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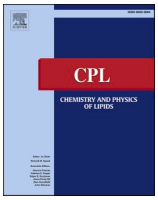

Lipidomic analysis identified potential predictive biomarkers of statin response in subjects with Familial hypercholesterolemia

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ABSTRACT

Familial hypercholesterolemia (FH) is a disorder of lipid metabolism that causes elevated low-density lipoprotein cholesterol (LDL-c) and increased premature atherosclerosis risk. Statins inhibit endogenous cholesterol biosynthesis, which reduces LDL-c plasma levels and prevent from cardiovascular events. This study aimed to explore the effects of statin treatment on serum lipidomic profile and to identify biomarkers of response in subjects with FH. Seventeen adult FH patients underwent a 6-week washout followed by 4-week treatment with atorvastatin (80 mg/day) or rosuvastatin (40 mg/day). LDL-c response was considered good (40–70 % reduction, n = 9) or poor (3–33 % reduction, n = 8). Serum lipidomic profile was analyzed by ultra-high-performance liquid chromatography combined with electrospray ionization tandem time-of-flight mass spectrometry, and data were analyzed using MetaboAnalyst v5.0. Lipidomic analysis identified 353 lipids grouped into 16 classes. Statin treatment reduced drastically 8 of 13 lipid classes, generating a characteristic lipidomic profile with a significant contribution of phosphatidylinositols (PI) 16:0/18:2, 18:0/18:1 and 18:0/18:2; and triacylglycerols (TAG) 18:2x2/18:3, 18:1/18:2/18:3, 16:1/18:2x2, 16:1/18:2/18:3 and 16:1/18:2/Arachidonic acid (p-adjusted <0.05). Biomarker analysis implemented in MetaboAnalyst subsequently identified PI 16:1/18:0, 16:0/18:2 and 18:0/18:2 as predictors of statin response with and receiver operating characteristic (ROC) areas under the curve of 0.98, 0.94 and 0.91, respectively. In conclusion, statins extensively modulate the overall serum lipid composition of FH individuals and these findings suggest that phosphatidyl-inositol molecules are potential predictive biomarkers of statin response.

Abbreviations: ACMG, American College of Medical Genetics and Genomics; Apo, apolipoprotein; AUC, area under the curve; CAD, coronary artery disease; CVD, cardiovascular disease; DLCN, Dutch Lipid Clinic Network; FC, fold change; FDR, false discovery rate; FH, Familial Hypercholesterolemia; HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein cholesterol; HMGR, 3-hydroxi-3-methylglutaryl Coenzyme A reductase; LC, liquid chromatography; LDL, low-density lipoprotein; LDL-c, LDL cholesterol; LDLR, low-density lipoprotein receptor; MetS, metabolic syndrome; MS, mass spectrometry; PC, phosphatidylcholine; PI, phosphatidyl-inositol; ROC, receiver operating characteristic; sPLS-DA, sparse partial least squares discriminant analysis; TAG, triacylglycerol; VIP, variable importance in projection; VLDL-c, very low-density lipoprotein cholesterol.

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1. Introduction

Familial hypercholesterolemia (FH) is a monogenic dyslipidemia that causes elevated plasma concentrations of low-density lipoprotein cholesterol (LDL-c), and increased risk of premature atherosclerotic cardiovascular disease (CVD) (Vallejo-Vaz et al., 2015).

FH is caused by deleterious mutations in genes involved in the homeostasis of intracellular cholesterol, such as *LDLR, APOB*, *PCSK9* and *LDLRAP1* (Sturm Amy et al., 2018; de et al., 2021). Metanalyses of 11 million subjects estimated prevalence of 1:313 (heterozygous) and 1:400,000 (homozygous), and consequently over 30 million individuals are affected worldwide (Beheshti et al., 2020).

Cholesterol-lowering is critical in primary and secondary prevention of CVD in FH patients and, when diet is insufficient to control LDL-c levels, statins are used as first-line treatment (Nordestgaard et al., 2013). They are competitive inhibitors of the 3-hydroxi-3-methylglutaryl Coenzyme A reductase (HMGCR), that reduce the synthesis of endogenous cholesterol and increase the clearance of LDL particles through stimulating LDLR expression in hepatocytes (Baigent et al., 2005). In this way, statins reduce the risk of cardiovascular events and the benefit is proportional to the magnitude of the reduction in LDL-c levels (Kizer et al., 2010). Moreover, statins are also recognized by their pleiotropic effects, a number of additional benefits beyond LDL-c reduction, such as reduction of mediators of inflammation, improving vascular-endothelial function, anti-oxidative and anti-proliferative activity, among others (Morofuji et al., 2022). By inhibiting HMGR, statins reduce the production of isoprenoids, intermediates in cholesterol biosynthesis, and the prenylation of small GTP-binding proteins (Zhou and Liao, 2010) that were proposed as mechanisms to promote additional effects; however, the molecular mechanisms of the pleiotropic effects of statins have not yet been fully elucidated.

Development of "omics" technologies in the last decades favors a deeper understanding of the mechanism of action and the pleiotropic and side effects of cardiovascular drugs with the aim of identifying new targets for future personalized medicine. In this way, the metabolomics of body fluids has been proposed as a tool for better characterization of the therapeutic response to drugs, a new research area known as pharmacometabolomics (Gianazza et al., 2023). Regarding lipid-lowering drugs, lipidomics, a comprehensive metabolomics tool to evaluate lipid profiling, is particularly important. However, only a few works have evaluated the influence of statins on plasma lipidome in dyslipidemic patients (Bergheanu et al., 2008; Kaddurah-Daouk et al., 2010; Meikle et al., 2015), differing in type of statin, time of exposure, target population or analytical lipidomic approach to quantify lipids. In this way, it is important to highlight that there are no studies evaluating the influence of statins on plasma lipidomic profile in FH patients. In addition, an important extent of this technology that it is also a resource to explore the potential of lipid species as biomarkers, which could imply the discovery of predictors of pharmacological response. In this way, it is noteworthy that the effectiveness of statin therapy in lowering LDL-c levels can substantially differ between individuals (Sun et al., 2023), with an estimate of only 20 % of patients with FH reaching the therapeutic goal (Vallejo-Vaz et al., 2015).

This study explored the effects of statin treatment on the overall serum lipid profile using a lipidomic approach and to identify biomarkers of response, for the first time, in a cohort of adult subjects with FH.

2. Materials and methods

2.1. Study design and participants

A group of 17 adult FH patients from the FHBGEP study protocol (Borges et al., 2021), diagnosed according to the Dutch Lipid Clinic Network (DLCN) modified criteria (de et al., 2021), was selected at the Medical Clinical Division Section of the Institute of Cardiology Dante Pazzanese, Sao Paulo, Brazil. An exon-targeted gene sequencing strategy was used to identify variants in FH-related genes, as previously described (Borges et al., 2021), and pathogenic variants were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015). Clinical, demographic, and anthropometric data of participants were obtained from interviews and medical records.

The FH patients were in primary cardiovascular prevention and were not taking statins for at least six weeks or were submitted to a 6-week washout period by suspending the statin treatment (Baseline). Afterwards, patients were treated with a high dose of atorvastatin (80 mg/day) or rosuvastatin (40 mg/day) during four weeks (Treatment).

Subjects with renal or liver diseases, thyroid or adrenal disorders, neoplasms, or HIV infection were not included in this study.

This study protocol was approved by the local Ethics Committees (CAAE #24618713.0.1001.5462, #24618713.0.1001.5462, #05234 918.4.0000.5462, and #24618713.0.3001.0067). The study was conducted according to good clinical practices and the Declaration of Helsinki guidelines (as revised in 2013). All subjects agreed to participate and signed an approved written informed consent.

2.2. Blood sampling and laboratory testing

Blood samples were obtained using the vacuum system tubes (Vacutainer™, Becton Dickinson Company, Plymouth, UK) from fastened patients (at least 8 h) for conventional laboratory tests and molecular study, as previously described (Borges et al., 2021; Dagli-Hernandez et al., 2022; Los et al., 2021; Barbosa et al., 2023; Los et al., 2022). Blood, plasma and serum samples were used to assess the lipid and glycemic profiles, thyroid-related hormones, liver function, and drug-induced muscle lesion. Serum fraction was also used for lipidomic analysis.

Glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-c), and triacylglycerols were analyzed by enzymatic methods. LDL-c and very low-density lipoprotein cholesterol (VLDL-c) were calculated using Friedwald's formula. Apolipoproteins AI (apo AI) and B (apo B) were determined by immunoturbidimetry. Glycated hemoglobin (HbA1c) was analyzed by high-performance liquid chromatography and insulin was measured by an automated chemiluminescence method. Creatine kinase and alanine and aspartate aminotransferases were determined by UV kinetic methods using a Roche automated system.

To perform a biomarker screening according statin response, FH patients were grouped based on LDL-c reduction. As detailed in results and in Suppl. Fig. 1, FH patients were classified as "good-responder" (LDL-c reduction $\geq$40.6 %; n = 9) or "poor-responders" (LDL-c reduction $\leq$33.5 %; n = 8).

2.3. Lipidomic analysis

2.3.1. Extraction of serum lipids

Total lipids were extracted from serum samples (50 μL) using a modified methyl-tert-butyl ether method for high-throughput lipidomics (Matyash et al., 2008). Samples were mixed with 50 μL of internal standards (10 μg/mL) before extraction. These standards were previously described (Queiroz et al., 2019) and included the detection of the following lipid classes and subclasses: 1) Fatty acids: Free fatty acid (FFA); 2) Glycerophospholipids: Phosphatidylethanolamine (PE), Plasmenyl-phosphatidylethanolamine (pPE), Phosphatidylcholine (PC), Lyso-Phosphatidylcholine (LysoPC), Plasmanyl-phosphatidylcholine (oPC), Plasmenyl-phosphatidylcholine (pPC), Phosphatidyl-dimethyle thanolamine (PDME), Phosphatidylinositol (PI); 3) Sphingolipids: Sphingomyelin (SM), Ceramide (Cer), Hexosylceramides (Hex-Cer); 4) Sterols: cholesterol (Ch); 5) Storage Lipids: Cholesteryl ester (CE), Triacylglycerol (TAG), Alkyldiacylglycerol (ADG). The extracted lipids were dissolved in isopropanol (100 μL) and centrifuged at 1500 x *g* for 3 min at 4 °C before analysis.

2.3.2. LC–MS/MS analysis and data processing

Lipid extracts were analyzed by an untargeted lipidomics approach using an ultra-high-performance liquid chromatography (LC) (Nexera, Shimadzu, Kyoto, Japan) combined with electrospray ionization tandem time-of-flight mass spectrometry (MS) (Triple TOF® 6600, Sciex, Concord, US). Chromatographic conditions and MS settings were detailed in previous studies (Queiroz et al., 2019; Chaves-Filho et al., 2019). Briefly, MS was operated using the Dependent Information Acquisition (IDA®) with scan range set a mass-to-charge ratio of 100–2000 Da. Data were collected every 1.05 s cycle with 100 ms acquisition time for MS1 scan and 25 ms to obtain MS/MS of the 36 main precursor ions. Data were acquired using Analyst® 1.7.1 with an ion spray voltage of –4.5 kV and 5.5 kV for negative and positive modes, respectively, and the cone voltage at 80 V. The curtain gas was set at 25 psi, nebulizer and heater gases at 45 psi and interface heater of 450 °C.

Lipid species were identified based on inspection of their MS/MS using PeakView® (Sciex, Concord, US), as previously described (Queiroz et al., 2019). Each lipid molecular specie was quantified by the peak area of the precursor ion normalized by the peak area of the corresponding internal standard using MultiQuant® (Sciex, Concord, US). The concentration was calculated based on the ratio between integrated MS data of the lipid specie and the volume of serum used for lipid extraction. Lipid concentration was expressed as mg/mL of serum.

2.3.3. Bioinformatic analysis

MetaboAnalyst v5.0 (Pang et al., 2021), a web based tool that provides different statistical tests for metabolomics and lipidomic analysis, was used for initial global screening using log transformed normalized data. An overall picture of the influence of statin treatment on serum lipidome was obtained by sparse partial least squares discriminant analysis (sPLS-DA), calculating the variable importance in projection (VIP) score which ranks the indicators in the order of their importance. The changes in lipid profiles after statin treatment were assessed using a volcano plot with fold-change >2.0 and p-value <0.05 using Wilcoxon test for paired data and false discovery rate (FDR) to avoid type I errors from multiple comparisons. sPLS-DA was further used to discriminate between "good-responders" and "poor-responders" among FH patients according to their LDL-c reduction after statin treatment. Finally, receiver operating characteristic (ROC) curves were constructed using the "biomarker analysis" tool in MetaboAnalyst v5.0 to discriminate serum lipids between both statin response groups.

2.4. Statistical analysis

Statistical analysis was performed using SPSS v. 22.0 (Chicago, IL, US). Significant results were considered for p-value <0.05. The Kolmogorov-Smirnov test was used to assess the distribution of the continuous variables. Variables with normal distributions were compared by paired *t*-test and shown as mean and SD, and those with skew distributions were compared by paired Wilcoxon test and shown as median and interquartile range. Categorical variables were compared by chi-square test.

3. Results

3.1. Characteristics of the participants

Clinical and laboratory data of the FH subjects are described in Table 1. These individuals were mainly women (76.5 %) and Caucasian (41.2 %) with mean age ~ 55 years. Most of them had hypertension (64.7 %), whereas obesity (47.1 %) and type 2 diabetes (11.8 %) were less frequent. Angina was a major cardiovascular event (52.9 %), with less frequency of acute myocardial infarction (5.9 %), coronary artery disease (11.8 %) and peripheral artery disease (17.6 %). FH-related characteristics were: corneal arcus (17.6 %), xanthoma (11.8 %) and xanthelasma (17.6 %). FH clinical diagnosis was defined or probable in 47.1 %, whereas molecular diagnosis was confirmed in 17.6 % of FH patients.

Table 1
Clinical and laboratory data of the FH patients before and after statin treatment.

Variables	Baseline	Treatment	*P-value*
Age, years	55 (49–66)		–
Sex (women), %	76.5 (13)		–
Ethnics (Caucasian), %	41.2 (7)		–
Hypertension, %	64.7 (11)		–
Body mass index, kg/m^2	27.9 (25.1–31.5)		–
Obesity, %	47.1 (8)		–
Type 2 diabetes, %	11.8 (2)		–
Corneal arcus, %	17.6 (3)		–
Xanthoma, %	11.8 (2)		–
Xanthelasma, %	17.6 (3)		–
FH clinical diagnosis[a] Possible, %	52.9 (9)		
Defined or probable, %	47.1 (8)		
FH molecular diagnosis[b] (%)	17.6 (3)		
Angina, %	52.9 (9)		–
Acute myocardial infarction, %	5.9 (1)		–
Coronary artery disease, %	11.8 (2)		–
Peripheral artery disease, %	17.6 (3)		–
Tobacco smoking, %	0		–
Family history of FH, %	82.4 (14)		–
CHD, %	82.4 (14)		–
Stroke, %	70.6 (12)		–
Total cholesterol, mg/dL	294 [269–325]	191 [167–241]	*<0.001*
LDL cholesterol, mg/dL	215 [187–242]	115 [105–152]	*<0.001*
HDL cholesterol, mg/dL	45 [37–55]	43 [39–49]	*0.344*
VLDL cholesterol, mg/dL	31 [29–39]	25 [15–28]	*0.012*
Triacylglycerols, mg/dL	155 [147–194]	125 [77–141]	*0.011*
Apolipoprotein AI, mg/dL	153 [130–163]	145 [131–170]	*0.619*
Apolipoprotein B, mg/dL	167 [139–189]	91 [84–119]	*<0.001*
Glucose, mg/dL	88 [84–94]	89 [86–99]	*0.244*
HbA1c, %	5.8 [5.8–6.0]	5.6 [5.4–6.0]	*0.567*
Insulin, μIU/mL	8.70 [5.40–11.70]	7.50 [6.40–10.30]	*0.513*

Number of subjects in parentheses. Continuous variables are shown as median and interquartile range and were compared using Wilcoxon paired test. Categorical variables were compared by chi-square test. $p < 0.05$ was considered significant. FH: familial hypercholesterolemia; CHD: coronary heart disease; HbA1c: glycated hemoglobin; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein. (a) Dutch Lipid Clinic Network modified criteria; (b) Pathogenic and likely pathogenic variants in FH-related gene according to ACMG guidelines. The confirmed variants were identified in *LDLR* only (rs121908026, rs121908031 and rs752596535).

As expected, FH subjects have altered serum lipid profile at baseline, and statin treatment reduced significantly total cholesterol ($p < 0.001$), LDL-c ($p < 0.001$), VLDL-c ($p = 0.012$), triacylglycerols ($p = 0.011$), and apo B ($p < 0.001$) (Table 1). Concentrations of HDL-c, apo AI, glucose, HbA1c and insulin were not influenced by 4-week statin treatment ($p > 0.05$).

3.2. Serum lipidomic profile

Lipidomic descriptive analysis identified 353 molecular species (Suppl. Table 1) grouped in 16 lipid subclasses, being TAG and glycerophospholipids those with higher number of molecular species among lipid subclasses (Fig. 1A). To evaluate the effect of treatment on lipid subclasses oPC/pPC/PC were pooled together into PC, as well as pPE/PE into PE (for a total of 13 lipid subclasses).

Statin treatment reduced concentrations of most serum lipid subclasses (8 of 13), including glucosylceramide, ceramide, phosphatidylcholines (oPC/pPC/PC) and lyso-PC, phosphatidylethanolamines (pPE/PE), phosphatidylinositol, sphingomyelin and free cholesterol lipid subclasses ($p < 0.05$; Fig. 1B).

A global lipidomic analysis using sPLS-DA revealed a clear

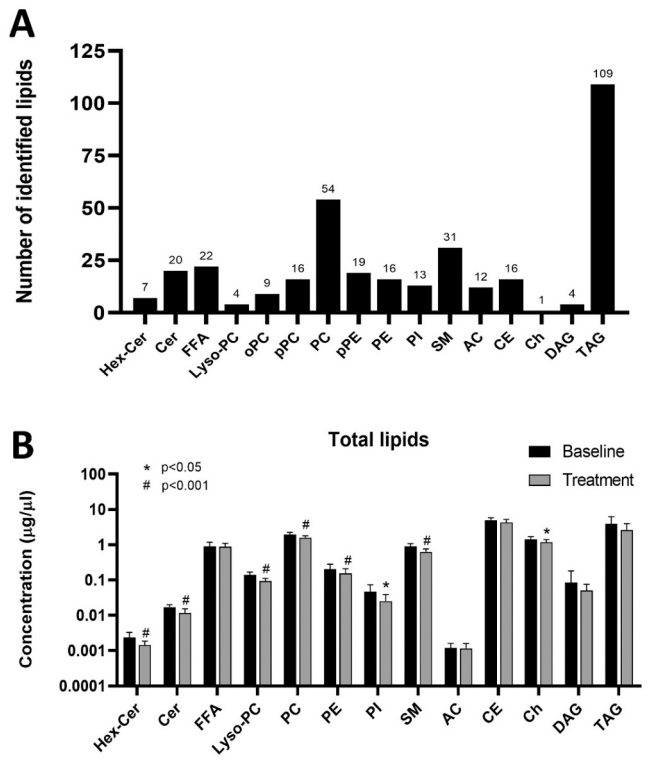

Fig. 1. Basal serum lipidomic profile of FH subjects. (A) Number of identified lipid species and lipid subclasses; **(B)** Concentration of lipid subclasses at baseline and after statin treatment. Values were compared by paired *t*-test or paired Wilcoxon test. FH: Familial hypercholesterolemia, Hex-Cer: Hexosylceramides, Cer: Ceramide, FFA: Fatty acids: Free fatty acid, LysoPC: Lyso-Phosphatidylcholine, oPC: Plasmanyl-phosphatidylcholine, pPC: Plasmenyl-phosphatidylcholine, PC: Phosphatidylcholine, pPE: Plasmenyl-phosphatidylethanolamine, PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, SM: Sphingomyelin, AC: Acylcarnitine, CE: Cholesteryl ester, Ch: free cholesterol, DAG: diacylglycerol, TAG: Triacylglycerol.

separation of serum lipid species at baseline and after statin treatment (Fig. 2A). Further evaluation of VIP scores identified the lipid species with major contribution for this profile. Fig. 2B shows top 15 lipid species with the highest VIP scores, with PI (16:0/18:2), TAG (18:2x2/18:3) and PI (18:0/18:2) showing the most relevant contributions for the mentioned segregation (VIP >2.1).

Fig. 3 represents a volcano plot of serum lipids, and most lipid species were reduced after treatment. Further analysis using FDR <0.05 and a 2-fold change (FC >2.0 or <0.5) as cut-off criteria, identified 8 lipid species including PI and TAG, all of them significantly diminished after statin treatment as detailed in the nested table of Fig. 3 (FC <0.5, FDR <0.05). The serum concentrations of these lipid species in FH patients before and after statin treatment are shown in Fig. 4, confirming a significant reduction after treatment ($p < 0.05$).

We further used sPLS-DA to identify lipid species associated to statin response. LDL-c reduction among FH ranged from 2.8 % to 70.9 % patients and they were classified as "good-responder" if the LDL-c reduction was ≥40.6 % (n = 9) or "poor-responders" if they had an LDL-c reduction ≤33.5 % (n = 8) (Suppl. Fig. 1). Discriminant Analysis considering three main components in MetaboAnalyst v5.0 reveled that serum lipid species at baseline could discriminate between "good-responders" and "poor-responders" FH patients (Fig. 5A), being PI species major contributors among identified lipid species in discriminating groups of statin response according to LDL-c reduction (Fig. 5B).

Further biomarker analysis through ROC curves performed in MetaboAnalyst v5.0 confirmed the potential of serum lipid species at baseline that could discriminate between "good-responders" and "poor-responders" FH patients. The analysis identified three PI with an area under the curve (AUC) higher than 0.9 (Fig. 6), the PI (16:1/18:2) (AUC: 0.986), PI (16:0/18:2) (AUC: 0.944) and PI (18:0/18:2) (AUC: 0.917). These lipids identified as potential predictors of statin response were reduced in the group of "poor-response".

4. Discussion

In this study, we evaluated changes in the lipidome profile of FH subjects treated with lipid-lowering drugs. Recent lipidomic approaches have identified hundreds of different circulating lipid species, both in family history and population-based hyperlipidemias, associated with high LDL-c or high TAG, and increased risk for coronary artery disease (CAD) (Rämö et al., 2019).

Here, using an untargeted lipidomic approach, the influence of statins on serum lipid species was assessed, as well as the potential of these lipids to predict the response of drug therapy, useful as minimal invasive biomarkers. Moreover, lipid signatures that reflect the drug response phenotype indicate potential mechanisms that provide insights about the underlying basis for individual variation in drug response, offering new possibilities for lipid-lowing pharmacological strategies in FH patients (Gianazza et al., 2023).

Traditional plasma lipid profile evidenced that statins successfully reduced apoB-containing cholesterol fractions and triglycerides after the 6-week treatment. Further lipidome profiling showed a clear reduction of most lipid classes in FH patients. Some studies have evaluated the influence of statins on lipidome profile with inconsistent results, however these studies differ in their analytical approaches for quantifying lipids, type of statin and time of treatment, as well as in the target study populations (Bergheanu et al., 2008; Kaddurah-Daouk et al., 2010; Meikle et al., 2015; Lee et al., 2018; Orsoni et al., 2016). In line with our findings, early work in a subset of the RADAR study in Netherlands reported that several plasma sphingomyelins and phosphatidylcholines correlate with different effects of treatment with atorvastatin (20–80 mg/day) and rosuvastatin (10–40 mg/day) for 6–18 weeks on the LDL-c/HDL-c ratio (Bergheanu et al., 2008). Using targeted lipidomics, the CAP study described reduction of plasma cholesteryl ester, free cholesterol, phosphatidylcholines, phosphatidylethanolamines and triacylglycerols in African-American and white individuals after a 6-week treatment with simvastatin (20 mg/day) (Kaddurah-Daouk et al., 2010). Hyperlipidemic patients treated with rosuvastatin (20 mg/day by 3–8 weeks) had reduced levels of phosphatidylcholines, whereas fatty acids were increased after drug treatment (Lee et al., 2018), a characteristic that was not observed in our work, where total FFA levels were not modified by statins. Although relevant differences in study design, subjects and lipid-lowering treatment, all these works described a clear clusterization of lipids species before and after treatment, as also was observed in this work. Moreover, Meikle et al (Meikle et al., 2015). reported that 180 days of pitavastatin treatment (4 mg/day) attenuated the abnormal plasma lipidome in a subgroup of twelve mixed dyslipidemic patients with metabolic syndrome (MetS) from the CAPITAIN trial. A further MS/MS analysis of lipoprotein lipid classes and subclasses in this study population demonstrated that pitavastatin modifies HDL3 content by enriching in polyunsaturated phospholipids and plasmalogens, and decreases the content of phospholipid hydroperoxides derived from LDL in patients with atherogenic mixed dyslipidemia (Orsoni et al., 2016).

Our results for both analytical approaches, the identification of important features from sPLS-DA (VIP scores) and the volcano plot, showed consistent results demonstrating that specific lipids species into phosphatidylinositol and triacylglycerol are the main lipids affected by statin treatment. PI (16:0/18:2) had the highest VIP score and also showed the more significant and steepest reduction (FC: 0.42; $p < 0.001$). PI (18:0/18/2) and PI (16:0/18:1) were also significantly reduced after statin treatment. Phosphatidylinositols are present in all tissues and cell types, but are particularly abundant in the brain tissue

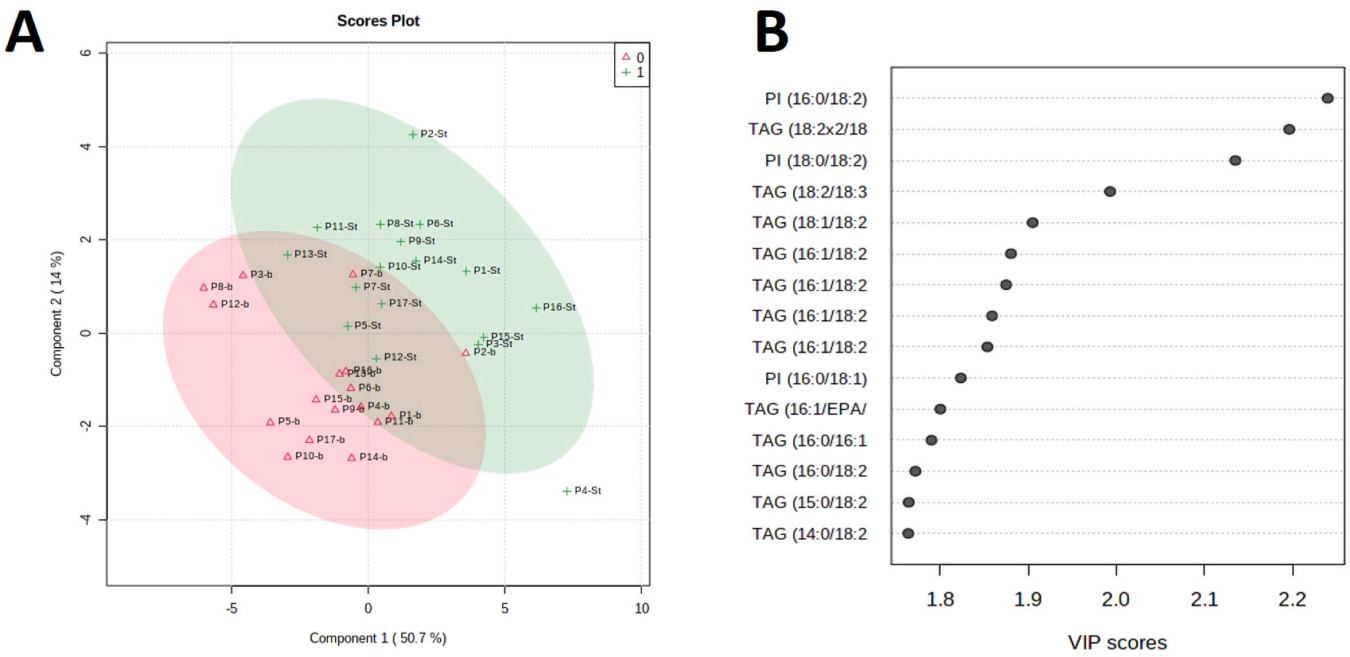

Fig. 2. Global lipidomic profile in FH subjects treated with statins. **(A)** Sparse partial least squares discriminant analysis (sPLS-DA) demonstrates that of serum lipids species clearly differentiated between baseline (Δ, pink diagram) and after statin treatment (+, green diagram) based on scores of two principal components; **(B)** Important features identified by sPLS-DA according VIP scores. The analysis was performed using MetaboAnalyst v5.0 as described in methods. Top 15 lipids species with the highest VIP scores are shown. FH: Familial hypercholesterolemia, PI: Phosphatidylinositol, TAG: Triacylglycerol; VIP, variable of importance in projection. In Fig. 1 B, oPC, pPC and PC were pooled together as PC; and pPE and PE were pooled together as PE.

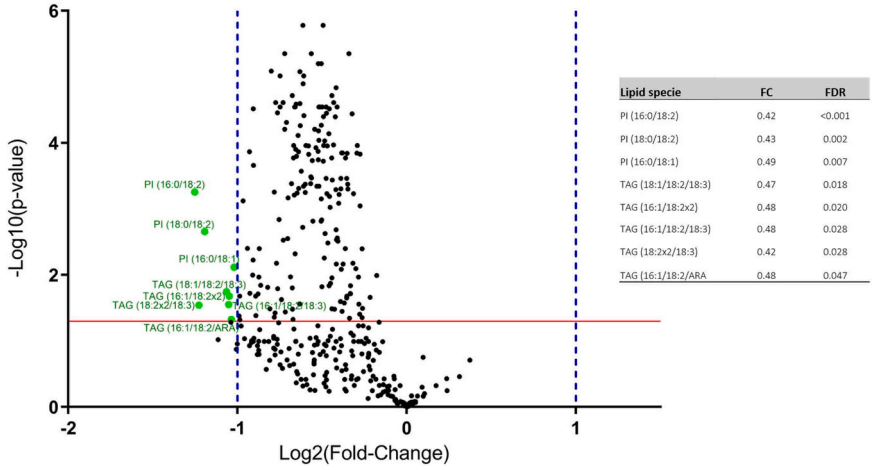

Fig. 3. Volcano plot of serum lipid species influenced by statin treatment. The analysis was performed in MetaboAnalyst v 5.0, using FDR < 0.05 and 2-fold change (FC >2.0 or <0.5) as cut-off criteria. Data of paired log transformed data was compared by Wilcoxon test. FDR: False discovery rate. ARA: Arachidonic acid; PI: Phosphatidylinositol, TAG: Triacylglycerol. IUPAC names and Human Metabolome Data Base ID number are provided in Suppl. Table 2.

(10 % of the phospholipids). They are anionic phospholipids consisting of a phosphatidic acid backbone linked to inositol through a phosphate group (Dickson and Hille, 2019). Phosphatidylinositols may contain different types of fatty acids, with different lengths and degrees of saturation, attached at the C-1 and C-2 positions. In particular, PI (16:0/18:2) has a palmitic acid chain at the C-1 position and a linoleic acid chain at the C-2 position. The inositol group is covalently linked to the phosphate group, which provides a bridge to the lipid tail. Phosphatidylinositols are synthesized by transfer of phosphodiacylglycerol from CDP-diacylglycerol to myoinositol, mediated by CDP-diacylglycerol-inositol 3-phosphatidyltransferase (Blunsom and Cockcroft, 2020). Therefore, statins may downregulate the phosphatidylinositol production, possibly by impairing the synthesis of CDP-diacylglycerol.

Phosphatidylinositol and their phosphorylated derivatives, phosphoinositides, regulate several cellular functions, with numerous evidences that indicate potential associations of PI-mediated signaling pathways with the etiology of various diseases, including CVD (Ghigo et al., 2012).

An early work evaluated the effects of rosuvastatin treatment (10 or 40 mg/d, for 5weeks) on the plasma phospholipidome of MetS subjects, and observed a dose-dependent reduction of phosphatidylinositol (–34 % and –40 %) compared to the placebo group (Ng et al., 2014). In that work, a significant reduction of all phosphatidylinositol lipid species was observed, including the PI (16:0/18:2) identified in this study. Recently, a lipidomic analysis of human liver cells (Hep3B) exposed to 5 μM simvastatin for 48 h revealed association of this induction of low-cholesterol environment with reduction of phosphatidylinositol

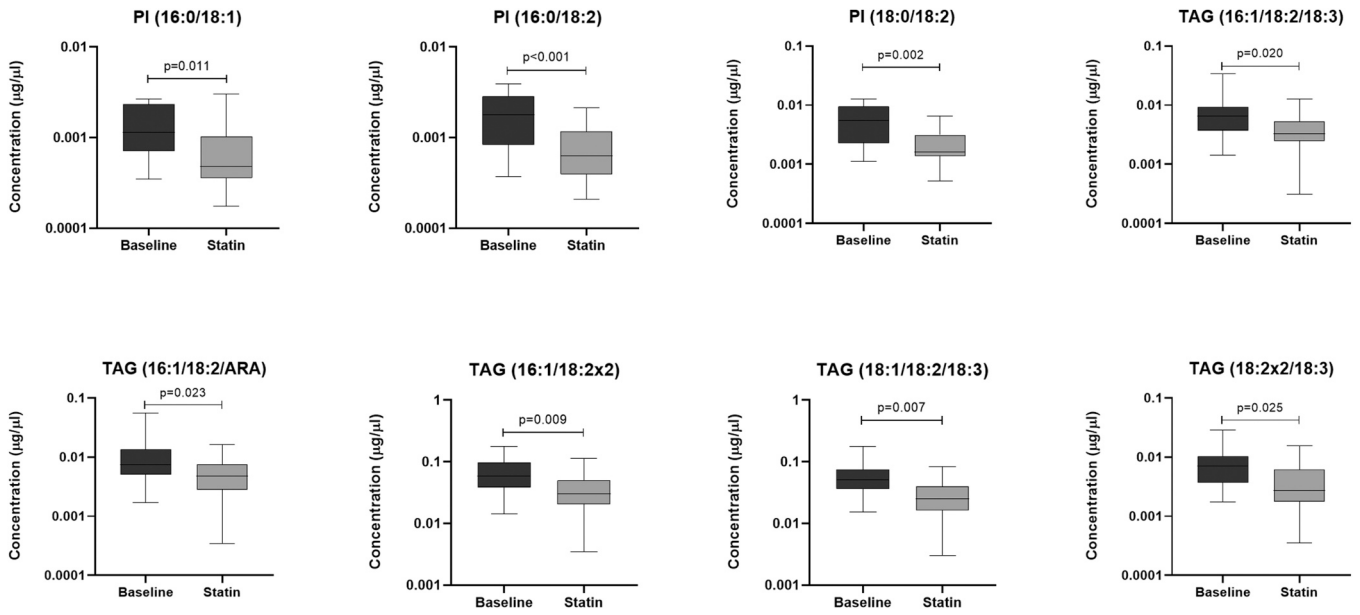

Fig. 4. Influence of statin treatment on serum concentration of lipid species in FH subjects. Data before and after statin treatment were compared by paired *t*-test and are shown as box plots with errors bars (5th and 95th percentile). FH: Familial hypercholesterolemia; ARA: Arachidonic acid; PI: Phosphatidylinositol, TAG: Triacylglycerol. IUPAC names and Human Metabolome Data Base ID number are provided in Suppl. Table 2.

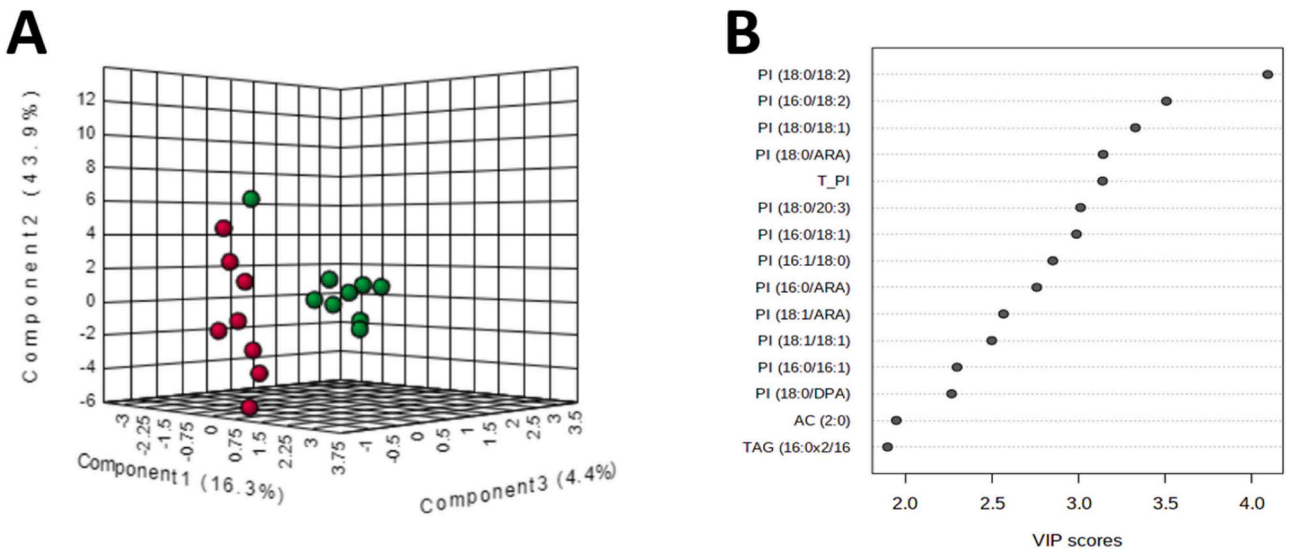

Fig. 5. Global lipidomic profile in FH subjects according to statin response. **(A)** Sparse partial least squares discriminant analysis (sPLS-DA) demonstrates that of serum lipids species clearly differentiated between "poor-responders" (red circles) and "good-responders" (green circles) based in the scores of three principal components; **(B)** Important features identified by sPLS-DA according to VIP scores. FH subjects were classified as "good-responder" (LDL-c reduction $\geq$ 40.6 %; n = 9) or "poor-responders" (LDL-c reduction $\leq$ 33.5 %; n = 8) before analysis. The analysis was performed using MetaboAnalyst v5.0 as described in methods. Top 15 lipids species with the highest VIP scores are shown. FH: Familial hypercholesterolemia; Arachidonic acid; DPA: Docosapentaenoic acid; LDL-c: low-density lipoprotein cholesterol; PI: Phosphatidylinositol, AC: Acylcarnitine, TAG: Triacylglycerol; VIP: Variable of importance in projection.

species and the PI(36:2)/PC(18:0_20:4) ratio (Schooneveldt et al., 2021). In the LIPID study, pravastatin treatment altered plasma lipidome, particularly change in PI(36:2)/PC(18:0_20:4) ratio mediated 58 % of the treatment effect, whereas change in LDL-c accounted for 32 % of the risk reduction for secondary coronary events (Jayawardana et al., 2019). Based in their findings, authors suggested that statins modulate alternative lipid pathways that could be involved in the pleiotropic effects of statins.

VIP scores from sPLS-DA and the volcano plot strategy also identified a reduction of triacylglycerol lipid species after statin treatment in the plasma of FH patients. TAG (18:1/18:2/18:3), TAG (16:1/18:2x2), TAG (16:1/18:2/18:3), TAG (18:2x2/18:3) and TAG (16:1/18:2/ARA) were significantly reduced by at least half of the baseline plasma concentration (FC <0.5). Triacylglycerols are glycerides with glycerol esterified with three groups of fatty acids, which can have different lengths and saturations, the most common being 16, 18 and 20 carbons. They are major components of chylomicrons and VLDL and play an important role in metabolism as energy sources and transporters of dietary fat (Mu and Porsgaard, 2005). Triacylglycerols, as lipid class, are largely associated with a high cardiometabolic risk, as previously showed in MetS individuals when a lipidomic analysis was performed, being elevated over 2-fold as compared to healthy controls (Meikle et al., 2015). Furthermore, in that work, treatment of MetS subjects with pitavastatin (4 mg/day/180 days) significantly reduced triacylglycerol, but this

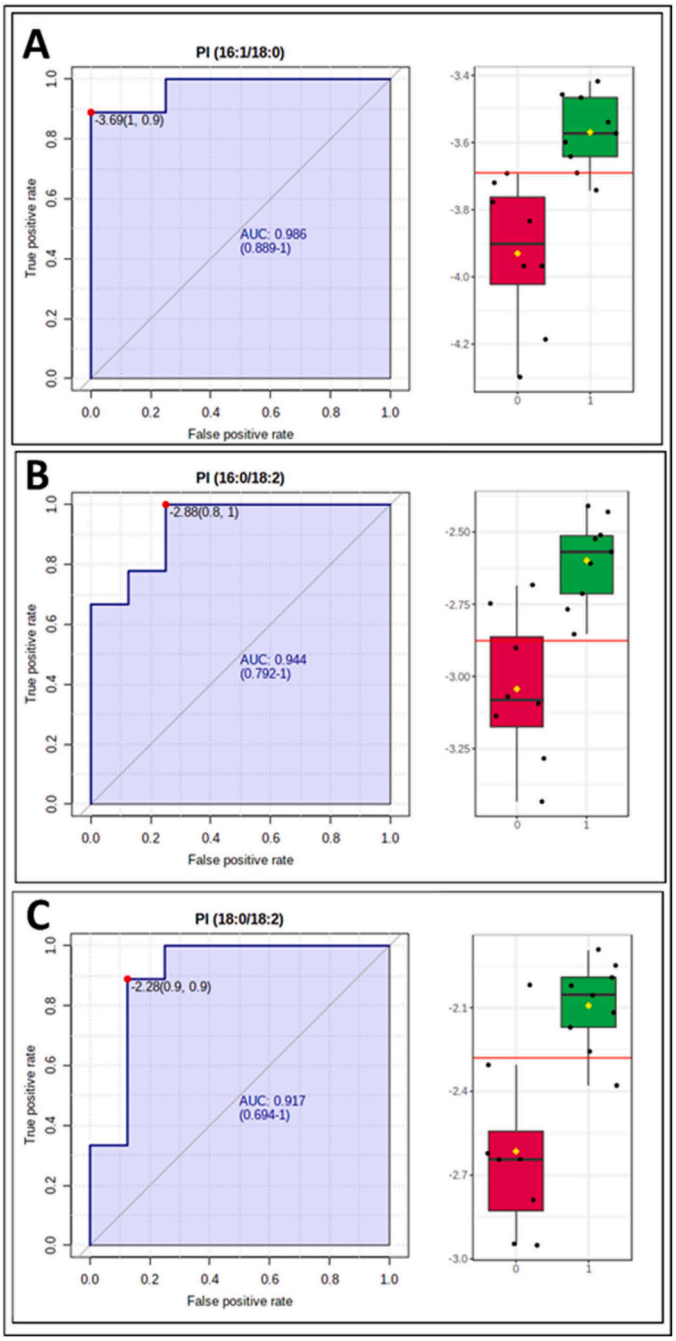

Fig. 6. Serum lipid species related to statin response in FH subjects. **Left panels** represent Receiver Operating Characteristic (ROC) curve. **Right panels** show box-plot of the concentration of **(A)** PI (16:1/18:0), **(B)** PI (16:0/18:2) and **(C)** PI (18:0/18:2) in "poor-responders" (red box-plot) and "good-responder" (green box-plot) FH subjects. Biomarker analysis to predict statin response was performed in MetaboAnalyst v5.0. FH subjects were classified as "good-responder" (LDL-c reduction $\geq$ 40.6 %; n = 9) or "poor-responders" (LDL-c reduction $\leq$ 33.5 %; n = 8). FH: Familial hypercholesterolemia, LDL-c: low-density lipoprotein cholesterol, PI: Phosphatidylinositol.

reduction was not reply at molecular triacylglycerol species, suggesting statins have targeted effects on biological pathways of both lipid and fatty acid metabolism (Meikle et al., 2015). Recently, it was demonstrated that simvastatin modulates plasma lipidomic profiles, including triacylglycerol lipid species, in metastatic castration-resistant prostate cancer men (Mak et al., 2022). Even the early evidence, the effect of statins on molecular species of triacylglycerols has been poorly studied. In this line, a recent work analyzed lipidomics data from mice and clinical patients and suggested that treatment with statins contributes to restoring the triacylglycerols homeostasis via the LXRα-ATGL/EPT1 axis, alleviating hepatic steatosis in MetS (Chen et al., 2023), which should be considered when interpreting the influence of statins on cardiovascular health, beyond their effects on cholesterol reduction.

An important extension of this work was the analysis of plasma lipidome as biomarkers of the cholesterol-lowering response to statins in patients with FH. The efficacy of statin treatment is highly variable in hypercholesterolemic subjects and different approaches have been explored in order to identify genetic, epigenetic or biochemical/metabolomic predictors (Borges et al., 2021). Our results suggest some plasma phosphatidylinositol – PI (16:1/18:0), PI (16:0/18:2) and PI (18:0/18:2) - are good predictors of LDL-c lowering in FH subjects, represented by an AUC $>$ 0.9 towards identification of FH individuals with poor cholesterol lowering response. Coincidently, two of the lipid species that were previously identified by being modulated by statin treatment, were also identified as biomarkers in our work –PI (16:0/18:2) and PI (18:0/18:2) -. The concentration of the three phosphatidylinositol lipid species, identified by Biomarker Analysis from MetaboAnalyst v5.0, had lower baseline concentrations in the "poor--responder" group. Nevertheless, scarce information is available regarding lipid species as predictors of statin response.

As mentioned above, a previous work suggests that the reduction of the PI(36:2)/PC(18:0_20:4) ratio by pravastatin could be linked to long term CVD risk reduction, however these parameters have been not yet explored as biomarkers (Schooneveldt et al., 2021). An early report from the CAP Study, designed to assess the influence of genetic and non-genetic factors on response to simvastatin treatment (40 mg/day/6-week), performed lipidomic analysis in a subset of 24 "good'' responders (top 10 %) matched with 24 "poor" responders (low 10 %) (Kaddurah-Daouk et al., 2010). Further, a correlation analysis showed that baseline concentrations of cholesterol ester and phospholipid metabolites correlate with variations in LDL-c response to treatment. However, the results from the targeted lipidomics platform reported by the research group did not include phosphatidylinositols among lipid classes evaluated. In this way, the evolution of analytical strategies and analysis platforms allows to access a more detailed information and identification of molecular species in lipidomics.

This study has some limitations due to its exploratory nature. A limitation is the small sample size of this cohort of FH patients, therefore, further studies with larger samples would contribute to validate our findings. Critical differences between statins that lead to variable cholesterol reduction should also be considered, limiting the extent of our conclusions and highlighting the need to explore other types of statins. Despite these limitations, this study has an important contribution to the research area of pharmacometabolomics of lipid-lowering drugs. Our work represents the first approximation to understand the influence of cholesterol-lowering by statins on the lipidomic profile in FH subjects. In this way, our research group performed a complete screening at molecular level (Borges et al., 2021), running this study protocol in a well-characterized cohort from Brazil.

In conclusion, our findings indicate that statins extensively modulate the overall composition of serum lipids in FH individuals, with some molecular species of phosphatidylinositol and triacylglycerols being particularly affected. Furthermore, phosphatidylinositol molecules have potential use as predictive biomarkers of pharmacological response.

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CRediT authorship contribution statement

Alvaro Cerda: Conceptualization, Investigation (Experimental procedures – data analysis and interpretation), Formal analysis (Statistical Analysis), Writing – original draft. **Raul H. Bortolin:** Conceptualization, Investigation (Experimental procedures – data analysis and interpretation), Writing – original draft. **Marcos Y. Yoshinaga**: Investigation (Lipidomic analysis), Writing – review. **Renata C. C. Freitas:** Investigation (Clinical data acquisition and analysis), Writing – review. **Carolina Dagli-Hernandez:** Investigation (Clinical data acquisition and analysis), Writing – review. **Jessica B. Borges:** Investigation (DNA sequencing), Formal analysis (DNA data analysis), Writing – review. **Victor F. Oliveira:** Formal analysis (DNA data analysis and Bioinformatics), Writing – review. **Rodrigo M. Gonçalves**: Investigation (Selection and follow-up of patients), Writing – review. **Andre A. Faludi:** Investigation (Selection and follow-up of patients), Writing – review. **Gisele M. Bastos:** Investigation (Clinical data acquisition and analysis), Writing – review. **Rosario D. C. Hirata**: Writing – original draft, Writing – review and editing. **Mario H. Hirata**: Conceptualization, Funding acquisition, Supervision, Writing – review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data are available in Supplementary Material.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.chemphyslip.2023.105348.

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