a previous article published [32], mainly cornified squamous epithelial cells were observed in the estrus phase, and mainly leukocytes in the diestrus phase.

Plasma samples collection

Five-month-old mice were anaesthetised with isoflurane (4 – 5%) and sacrificed by cervical dislocation. Blood samples were taken by decapitation. To prevent clotting, heparin was added to the tubes before sampling. To obtain plasma, blood samples of approximately 1 mL were centrifuged in an EDTA microtainer tube (Aquisel, Barcelona, Spain) for 15 min at 1500 × g. Thereafter, nearly 400 µL of the supernatant plasma were collected and stored at –80 °C until the analysis.

Plasma samples treatment

Lipid extraction was performed by adding 150 µL of isopropanol to 50 µL of the plasma previously thawed, vortexed and left to stand for 20 min at -20 °C. Later, it was centrifuged at maximum power (13,000 g) for 10 min at 4°C and the supernatant was collected. Then, 300 µL of isopropanol were added to 50 µL of the extract, from which 90 µL were transferred to a 96-well injection plate. After that, 10 µL of the internal standard (IS) mix solution ((MG(17:0), LPC(17:0), Cer(d18:1/17:0), DG(17:0/17:0), PE(17:0/17:0), PC(17:0/17:0), TG(17:0/17:0/17:0) CE(17:0) for the positive pole, and LPC(17:0), Cer(d18:1/17:0), PE(17:0/17:0), PC(17:0/17:0), PS(17:0/17:0) for the negative pole) (3 µg mL^{-1}, each compound) were added to each sample. The IS mix is used as quality control to monitor its response along the sequence, as well as to correct potential injection or chromatographic errors. For this, each individual lipid is corrected with the IS corresponding to the same lipid class (lipid signal/IS signal).

Liquid chromatography coupled to mass spectrometry analytical method

For the analysis, Ultra-Performance Liquid Chromatography equipment (UPLC) coupled to a high-resolution mass spectrometer (MS) with Orbitrap UPLC-QExactive Plus detector (UPLC-TOF/MS-Orbitrap QExactive Plus MS) available at the Analytical Unit of the Instituto de Investigación Sanitaria La Fe (IISLaFe, Valencia, Spain) was used.

The chromatographic and mass spectrometric conditions were the same as those used in the previous work [32]. Concisely, Acquity UPLC CSH C18 column (100 × 2.1 mm, 1.7 µm) from Waters. As for the mobile phase in the positive ionization mode, it was acetonitrile/water (60:40, v/v) with ammonium formate (10 mM) (A) and isopropyl alcohol/acetonitrile (90:10, v/v) with ammonium formate (10 mM) (B); in the negative ionisation mode, it was acetonitrile/water (60:40, v/v) with ammonium acetate (10 mM) (A) and isopropyl alcohol/acetonitrile (90:10, v/v) with ammonium acetate (10 mM) (B). The flow rate was 400 µL min-1, the column temperature was 65 °C, and the injection volume was 5 µL.

To ensure the quality and reproducibility of the analysis, as well as to avoid intra-batch variability, an injection in random order, an analysis of at least 5 quality controls (QC_{cond}) to condition the column and equipment, a QC analysis in MS mode (Full MS) every 8 samples and a QC analysis in DIA (Data Independent Fragmentation) and DDA (Data Dependent Fragmentation) modes were performed at the beginning, middle and end of the sequence. Finally, the lipid species annotations with the LipidMSv3 annotation package were carried out [33].

Processing of lipidomic data

Data processing, peak picking, retention time alignment and peak integration were carried out with the LipidMSv3 R package. Once the data were processed, they were then filtered and normalised using a filtering and normalisation script developed in the Analytical Unit of the Instituto de Investigación Sanitaria de La Fe (Valencia, Spain). Briefly, the data were normalised by applying the Median Fold Change method with R package. Then, those variables whose coefficients of variation (CV%) in the QCs were > 30% and those variables that had more than 60% of zeros were eliminated; also, outliers (deviated more than zscore x mean) were replaced by the value of the mean ± zscore within each group [34]. Finally, the data were analysed by grouping lipid species

and summing the analytical responses for each class. Subsequently, based on the adduct of each variable, they were classified into positive and negative adducts. One database was created for species with positive adducts and another for negative adducts. Those analytical responses of duplicate species with the same retention time and the same or similar mass-to-charge ratio were added together. A new database was made to join the species with positive and negative adducts, and the duplicate species were checked and removed. For the species with both positive and negative adducts, the highest responses were screened and adducts with the lowest response were eliminated.

Brain lipidomic data

The relationship between lipids in plasma and in different brain areas (cerebellum, amygdala, hippocampus, cortex) was carried out in the same mouse specimens. For this, the raw data obtained in the previous published work were used [32]. These data were processed in the same way as explained previously "Processing of lipidomic data" section. Then, the same lipids previously identified in plasma were searched in brain samples.

Statistical analysis

IBM Statistical Package for the Social Sciences software version 23.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the univariant statistical analysis. Categorical variables were expressed as frequencies and percentages (%), and numeric variables were expressed as medians and interquartile ranges (IQR). Differences between medians were analysed by using the non-parametric test (Mann–Whitney U test). Furthermore, the *p*-values ≤ 0.05 were considered statistically significant. Volcano plot, their values and the heatmap were created using MetaboAnalyst version 4.0 platform. In order to identify a wider range of possible changes that may occur in lipids, the significance level was set at $p < 0.10$ in the volcano plot analysis.

The multivariate analysis was performed using R (version 4.3.1.), R packages MDA tools (version 0.14.1), and cut point (version 1.1.2) with IDE R-Studio (version 2023.06.0). For the multivariant analysis, a partial least-squares discriminant analysis (PLS-DA) was carried out using all the plasma lipids, as predictor variables; and the mice group WT (-1) and TG (+1) as response variable. The predictor variables were standardized to obtain the model standardized coefficients, which offer a scaled measure of how each predictor variable influences the response variable, adjusted for the latent structure of the model, integrating information from both weights and loadings. The PLS-DA was built to describe the potential different lipid profile associated to AD, and to carry out inferences about the most important variables (VIP scores). The variables with VIP score > 2 were selected, in order to reduce dimensionality, and another PLS-DA model was developed from these selected variables.

The permutation test (10,000 permutations) was applied to the proposed PLS-DA model, recording both the calibration R^2 and cross-validation R^2 for each case. The predicted values obtained by leave-one-out cross validation were employed in the performance model evaluation. The corresponding Area under the ROC Curve was calculated and the cut-off was established in zero.

SPSS software was also used to assess the relationship between plasma and brain by Pearson correlations, and to study the relationship between plasma lipids and the estrous cycle of mice applying the Mann–Whitney U-test for differences between medians, with *p*-value ≤ 0.05 being considered statistically significant.

Results

Animal model description

As can be seen in Table 1, there were no differences between WT and TG in terms of age (*p*-value 0.58), weight (*p*-value 0.08) and the mouse estrous cycle (*p*-value 0.37).

Table 1 Variables for the mouse model description

Variable		WT (*n* = 10)	TG (*n* = 10)	*p*-value (Mann Whitney U Test)
Age (days, median (IQR))		164 (161 – 164)	162 (161 – 164)	0.58
Age (months, median (IQR))		5.39 (5.29–5.39)	5.32 (5.29–5.39)	0.58
Weight (grams, median (IQR))		42.25 (37.98 – 44.35)	37 (27.35 – 41.73)	0.08
Estrous Cycle (n, (%))	Estrus	4 (40%)	6 (60%)	0.37
	Diestrus	6 (60%)	4 (40%)	

IQR inter-quartile range

Table 2 Percentage (mean) of lipids grouped by saturation degree in plasma and group of mice

Saturation degree	WT	TG	*p*-value
SATs (%)	10.16	10.12	0.684
MUs (%)	16.26	15.07	0.280
PUs (%)	73.58	74.81	0.912

SAT saturated lipid, *MU* monounsaturated lipid, *PU* polyunsaturated lipid

Table 3 Percentage (mean) of lipid family in plasma and group of mice

Lipid family	WT	TG	*p*-value
FA (%)	4.06	4.88	0.143
GL (%)	20.81	22.46	0.684
GP (%)	69.79	66.65	0.315
SP (%)	1.84	1.79	0.853
ST (%)	3.50	4.22	0.247

FA fatty acid, *GL* glycerolipid, *GP* glycerophospholipid, *SP* sphingolipid, *ST* sterol lipid

Univariate lipid analysis

The results obtained from the lipid saturation degree are summarized in Table 2. As can be seen, the major group corresponded to the polyunsaturated lipids (PUs), followed by monounsaturated lipids (MUs), and saturated lipids (SATs). It should be noted that there were no statistically significant differences between WT and TG in this classification.

Regarding the classification of lipid families, the higher presence corresponded to glycerophospholipids (GP), followed by glycerolipids (GL), fatty acids (FA), sterol lipids (ST) and sphingolipids (SP). As shown in Table 3, it should be noted that a non-significant increase in FA, GL and ST levels was observed in TG mice.

As can be seen in Fig. 1, a total of 5 lipid families, including 330 lipid species were detected by the LipidMSv3 R package. Specifically, they were grouped into FA ($n=12$), GL ($n=100$; 6 monoacylglycerols (MAG), 6 diacylglycerols (DAG), 88 triacylglycerols (TAG)), GP ($n=179$; 78 phosphatidylcholines (PC), 14 phosphatidylethanolamines (PE), 9 phosphatidylinositols (PI), 20 lysophosphatidylcholines (LPC), 3 lysophosphatidylethanolamines (LPE), 2 lysophosphoinositols (LPI), 32 ether-linked phosphatidylcholines (PCo), 25 ether-linked phosphatidylethanolamines (PEo)), SP ($n=28$; 8 ceramides (Cer), 20 sphingomyelins (SM)), and ST (7 cholesteryl esters, CE).

The results from the univariate analysis showed that the levels of 18 lipids were significantly different between WT and TG mice, 10 were increased and 8 were decreased in APP/PS1 mice (see Table 4). All lipids belonging to the GL (5 TAG) and ST (1 CE) families

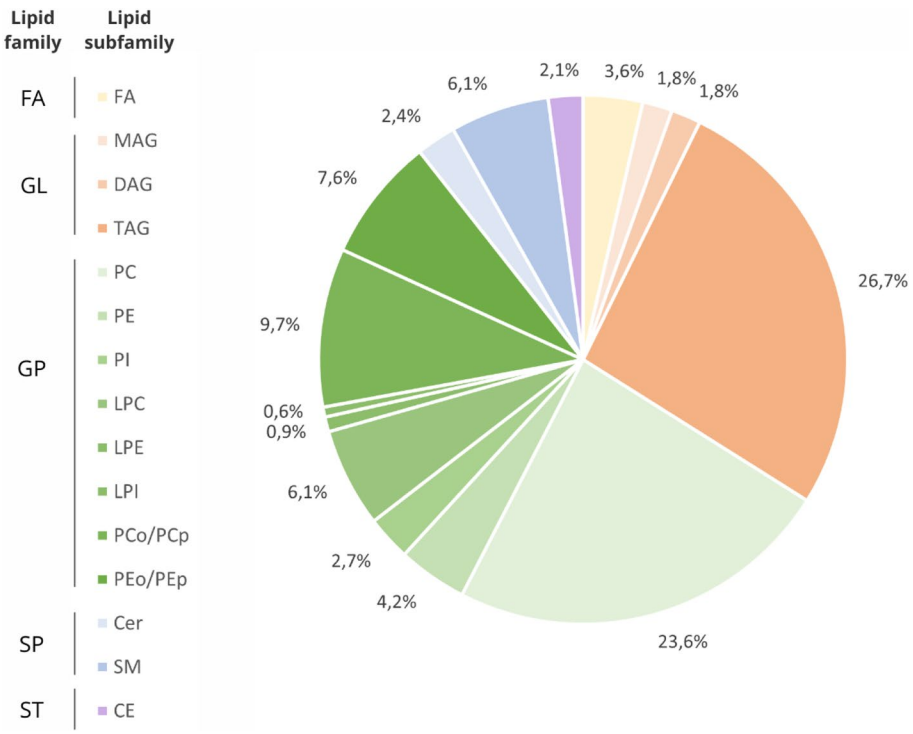

Fig. 1 Percentages of lipid subfamilies detected by the LipidMSv3 R package grouped by lipid family

Table 4 Statistically significant lipid variables that increased and decreased in the AD mouse model

Lipid	WT ($n=10$) (median (IQR)) ×10^7	TG ($n=10$) (median (IQR)) ×10^7	*p*-value (Mann Whitney test)	In AD
TAG(58:2)	11.3 (9.4 – 20.4)	20.2 (12.2 – 31.1)	0.035	↑
TAG(60:2)	4.8 (4.2 – 8.4)	9.0 (5.3 – 13.9)	0.015	↑
TAG(62:2)	1.6 (1.4 – 2.4)	2.8 (1.7 – 4.5)	0.023	↑
TAG(62:3)	1.9 (1.1 – 3.8)	4.3 (2.2 – 6.3)	0.029	↑
TAG(64:1)	0.6 (0.4 – 0.9)	1.1 (0.7 – 2.0)	0.019	↑
PC(18:1/18:0)	3472.1 (3159.5 – 4242.9)	2477.3 (1904.0 – 3466.3)	0.043	↓
PC(35:1)	1.7 (1.5 – 2.0)	2.3 (1.9 – 2.8)	0.005	↑
PC(36:1)	126.8 (117.4 – 136.9)	112.5 (104.0 – 121.5)	0.043	↓
PC(40:4)	5.6 (5.1 – 5.9)	4.6 (4.1 – 5.4)	0.023	↓
PC(40:7)	110.8 (97.7 – 148.8)	79.6 (64.8 – 116.9)	0.043	↓
PC(44:12)	0.3 (0.3 – 0.4)	0.2 (0.1 – 0.3)	0.010	↓
PE(18:0/22:6)	9.2 (5.3 – 11.4)	5.1 (3.6 – 7.9)	0.023	↓
LPC(15:0)	1.6 (1.4 – 1.9)	2.0 (1.8 – 2.0)	0.023	↑
PCo(32:0)	1.6 (1.4 – 2.0)	2.2 (1.9 – 2.8)	0.007	↑
PCo(34:2)|PCp(34:1)	0.8 (0.7 – 0.9)	1.0 (0.9 – 1.2)	0.035	↑
PEo(18:2/20:4)|PEp(18:1/20:4)	6.0 (5.5 – 6.9)	5.1 (4.2 – 5.5)	0.029	↓
Cer(d18:1/16:0)	11.4 (10.6 – 14.7)	9.0 (8. – 11.2)	0.035	↓
CE(16:0)	20.8 (14.8 – 30.8)	36.6 (27.0 – 43.5)	0.035	↑

Abbreviations: *CE* cholesteryl ester, *Cer* ceramide, *IQR* Inter-quartile range, *LPC* lysophosphatidylcholine, *PC* phosphatidylcholine, *PCo/PCp* ether-linked phosphatidylcholine, *PE* phosphatidylethanolamine, *PEo/PEp* ether-linked phosphatidylethanolamine, *TAG* triacylglycerol

increased in AD plasma. Regarding the GP family, 5 PCs and 1 PE decreased in AD, except 1 PC. On the other hand, 1 LPC and 2 PCos increased in AD. Finally, the only significant SP belonging to the Cer subfamily decreased in AD.

From the volcano plot analysis, 9 lipid species were selected showing statistically significant differences between genotypes, being the species CE(16:0), TAG(20:4/20:4/20:4), PCo(20:0/22:6)|PCp(42:5), Cer(d18:1/22:0), Cer(d18:1/16:0), FA(20:0) significantly higher in the TG group; and PE(40:6), PCo(34:2)|PCp(34:1), TAG(42:1) significantly lower in the TG group (see Figure S1 at supporting information). Moreover, Table 5 shows the lipids identified by volcano, with fold changes (FC) from 0.29 to 3.81, and *p*-values from 0.001 to 0.096. From these lipids, a heatmap analysis was carried out with clustering, revealing that most of the TG mice showed higher levels for CE(16:0), TAG(20:4/20:4/20:4), PCo(20:0/22:6)|PCp(42:5), Cer(d18:1/22:0), Cer(d18:1/16:0), and FA(20:0) (Fig. 2).

Table 5 Important features identified by volcano plot ordered in ascending order of *p*-value

Lipid	FC	*p*-value
CE(16:0)	2.5883	< 0.001
TAG(20:4/20:4/20:4)	3.8134	0.001
PE(40:6)	0.2921	0.013
PCo(20:0/22:6)|PCp(42:5)	3.4204	0.030
PCo(34:2)|PCp(34:1)	0.4940	0.035
Cer(d18:1/22:0)	2.4626	0.061
Cer(d18:1/16:0)	2.4139	0.067
TAG(42:1)	0.4279	0.085
FA(20:0)	2.3435	0.096

Abbreviations: *CE* cholesteryl ester, *Cer* ceramide, *FA* fatty acid, *FC* fold change, *LPC* lysophosphatidylcholine, *PC* phosphatidylcholine, *PCo/PCp* ether-linked phosphatidylcholine, *PE* phosphatidylethanolamine, *TAG* triacylglycerol

Multivariate lipid analysis

For exploratory data analysis, PLS-DA was applied to distinguish the WT and TG groups according to the identified lipid profiling dataset. The PLS-DA score plot results displayed a separation between the WT and TG groups, with a principal component explaining 57.6% of the total variance. From this PLS-DA model, 7 lipid variables (6 GPs and 1 GL) with a VIP score > 2 were selected to develop a second PLS-DA. The *p*-value obtained for the permutation test on the calibration results was 0.0012. The *p*-value obtained

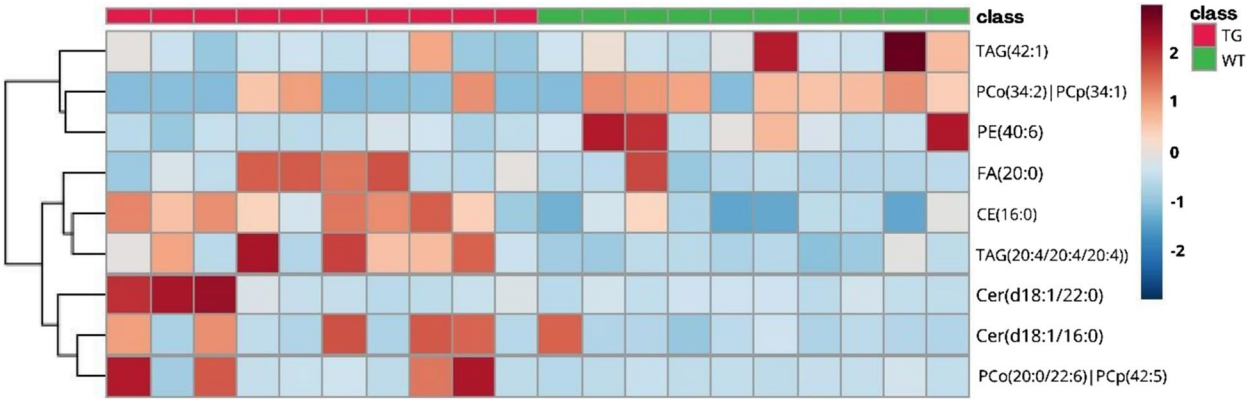

Fig. 2 Heatmap of the 9 most important lipids responsible for classification based on the volcano plot analysis. Positive Z-score values are shown in red, while negative Z-score values are shown in blue

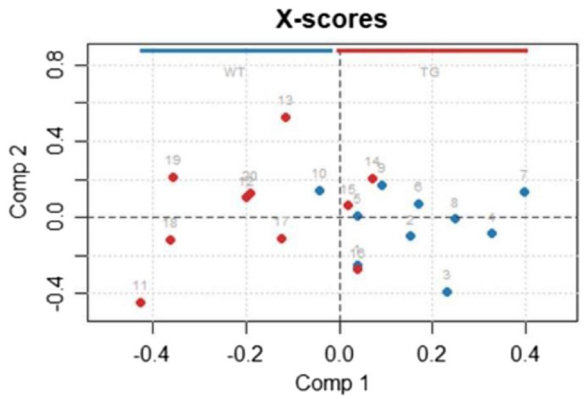

Fig. 3 PLS-DA score plot show the separation between groups (TG in red and WT in green) from the 7 selected lipid variables

Table 6 PLS-DA model standardized coefficients obtained for the lipid variables selected

Lipid	Model coefficients
TAG(64:1)	0.1311
PC(35:1)	0.1473
PC(40:4)	-0.1328
PC(44:12)	-0.1534
PI(38:4)	-0.1296
Total PI	-0.1322
PCo(32:0)	0.1475

Abbreviations: *PC* phosphatidylcholine, *PCo/PCp* ether-linked phosphatidylcholine, *PI* phosphatidylinositol, *TAG* triacylglycerol

for the permutation test on the cross-validation results was 0.0003. Therefore, significant *p*-values in the permutation test (PLS-DA) implies that the model's performance is unlikely due to random chance, indicating that the model is not overfitted and has genuine predictive power.

As can be seen in Fig. 3, a clear separation between groups (WT and TG) was obtained. The corresponding standardized coefficients for this model are shown in Table 6, noting that the positive coefficients indicate that the higher levels in plasma, the greater AD risk; while the negative coefficients indicate that the lower levels in plasma, the greater AD risk. This validated model ($n=20$) showed an AUC-ROC of 0.9, sensitivity of 70% (CI 95%: 39.7 – 89.2%), specificity of 90% (CI 95%: 59.6 – 98.2%), positive predictive value of 87.5% (CI 95%: 52.9 – 97.8%), negative predictive value of 75% (CI 95%: 46.8 – 91.1%) and accuracy of 80% (IC 95%: 58.4 – 91.9%). For the calibrated model ($n=20$) the AUC-ROC was 0.93, and the other indexes were the same as in the validated model.

Assessment of plasma and brain lipids relationship

In a previous lipidomic study in different mouse brain areas (cerebellum, amygdala, hippocampus, cortex) [32] of the same specimens, 319 lipid species were detected, of which 114 matched with the lipids found in the present plasma study (see Fig. 4). In order to see which lipids correlate in the pathology between plasma and brain, only the lipids that were detected simultaneously in plasma and brain samples were evaluated, observing that some lipids correlated positively or negatively between plasma and different brain areas. Specifically, 2 total lipid subfamilies and 17 lipids correlated positively and 8 lipids correlated negatively between plasma and 4 brain areas (see Table 7). Of these, the species PC(40:5) and PC(40:7) correlated between plasma and two different brain areas simultaneously. In addition, 6 lipids showed statistically significant differences in brain between WT and TG (see Table 7) [32]. Specifically, DAG(18:1/18:2) and PEo(36:4)|PEp(36:3) in the cerebellum, MAG(22:3) and Cer(d18:1/16:0) in the amygdala, and PC(40:7) and PE(18:0/18:2) in the cortex.

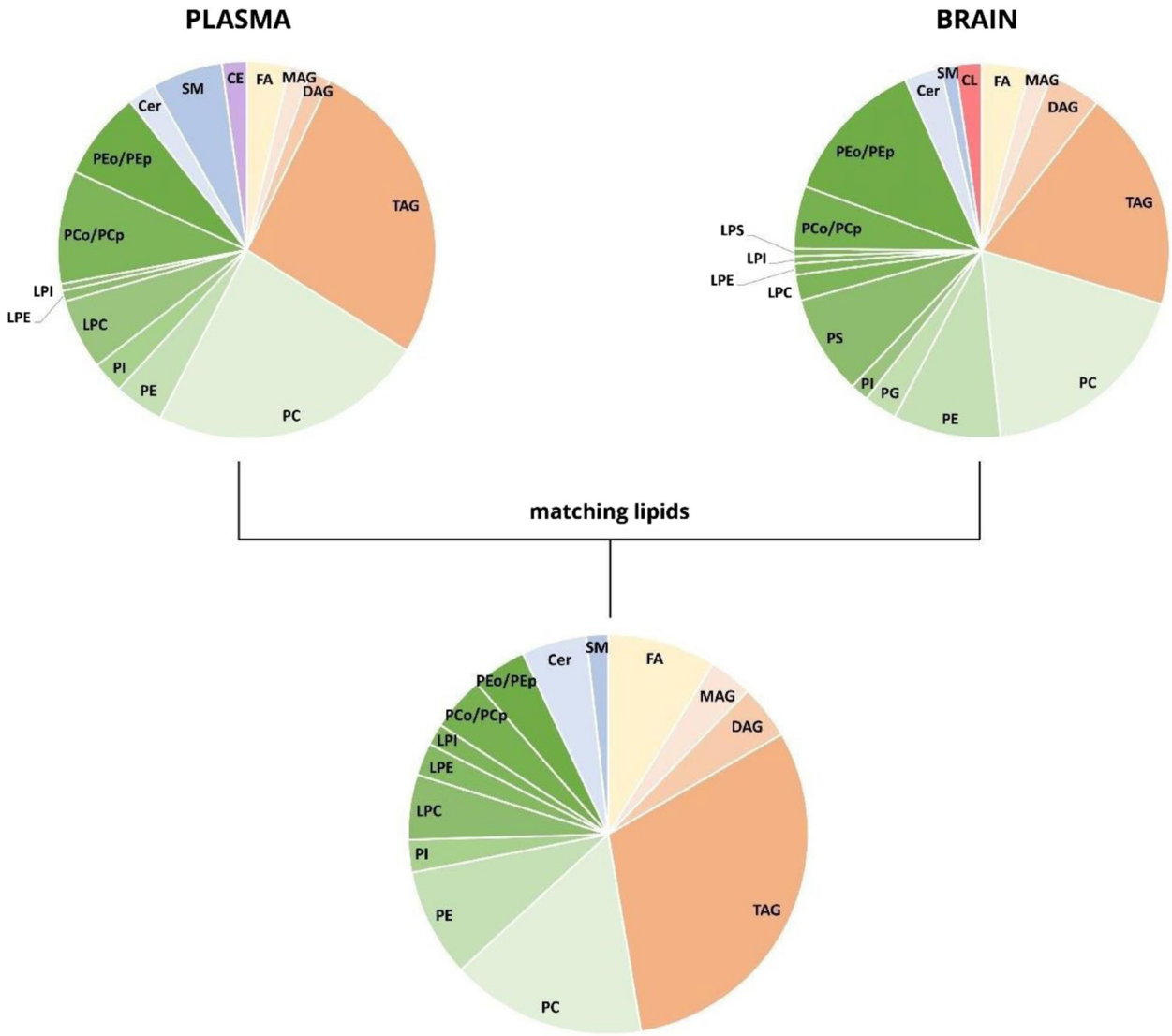

Fig. 4 Lipid families detected from plasma samples and brain samples, showing the matching lipids between both biological sample types

Study of the relationship between lipidomics and estrous cycle

From the vaginal cytology test, it was found that all the female mice ($n=20$) were at the longest estrous cycle stages, these are, estrus (12–48 h, $n=10$) and diestrus (48–72 h, $n=10$) [35]. Comparing plasma lipids levels between both groups (estrus, diestrus), significant differences were observed for 5 lipids belonging to the subfamilies LPC, PCo, PEo and SM (see Table S1 at supporting information). Specifically, LPC(24:1) and SM(d18:1/15:0) showed higher levels in estrus phase, and PCo(36:3)|PCp(36:2), PEo(18:1/18:2) and PEo(38:1) showed higher levels in diestrus phase.

Comparing lipid levels in each brain area (cerebellum, amygdala, hippocampus, cortex) between both groups (estrus and diestrus), numerous significant differences were observed for lipids belonging to different subfamilies (see Table S2-S5 at supporting information). Specifically, lipids of the LPC, LPE, LPI and LPS subfamilies showed higher levels in cerebellum, amygdala and hippocampus in estrus phase, as well as SM in hippocampus and cortex; while some lipids of the PCo and PEo subfamilies showed higher levels in amygdala, cerebellum and cortex in diestrus phase.

Discussion

Due to the strong need to identify early-stage changes in AD, this study was conducted in 5-month-old WT and TG mice from the same litter. In addition, due to the underrepresentation of studies in the female sex, this work was based on a female mice model, observing

Table 7 Statistically significant correlations between lipids levels found in plasma and different brain areas (cerebellum, amygdala, hippocampus, cortex)

Lipid subfamily	Lipid species	Plasma *vs* cerebellum r (*p*-value)	Plasma *vs* amygdala r (*p* value)	Plasma *vs* hippocampus r (*p* value)	Plasma *vs* cortex r (*p* value)
FA	FA(16:0)	-	-	0.457 (0.043)	-
	FA(18:0)	-	-	0.561 (0.010)	-
	FA(18:2)	-	-	0.480 (0.032)	-
	FA(20:0)	-	-	0.532 (0.016)	-
	Total FA	-	-	0.517 (0.020)	-
MAG	MAG(20:2)	-	-	0.479 (0.032)	-
	MAG(22:3)[a]	-	0.471 (0.042)	-	-
DAG	DAG(18:1/18:2)[a]	-0.486 (0.030)	-	-	-
	DAG(18:2/18:2)	-	-	0.467 (0.038)	-
TAG	TAG(49:1)	-0.507 (0.023)	-	-	-
	TAG(52:0)	-	-	0.597 (0.005)	-
	TAG(59:2)	-	-0.505 (0.027)	-	-
PC	PC(18:0/20:4)	-	-	-	0.449 (0.047)
	PC(18:0/22:4)	-0.578 (0.008)	-	-	-
	PC(18:0_20:3)	-0.524 (0.018)	-	-	-
	PC(36:5)	-	-	0.508 (0.022)	-
	PC(40:5)	0.572 (0.008)	-	-	0.722 (< 0.001)
	PC(40:7)[a]	-	-	-0.507 (0.022)	-0.472 (0.036)
PE	PE(16:0/20:4)	-	-	0.470 (0.036)	-
	PE(18:0/18:2)[a]	-	-	-	0.465 (0.039)
	PE(34:2)	0.599 (0.005)	-	-	-
LPC	LPC(18:1)	-	0.510 (0.026)	-	-
PEo/PEp	PEo(36:4)|PEp(36:3)[a]	-0.623 (0.003)	-	-	-
Cer	Cer(d18:1/16:0)[a]	-	0.484 (0.036)	-	-
	Cer(d18:1/22:0)	-0.477 (0.033)	-	-	-
	Total Cer	0.545 (0.013)	-	-	-
SM	SM(d38:1)	0.586 (0.007)	-	-	-

r Pearson coefficient correlation

Abbreviations: *DAG* diacylglycerol, *FA* fatty acid, *LPC* lysophosphatidylcholine, *MAG* monoacylglycerol, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PEo/PEp* ether-linked phosphatidylethanolamine, *SM* sphingomyelin, *TAG* triacylglycerol

[a] lipids showing significant differences between WT and TG mouse brains

that the estrous cycle phases did not show differences between WT and TG groups. Moreover, plasma lipid levels in the early stages of AD and their correlations with previous results in brain levels [32], were evaluated in the same mice. In this sense, some plasma lipid variables, closely related to brain, were identified as potential and minimally invasive AD biomarkers. To our knowledge, this is the first lipidomics study for plasma and brain relationship, monitoring the estrous cycle.

Regarding lipidomics, some brain and blood studies can be found in literature [36–40]. In the present study, some lipid species showed statistically significant differences in plasma levels. According to the different lipid families, FAs showed some discrepancies. In fact, dietary supplementation containing specific FAs could help to improve cognitive function in patients with very mild AD [41], because these levels might be decreased in AD (serum, plasma, brain) [39, 42, 43]. However, another study in patients reported that high levels of these circulating compounds are associated with Aβ-mediated neurotoxicity [44]. Similarly, in the present study, we found an increase in the FA(20:0) lipid in the TG group. With respect to GLs, the present work showed a generalised increase in TAG levels in the TG mice compared to the WT mice. Similarly, it was observed in other studies performed on serum and plasma from the same female mouse model, with other analytical techniques and at the same age [45]; also, at 9-month-old [46], and even in females in the ApoE4-TR mouse model at an older age [37]. Concerning GPs, PCs and PEs, they are

the main form of phospholipids in cell membranes and play essential roles involved in neuronal membrane formation, signal transduction, autophagy, and maintenance of mitochondrial morphology [47, 48]. In lipidomic studies performed in brain and plasma from different mouse models and sex, decreased levels of PC and PE were found in early and late stages of AD [36, 49–53]. Specifically, in the present study decreased levels of PC(36:1) and PC(40:4) in AD plasma were observed. Also, decreased levels of PCs and PEs were detected in lipidomic studies performed in multiple regions of human brains with mild cognitive impairment (MCI) and AD, mainly in the two regions most affected by the disease, hippocampus and cortex [54, 55]. About LPLs, as in previous studies carried out in plasma and serum of mice and human, the present study found increased levels in the AD group [56–58]. With respect to SPs, it is known that their metabolism may contribute to the pathogenesis of diverse neurodegenerative diseases. In particular, ceramides are thought to be associated with pro-apoptotic cellular processes that promote neurodegeneration and neuroinflammation [59], being related to oxidative stress and Aβ accumulation [60]. In the present work, we found a significant reduction in Cer(d18:1/16:0) in plasma in the AD group; and in the previous brain study with the same mice, an increase of this ceramide was observed in AD. Nevertheless, the reason why only this Cer shows correlation is not clear from the lipidomics data obtained in the present work, and further studies are required. However, in the work carried out by Zhant et al. performed in brain from the same female mouse model, a decrease of Cer(d18:1_18:1) levels was observed at 2—3 months of age, but an increase at 7 months [39]. Probably it could be explained by the increase from a certain disease stage. On the other hand, in the study reported by Mielke et al., it was observed that elevated levels of serum ceramide in older women, especially Cer(d18:1/16:0) and Cer(d18:1/24:0), were associated with the risk of developing late-onset sporadic AD [61]. Also, plasma levels increase has been associated with MCI in middle-aged men [62]. Other studies observed increased levels of ceramides in plasma and increased expression of Cer(d18:1/16:0) synthase enzyme in astrocytes from patients with late-onset AD, frontotemporal lobar dementia and amyloid angiopathy [40]. This might suggest that Cer, specifically Cer(d18:1/16:0) could be a good early biomarker of AD, as a decrease is observed in brain and plasma from early AD mouse specimens and an increase in blood from late AD patients. Among the sterol family, in the present study it was observed a significant increase in the levels of the lipid species CE(16:0) in AD mice, matching an increase of two CE (CE(20:5), CE(16:1)) in the previously mentioned study using the same mouse model at 7 months of age [39]. However, some small differences were observed within the same group, which may be due to their weight, stage of the estrous cycle or litters.

Considering a multivariate approach, most of the lipidomics studies found in literature to develop predictive models of AD are based on humans. In the present work, the regression model selected lipids belonging to the TAG, PC and PI subfamilies providing satisfactory diagnosis indexes (70% of sensitivity, 90% of specificity and an 80% of accuracy). In a previous study carried out in human plasma samples, following omics integration between microRNAs and lipids and using a regression model, the lipids selected were from the same subfamilies, and specifically FA showed the higher loadings [63]. Other recent studies carried out in patients have selected certain lipids in blood and cerebrospinal fluid for the prediction of the risk of clinical progression, showing prediction efficiencies with AUC values between 0.72 – 0.88 [28, 64–67]. In the present study, the lipids selected by the PLS-DA model are species belonging to the TAG, PC, PI and PCo subfamilies; specifically, TAG(64:1), PC(35:1), PC(40:4), PC(44:12), PI(38:4), PCo(32:0). In general, the developed multivariate model could be a relevant tool to identify patients at risk of developing AD.

As regards the relationship between plasma and brain lipidomics in mice, potential biomarker similarities between brain and plasma could provide insight into individual metabolic status and support the identification of potential early and minimally invasive disease biomarkers. Nevertheless, few studies have focused simultaneously on plasma and brain lipids from the same mouse specimens, and even less have looked at their relationship with different brain areas [68]. Since most published lipidomic studies have homogenised the whole brain and therefore have not distinguished between areas [39] or have been performed specifically on one [69] or two brain areas [70]; but have not carried out correlations between these areas and plasma. In the present study, the significant changes in plasma lipid species and their relationship with the levels in different brain areas were evaluated, observing that 4 FAs and total FAs increased concomitantly in plasma and hippocampus of female 5-month age AD *vs* WT mice. Also, MAGs increased simultaneously in plasma as well as hippocampus and amygdala, PEs increased in cerebellum, hippocampus and cortex; LPCs increased in the amygdala; and 1 SM increased in the cerebellum. In contrast, levels of a PEo decreased in plasma, while they increased in cerebellum. On the other hand, other subfamilies such as DAG, TAG, PC and Cer, showed some disparity, since some lipid species increased or decreased within the same subfamily, as observed in previous studies [36, 38, 39, 53, 57, 71].

However, regarding individual lipids in the present work, MAG(22:3), DAG(18:1/18:2), PC(40:7), PE(18:0/18:2), PEo(36:4)|PEp(36:3) and Cer(d18:1/16:0) showed statistically significant differences between TG and WT in both plasma and brain samples, suggesting that they would be promising minimally-invasive biomarkers related to amyloid accumulation, and other unspecific mechanisms such as inflammation or cognitive decline, which are closely related to AD development. On the other hand, both groups (WT, TG) did not show differences for the estrous cycle phases, so the effect of this variable on lipidomics was not relevant in the present study.

In literature, the estrous cycle has been evaluated in behavioural animal studies, showing some discrepancy, as several studies found significant differences in the behaviour of female mice, while others did not [72, 73]. A previous work reported some worsening of the network and cognitive dysfunction in the estrus phase, whereas the deficits are attenuated in the diestrus phase [74]. Other studies suggested that females showed more anxiety-like behaviours during the diestrus phase [75, 76], and an increased reactivity [77], but others did not showed significant influence [78, 79]. In addition, mice in diestrus phase were found to have higher weight compared to those in metestrus and estrus [80]. In rats, it was observed a decrease in body weight and respiratory quotient, and increased energy expenditure in the days associated with estrus phase compared to diestrus phase [81]. In general, these previous results suggest that the cycle phase could also affect the metabolism, specifically lipid metabolism. In this sense, the present study showed significant differences in lipids levels in both plasma and brain, regardless of mouse genotype. To our knowledge, there are no studies in literature that have explored differences in lipid levels in female mice according to the estrous cycle. However, some lipids levels are increased or decreased throughout the cycle. Specifically, in plasma, a few differences have been found between estrus and diestrus, while at brain level many differences have been found, highlighting the cerebellum, amygdala and hippocampus areas with the greatest significant differences between mice groups (estrus, diestrus). Nevertheless, further research including the estrous cycle as variable in female models is needed, especially if the study groups are not equally distributed according to the phase of the cycle.

Among the study limitations, this lipidomic study was performed on 5-month-old female mice, it would have been interesting to compare these present results with males of the same age and littermates; as well as to compare with older specimens to see the progression of the disease. However, the work focused on female and early AD mouse model, which is widely underrepresent in literature. Furthermore, the results obtained in mice are not directly transferable to patients, as there are significant differences in anatomy, metabolism and physiology; but given the similarity of results with other studies performed in humans, it could suggest that these preliminary results could be validated in human samples.

Conclusions

This plasma lipidomic study carried out in the female APP/PS1 mouse model allowed to identify the main lipids altered in early AD, their relationship with brain lipidome, and the effect of the estrous cycle phase over lipid metabolism in the same specimens. Specifically, in plasma samples, lipids from TAG, PCo, LPC and CE subfamilies showed a significant increase in AD; while lipids from PC, PE, PEo and Cer subfamilies showed a significant decrease in AD. This suggests that lipid alterations can be detected in plasma at the earliest stages of AD. Furthermore, significant correlations were found between plasma and brain lipid levels (FA, MAG, PE, PEo, PEo, LPC and SM subfamilies). In general, this study shows a few relationships between lipid metabolism in plasma and brain areas, identifying six individual lipids (DAG(18:1/18:2), PEo(36:4)|PEp(36:3), MAG(22:3), Cer(d18:1/16:0), PC(40:7) and PE(18:0/18:2)) as potential early and minimally-invasive AD biomarkers closely related to brain pathology. Nevertheless, further work is required to validate these potential biomarkers in plasma human samples. In addition, it is important to highlight that the estrous cycle monitoring could be relevant in metabolism female studies.

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid-β
BBB	Blood-brain barrier
CE	Cholesteryl ester
Cer	Ceramide
CV	Coefficients of variation
DAG	Diacylglycerol
FA	Fatty acid
FC	Fold change
GL	Glycerolipid
GP	Glycerophospholipid
IQR	Inter-quartile range
IS	Internal standard
LPC	Lysophosphatidylcholine
MAG	Monoacylglycerol
MS	Mass spectrometer
MU	Monounsaturated lipid
PC	Phosphatidylcholine
PCo/PCp	Ether-linked phosphatidylcholine
PE	Phosphatidylethanolamine
PEo/PEp	Ether-linked phosphatidylethanolamine
PI	Phosphatidylinositol
PLS-DA	Partial least-squares discriminant analysis
PU	Polyunsaturated lipid
QC	Quality controls
SAT	Saturated lipid
SM	Sphingomyelin

SP	Sphingolipid
ST	Sterol lipid
TAG	Triacylglycerol
TG	APPswe/PS1dE9 transgenic
UPLC	Ultra-Performance Liquid Chromatography
WT	Wild-type and

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
L.F-G. carried out the methodology, samples collection, treatment and analysis, and writing. A.B. carried out data accuracy and statistical analysis. M.R. carried out analysis of biological samples and data accuracy. A.F. carried out biological samples collection. A.L. carried out conceptualization and supervision. C.C-P. carried out conceptualization, methodology, writing and supervision.

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Availability of data and materials
The data of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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