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Analysis of serum lysophosphatidylethanolamine levels in patients with non-alcoholic fatty liver disease  
by liquid chromatography-tandem mass spectrometry

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## Abstract

Lysophosphatidylethanolamines (LysoPEs) are the partial hydrolysis products of phosphatidylethanolamine. Despite the unique in vitro bioactivities of LysoPEs, there are limited reports on the pathophysiological role for LysoPEs in the serum, due to the lack of sensitive analytical methods for determination of each molecular species in clinical samples. Herein, we developed a highly sensitive quantitative method to profile the serum LysoPE species by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with selected reaction monitoring (SRM). The internal standard (IS), chemically synthesized in-house and the lineup of seven major LysoPE species were used in this study. The limits of detection and quantification for each LysoPE species ranged within 0.5-3.3 pmol/mL and 1.0-5.0 pmol/mL, respectively. The combined concentration of LysoPEs in the serum from healthy subjects (n=8) and the patients with non-alcoholic fatty liver diseases (NAFLD) including simple steatosis (SS, n=9) and non-alcoholic steatohepatitis (NASH, n=27) were  $18.030 \pm 3.832$ ,  $4.867 \pm 1.852$ , and  $5.497 \pm 2.495$  nmol/mL, respectively. The combined and individual concentration of LysoPEs, except for LysoPE 18:0, significantly decreased in the patients with NAFLD compared with those for the healthy subjects. However, no significant difference was observed between the SS and NASH groups. Our proposed LC-MS/MS method is valid and has advantages of small sample volume, high sensitivity and simultaneous absolute quantitation for multiple molecular species. This method may enable diagnostic evaluation and elucidation of yet uncovered pathophysiological role for LysoPEs.

**Keywords**

lysophosphatidylethanolamine; LysoPE; liquid chromatography-tandem mass spectrometry; chemical synthesis; non-alcoholic fatty liver disease.

## Abbreviations

AcOH, acetic acid; Bu<sub>2</sub>SnO, dibutyltin oxide; CID, collision-induced dissociation; (COCl)<sub>2</sub>, oxalyl chloride; CV, coefficient of variation; DCM, dichloromethane; DHA, docosahexaenoic acid; DMF, *N,N*-dimethylformamide; DPPE, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; ESI, electrospray ionization; HPLC, high performance liquid chromatography; iPrOH, isopropanol; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOD, limits of detection; LOQ, limits of quantification; LPCAT, lysophosphatidylcholine acyltransferase; LPEAT, lysophosphatidylethanolamine acyltransferase; LysoPA, lysophosphatidic acid; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; LysoPG, lysophosphatidylglycerol; LysoPI, lysophosphatidylinositol; LysoPLs, lysophospholipids; LysoPS, lysophosphatidylserine; MAPK, Mitogen-activated protein kinase; MeOH, methanol; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NMR, Nuclear magnetic resonance; PE, phosphatidylethanolamine; PLA1, phospholipase A1; PLA2, phospholipase A2; SD, standard deviation; SS, simple steatosis; Tr-DPPE, trityl 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; Tr-GPEA, trityl *sn*-glycerol-phosphoethanolamine; Tr-LysoPE, trityl lysophosphatidylethanolamine.

## 1. Introduction

Lysophospholipids (LysoPLs) are deacylated products of phospholipids with single fatty acid chain and are produced by the action of phospholipase A (PLA1 or PLA2). Several types of LysoPLs were identified in biological samples such as plasma, with a change in their head groups, including lysophosphatidylcholine (LysoPC), lysophosphatidylethanolamine (LysoPE), lysophosphatidylinositol (LysoPI), lysophosphatidylserine (LysoPS), lysophosphatidylglycerol (LysoPG) and lysophosphatidic acid (LysoPA). Their biological activities are considered to be significantly different depending on their chemical nature as for example LysoPA is known to act as a lipid mediator [1, 2]. LysoPLs in plasma are generally bound to albumin or lipoproteins [3]. Numerous studies have suggested that changes in plasma levels of LysoPLs are linked to several diseases such as ovarian cancer, acute kidney injury, non-alcoholic fatty liver disease (NAFLD) and obesity [4-7]. LysoPC is the most abundant LysoPL in human plasma, and its levels are reported to be about several hundred micromoles [8] and its known to involve in several biological functions such as vascular endothelial dilation, cellular immune response and insulin secretion [9-11]. However, the *in vivo* roles and physiological functions of LysoPEs [12] were not well studied. Several *in vitro* studies demonstrate the LysoPEs as a neurotrophic activator via activation of mitogen-activated protein kinase (MAPK) signaling [13], increase intracellular calcium levels, and stimulates chemotactic migration and cellular invasion in cancers [14]. Clinical studies also demonstrate the decreased LysoPE levels in serum samples of migraine patients [15]. Despite the significant biological importance of

LysoPEs, most of the studies were focused on measurement of their relative concentrations [15, 16]. Although semi-quantitative analysis is useful as comprehensive one, it has fundamental drawbacks such as the inability to obtain an accurate concentration and less sensitive. So far, an absolute quantitative method for LysoPE determination is limited, due to the lack of authentic standards and optimal internal standard (IS) for LC-MS/MS.

Herein, we chemically synthesized the authentic LysoPEs (LysoPE 18:2, 20:4, 20:5 and 22:6) and an odd chain IS (LysoPE 15:0), which are not commercially available and aimed to develop an absolute quantitative method for LysoPE species by a simple liquid-liquid extraction followed by highly sensitive targeted LC-MS/MS analysis. Seven LysoPE molecular species prepared in this study are major molecular species in previous studies [17, 18]. Furthermore, we employed our method as a clinical application to profile the concentration of LysoPEs in the serum from patients with non-alcoholic fatty liver disease (NAFLD) and healthy subjects. NAFLD is a progressive hepatic disease characterized by massive triglyceride (TG) accumulation in the liver and can range from simple steatosis (SS) to non-alcoholic steatohepatitis (NASH) [19].

## 2. Materials and Methods

### 2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), trityl bromide, *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), linoleic acid, pentadecanoic acid and arachidonic acid were purchased from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). Dehydrated solvents {dichloromethane, pyridine, and *N,N*-dimethylformamide (DMF)}, triethylamine and sodium methoxide were purchased from FUJIFILM Wako Pure Chemical Corporation, (Osaka, Japan). Chloroform, methanol (MeOH) and oxalyl dichloride were purchased from KANTO CHEMICAL CO., INC., (Tokyo, Japan). Eicosapentaenoic acid (EPA) was purchased from Cayman Chemicals, (Michigan, USA). Acetic acid (AcOH) was purchased from NACALAI TESQUE, INC., (Kyoto, Japan).

The LysoPE species analyzed in the present study included 1-pentadecanoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 15:0), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 16:0), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 18:0), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 18:1), 1-linoleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 18:2), 1-arachidonoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 20:4), 1-eicosapentaenoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 20:5), 1-docosahexaenoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LysoPE 22:6). Among them, LysoPE 16:0, 18:0, and 18:1 were purchased from Avanti POLAR LIPIDS, INC. (Alabama, USA),

while the other LysoPE species were synthesized in our laboratory following the method below.

## 2.2. General procedure for synthesis of LysoPEs

The synthetic route for the preparation of LysoPEs is shown in **Fig. 1**. The LysoPE standards were synthesized by a method earlier established in our laboratory with minor modifications [17, 20]. Briefly, the protection of an amine moiety of DPPE with trityl bromide gives compound **2** trityl 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (Tr-DPPE) and subsequent deacylation using sodium methoxide gives compound **3** trityl sn-glycerol-phosphoethanolamine (Tr-GPEA) with 69% yield [17]. The compounds **4a-e** trityl lysophosphatidylethanolamine (Tr-LysoPE 15:0, 18:2, 20:4, 20:5 and 22:6) were obtained by direct acylation of Tr-GPEA using oxalyl dichloride and pyridine instead of dibutyltin oxide ( $\text{Bu}_2\text{SnO}$ ) and isopropanol (iPrOH), which often gives poor yield or no reaction [21]. In brief, the respective fatty acids (15:0, 18:2, 20:4, 20:5 and 22:6) were activated by converting them into acid chlorides by adding oxalyl chloride (2 eq) in DCM: DMF (1000:1) and stirring at room temperature for 3 hours. The activated acid chlorides were redissolved in DCM having 0.1M pyridine and reacted with Tr-GPEA for an overnight at 0°C-room temperature. The reaction was monitored by thin-layer chromatography, and the reaction mixture is extracted with chloroform and washed with 1N hydrochloric acid and a brine solution. The chloroform layer was dried in a vacuum and respective Tr-LysoPEs were purified by silica gel column chromatography with a yield about 21%-55%. Finally, the compounds **5a-e** (LysoPE 15:0, 18:2, 20:4, 20:5

and 22:6) were obtained by the deprotection of trityl group using 90% aqueous AcOH [17]. The IS, compound **5e**, LysoPE 15:0 was synthesized for the first time in this study. The detailed experimental protocols, high-resolution massed, and their spectroscopic characterizations ( $^1\text{H}$  and  $^{13}\text{C}$ -nuclear magnetic resonance (NMR)) were provided in the supplemental information (Supplemental Fig. 1-7).

### **2.3. Sample collections**

In order to obtain serum samples from healthy subjects, blood samples were collected from 8 young healthy volunteers, and are stored at  $-80^\circ\text{C}$ . The serum from NAFLD patients in which SS (n=9) and NASH (n=27), are obtained from Okayama University Hospital and are stored at  $-80^\circ\text{C}$  prior to analysis. The NAFLD patients were diagnosed histologically by liver biopsy using NAS score and Brunt classification. The details about the classification of the serum from NAFLD patients are provided in our previous paper [22]. Clinical parameters of both healthy subjects and NAFLD patients were provided in

#### **Table 1.**

### **2.4. Lipid extraction**

Lipid extraction was performed according to the Bligh and Dyer method with minor modifications [23]. Briefly, the serum sample (10  $\mu\text{L}$ ) was added into 400  $\mu\text{L}$  of MeOH {with 62.5 nmol/mL of IS (LysoPE 15:0)}. Then, 400  $\mu\text{L}$  of chloroform and 190  $\mu\text{L}$  of Milli-Q were added into the mixture and

vortexed at 3500 rpm for 5 min (MSV-3500, Funakoshi Co., Ltd., Tokyo, Japan). After centrifugation at 9000 rpm, 4°C for 5 min (CF-15R, HITACHI Co., Ltd., Tokyo, Japan), the chloroform layer was collected and dried during re-extraction, and the upper aqueous layer was re-extracted with 400 µL of chloroform. Next, combined organic layer was dried under vacuum for 30 min with centrifugal concentrator (CC-105, Tomy Seiko Inc., Tokyo, Japan). The obtained lipid extract was redissolved in 200 µL of MeOH, centrifuged at 15000 rpm, 4°C for 15 min to remove any residues and transferred to LC-MS vials.

## **2.5. LC-MS/MS conditions**

High performance liquid chromatography (HPLC) system (Thermo Fisher Scientific Inc., Massachusetts, USA) equipped with Hypersil GOLD column (50 × 2.1 mm, 1.9 µm, Thermo Fisher Scientific Inc., Massachusetts, USA) was used for separation of LysoPE. The oven temperature was maintained at 35°C, the sample tray was kept at 4°C, and the injection volume was set at 5 µL. The mobile phase consisted of 5 mmol/L aqueous ammonium acetate (A), iPrOH (B), and MeOH (C) at a flow rate of 200 µL/min. The following gradient elution was applied: The first 0.5 min, 20% A, 40% B, and 40% C; 0.5-5.0 min, gradually changed to 10% A, 40% B, and 50% C; then, 5.0-5.5 min, 5% A, 50% B, and 45% C, and kept this ratio until 13.0 min; the final 13.0-13.5 min, returned to the initial ratio, and kept until 15.0 min for re-equilibration.

TSQ Quantum Access mass spectrometer (Thermo Fisher Scientific, Inc., Massachusetts, USA)

was used in Electrospray ionization (ESI) negative ion mode. The capillary temperature was 200°C. The spray voltage was 4000 V. The sheath gas (nitrogen) pressure was 30 psi, the ion sweep gas (nitrogen) pressure was 4 psi, and the aux gas (nitrogen) pressure was 35 psi. The selected reaction monitoring (SRM) by collision-induced dissociation (CID) was optimized for each analyte, and the detailed parameters are listed in **Table 2**.

## **2.6. HPLC-Orbitrap MS conditions**

The semi-quantitative profiling of phosphatidylethanolamine (PE) for confirming its changes was carried out according to our previous method [24] by using an HPLC-Orbitrap MS (Thermo-Fisher Scientific Inc.). Chromatographic separation was conducted by a T3 C18 column (2 × 150, 3 μm, Waters, Milford, MA) with the mobile phase consisted of 10 mM aqueous ammonium acetate (A), iPrOH (B), and MeOH (C) following the optimized gradient elution. High-resolution MS was utilized for detection, with ranges of  $m/z$  150-1100 and 220-1650 for ESI-positive mode. While MS/MS fragmentation was acquired by collision induced dissociation in ion-trap mode. The raw data were processed by Xcalibur 2.3 (Thermo-Fisher Scientific Inc.) within the mass tolerance of 5.0 ppm. The identification of fatty acyl composition was executed with the LIPIDMAPS database [25].

## **2.7. Method validation**

A series of diluted LysoPE standard solutions was prepared, with the concentration 0.005, 0.01, 0.05, 0.25, and 2.0 pmol/ $\mu$ L for each standard. The limits of quantification (LOQ) and the limits of detection (LOD) were evaluated as the signal-to-noise ratio (S/N) as 10 and 3, respectively (Except for LysoPE 20:5, of which the S/N for LOQ was 4). Reproducibility was tested using serum from a healthy subject in intraday-assay for 10 replicates and interday-assay for 3 days. The recovery test was conducted by adding three levels of LysoPE species into the serum from a healthy subject with n=6 replicates.

## **2.8. Statistical analysis**

GraphPad Prism 7.0e (GraphPad Software Inc., La Jolla, CA, USA) was used as the software for all statistical analysis. The one-way ANOVA test followed by the Tukey-Kramer test for multiple comparison was applied in all clinical studies except the analysis of the Female/Male ratio in clinical parameters. The Chi-square test was applied in the analysis of the Female/Male ratio in clinical parameters.  $P < 0.05$  was considered as statistically significant. All results were expressed as means  $\pm$  standard deviation (SD).

## **3. Results**

### **3.1. Chemical synthesis of authentic LysoPEs and their characterizations**

The LysoPE authentic standards which are commercially unavailable were synthesized in our

laboratory by the method earlier established in our laboratory with minor modifications. In particular LysoPE standards such as LysoPE 18:2 (**5a**), LysoPE 20:4 (**5b**), LysoPE 20:5 (**5c**), LysoPE 22:6 (**5d**) and LysoPE 15:0 (**5e**: IS) are obtained in a four step reaction starting from commercially available DPPE (**1**). The protection of an amine group of DPPE was achieved with trityl bromide to give Tr-DPPE (**2**) with 90% yield and further the fatty acyls of **2** were hydrolyzed by sodium methoxide to give Tr-GPEA with 69% yield. To obtain the required LysoPEs, desired fatty acids of each molecular species were activated by converting them to acyl chlorides using carbonyl dichloride in pyridine and re-acylated to Tr-GPEA to yield respective Tr-LysoPEs, **4a-e** with a yield (21-53%). Earlier Bu<sub>2</sub>SnO and iPrOH were used to prepare Tr-LysoPE, but in this study, we found that use of carbonyl dichloride improved the reaction yield. Finally, deprotection of trityl moiety of Tr-LysoPEs using aqueous acetic acid gives the LysoPEs (**5a-e**) with a 30-97% yield. All the prepared standards were characterized by their high-resolution masses and structural confirmation was carried out by <sup>1</sup>H and <sup>13</sup>C-NMR. The detailed NMR assignments were provided in the supplemental information (Supplemental Fig. 1-7).

### 3.2. Method validation

#### 3.2.1. Linearity and sensitivity

Under the optimized conditions, the linearity of each LysoPE was evaluated (shown in **Table 3**). For all the LysoPE analytes, the R<sup>2</sup> were more than 0.9972, showing good linearity. The LOQ for all the

LysoPE ranged from 0.001 pmol/ $\mu$ L to 0.005 pmol/ $\mu$ L, and the LOD ranged from 0.0005 pmol/ $\mu$ L to 0.0033 pmol/ $\mu$ L, showing sufficient sensitivity.

### 3.2.2. Reproducibility and recovery

The results of reproducibility and recovery tests are listed in **Table 4**. The coefficient of variations (CVs) of intra-day and inter-day for all LysoPE species were within 12.6% and 9.6%, respectively. While the recoveries for all the LysoPE ranged from 87.3-111.1%, of CVs within 6.4%.

### 3.3. Freezing and thawing test and stability test

In order to check the stability of serum samples, the serum from n=3 healthy subjects were frozen and thawed three times within 3 days for freezing and thawing test, and measured a week, 4 weeks and 8 weeks after serum collection for stability test. During the freezing and thawing test, there was no significant influence on the LysoPE concentration (Supplemental Table 1). And in the stability test, between week 0 and week 8, there was also no significant influence on it (Supplemental Table 2).

### 3.4. LysoPE variation among healthy subjects, SS and NASH serum

By using the developed method, we acquired the LysoPE molecular species in serum from healthy subjects, SS, and NASH groups. Although, we have synthesized LysoPEs of *sn-1* type and

performed analysis of serum, we have not distinguished *sn*-1 or *sn*-2 in the samples. Hence, the analysis results are shown by omitting the appropriate stereochemistry because *sn*-1 and *sn*-2 LysoPE are quickly converted to each other and *sn*-1 types of LysoPE account for almost all LysoPEs in the plasma [18]. The LysoPE profile in healthy subjects showed the highest concentration of LysoPE 18:2 of 4.485 nmol/mL, followed by LysoPE 22:6 of 3.249 nmol/mL and LysoPE 20:4 of 3.020 nmol/mL (**Fig. 2**). In addition, both SS and NASH significantly decreased in LysoPE molecular species except for LysoPE 18:0 when compared with healthy subjects. However, LysoPE 18:0, concentrations shown to have no significant difference between three groups.

Combined LysoPE concentration in serum was found to be 18.030 nmol/mL in healthy subjects, 4.867 nmol/mL in SS and 5.497 nmol/mL in NASH (**Fig. 2**). There was a significant difference between healthy subjects and SS or NASH was observed, though there was no significant difference on combined LysoPE concentration between SS and NASH groups of any molecular species.

### **3.5. PE variation among healthy subjects, SS and NASH serum by using HPLC-Orbitrap MS**

We measured PE species, as they are the precursors of LysoPEs in serum from healthy subject, SS and NASH using HPLC-Orbitrap MS and their semi-quantitative profiles are shown in **Fig. 3**. PEs having polyunsaturated fatty acyl chain in serum from SS and NASH were significantly lower than those of the healthy subjects.



#### 4. Discussion

The phospholipids are the most abundant class of membrane lipids, which undergo hydrolysis by the action of PLA1 or PLA2 to produce LysoPEs. Previous studies have demonstrated the involvement of LysoPEs in cell signaling process, act as a neurotrophic activator [13], increase the intracellular calcium levels [14] and biomarkers for migraine [15]. Most of the reported LysoPE measurement methods are semi-quantitative, less sensitive and often represent only the variation trends. To know the physiological concentration and significance of LysoPEs absolute quantitation methods are necessary, LC-MS/MS with SRM methods are most promising as they are more sensitive and accurate. So far, absolute quantitative methods are limited in the literature due the lack of authentic standards and hence, role of LysoPEs in clinical science were a few. To overcome this problem in this study, we synthesized the authentic standards by a four-step chemical reaction along with a novel, odd uncommon LysoPE 15:0 molecular species as an internal standard. A simple oxalyl chloride mediated acylation step was employed in this study to prepare the good yields of LysoPEs in in the third step (**Fig. 1**), which is more efficient and simpler than the previous report involving  $\text{Bu}_2\text{SnO}$  [17].

The data of LOQ ranges from 0.001-0.005 nmol/mL and LOD from 0.0005-0.0033 (**Table 3**) showed that our method is highly sensitive enough to detect the LysoPEs in serum samples and it is about 10 times more sensitive and requires less sample volume than a previous report [18]. This sensitivity enables us to detect small amount of each LysoPE species in the serum from NAFLD patients. Further the

comprehensive method validation was performed as demonstrated in **table 4** showed that, the recovery rate is > 90% for most of the LysoPEs, with a low coefficient of variation and suggests the high precision of our methodology. Previously, the values of LysoPE were evaluated by semi-quantitative method or method using inappropriate internal standard, but not absolute quantitative one as shown in the present study. To overcome this problem, we have completed a novel unnatural IS (LysoPE 15:0) and seven standards, and established a more accurate and absolute measurement method for LysoPEs using sensitive targeted LC-MS/MS.

The results demonstrated in the **Fig. 2**, showed that most of the LysoPE molecular species serum concentrations were decreased in NAFLD (including SS and NASH) compared to healthy controls and these results are consistent with a previous report which showed the decreased total LysoPE levels in patients with NAFLD [6]. To our knowledge, this is the first report to profile individual molecular species variations in the serum from NAFLD patients. However, the reason behind the decreased serum concentration of LysoPEs and molecular mechanisms involving it remains unknown and needs to be elucidated. There are two possible reasons for lowering serum LysoPE in NAFLD patients. The first reason for lowering serum LysoPE might be lysophospholipid acyltransferase. It has been reported that the hepatic levels of lysophosphatidylcholine acyltransferase (LPCAT 1, 2, 3 and 4), which is a specific enzyme to metabolize LysoPC to phosphatidylcholine, were increased with an increase of inflammation cytokines, tumor necrosis factor- $\alpha$  and transforming growth factor- $\beta$ 1, thereby leads to reductions in the amount of

LysoPC in the serum [26]. Similar to these enzymes, LPCAT3, 4 and lysophosphatidylethanolamine acyltransferase 1 (LPEAT 1) were identified as specific lysophospholipid acyltransferase to metabolize LysoPE to PE in the liver [27]. Because both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were over normal ranges in SS and NASH patients recruited in this study, the liver inflammation might induce the increase of the expression of LPCAT3, 4 and LPEAT 1 followed by the decreases of combined LysoPE levels in serum. However, acyltransferases were not measured in this study, so it may be necessary to demonstrate them in mice in the future since human liver is difficult to obtain.

The second reason for lowering serum LysoPE might be decreasing of PEs in the serum. Semi-quantitative analysis results of PEs demonstrated the decreased levels of PE enriched in linoleic acyl, arachidonic acyl, and DHA {e. g. PE 34:2 (16:0/18:2), PE 36:2 (18:0/18:2), PE 38:4 (18:0/20:4), PE 38:5 (18:1/20:4), and PE 40:6 (18:0/22:6)} in NAFLD group. While PE 34:0, PE 34:3, PE 36:1, and PE 36:2 were different between SS and NASH, might be potential biomarkers for NASH (**Fig. 3**). And the decrease of serum PEs in NAFLD patients could be linked to the reduction of serum LysoPEs. It was considered that there are two possible reasons on the decreases of these PE molecular species. First, since polyunsaturated fatty acids are generally susceptible to oxidation [28], the PE may be degraded due to the oxidative stress toward its containing linoleic acid, arachidonic acid, and DHA. Second, since PE is mainly biosynthesized in the Kennedy pathway of the endoplasmic reticulum (ER) [29], total PE production might be possibly

reduced by ER stress [30]. It has been reported that ER stress is induced in NAFLD along with cellular fat accumulation, followed by the decreased levels of PE [31]. And finally, LysoPEs in the serum from NAFLD might be decreased.

Thus, our proposed highly sensitive method could be useful for future research on the analysis of LysoPE species. Since the receptor for LysoPE is unknown and the LysoPE metabolism in the cell and the blood is unclear [32], it was difficult to explain the decrease of LysoPE levels both in SS and NASH clearly.

## **5. Conclusion**

We successfully established the method for absolute quantitation of seven major LysoPE species.

The proposed method will be a powerful tool for obtaining the profile of the LysoPE species as well as the comparisons in the concentrations of LysoPE species. Our proposed method has some advantages such as small sample volumes, multiple species measurement, highly sensitive and could contribute to elucidating metabolism of LysoPEs. The detailed molecular mechanisms behind the change in PE and LysoPE concentration in SS and NASH patients will be of our future interest.

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## **Compliance with Ethical Standards**

Conflicts of Interest

The authors declare that there is no conflict of interest.

#### Ethics approval

All experimental protocols were carried out with a prior approval from Okayama University (No. 1604-011), Okayama City Hospital (No. 30-2) and Hokkaido University (No. 16-91-1; 19-53) Ethics Review Committee.

#### Consent to participate

All volunteers have provided written informed consents to participate in this research.

#### Consent for publication

All volunteers have provided written informed consents for the publication of this research.

#### **Author Contributions**

YY, TS, HC and SPH designed this research. YY and TF, SGBG, Yuki Yoshikawa involved in synthesizing all LysoPEs. KN and YF collected patients' samples. YY, ZC and YW analyzed data on LC-MS/MS. TS measured clinical data. YY, TS, ZC, SGBG, HC and SPH wrote manuscript. All authors read and approved the final manuscript.

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**Fig. 1** Synthetic scheme for each LysoPE standards and internal standard (IS) [17]. Reagents for the process are shown above the arrow, and solvents are shown below. The yield is shown in parentheses. Compound **5e** (R = pentadecanoic acid) is IS. AcOH, acetic acid; (COCl)<sub>2</sub>, oxalyl chloride; DCM, dichloromethane; DHA, docosahexaenoic acid; DMF, *N,N*-dimethylformamide; DPPE, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; EPA, eicosapentaenoic acid; LysoPE, lysophosphatidylethanolamine; Tr-DPPE, trityl 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; Tr-GPEA, trityl *sn*-glycerol-phosphoethanolamine; Tr-LysoPE, trityl lysophosphatidylethanolamine

**Fig. 2** Quantitation of LysoPE molecular species in human serum samples. Concentrations of seven molecular species of LysoPEs and combined LysoPEs in the serum from healthy subject, SS and NASH patients. LysoPE 20:4 in the serum from both patients were undetected. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. n. s. means not significant. LysoPE, lysophosphatidylethanolamine; SS, simple steatosis; NASH, non-alcoholic steatohepatitis

**Fig. 3** Profiling of PEs in serum from healthy subjects, SS and NASH patients by using HPLC-Orbitrap MS. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. SS, simple steatosis; NASH, non-alcoholic steatohepatitis; PEs, phosphatidylethanolamines



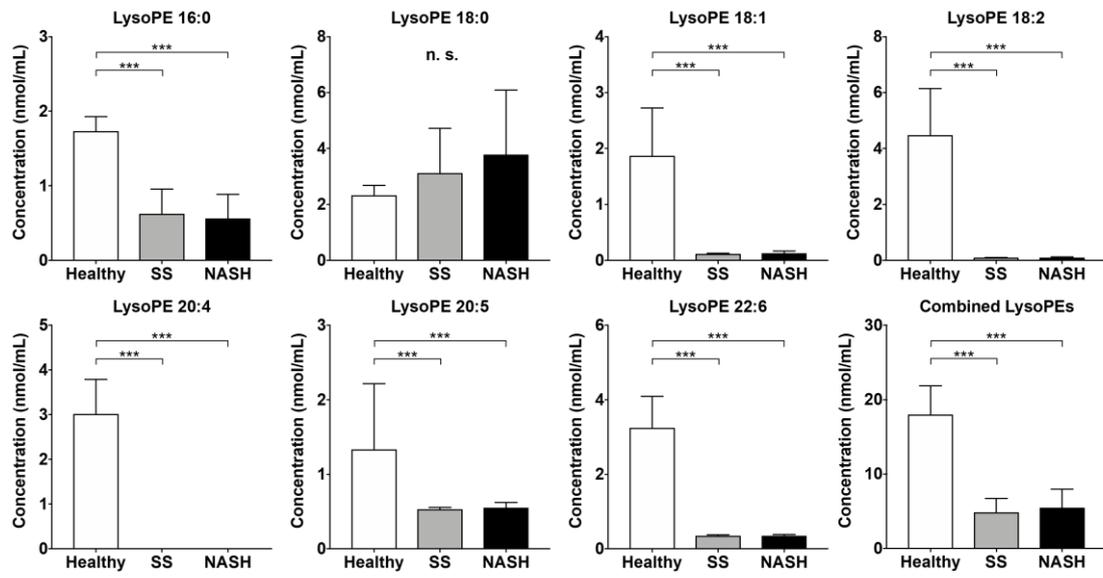


Fig. 2

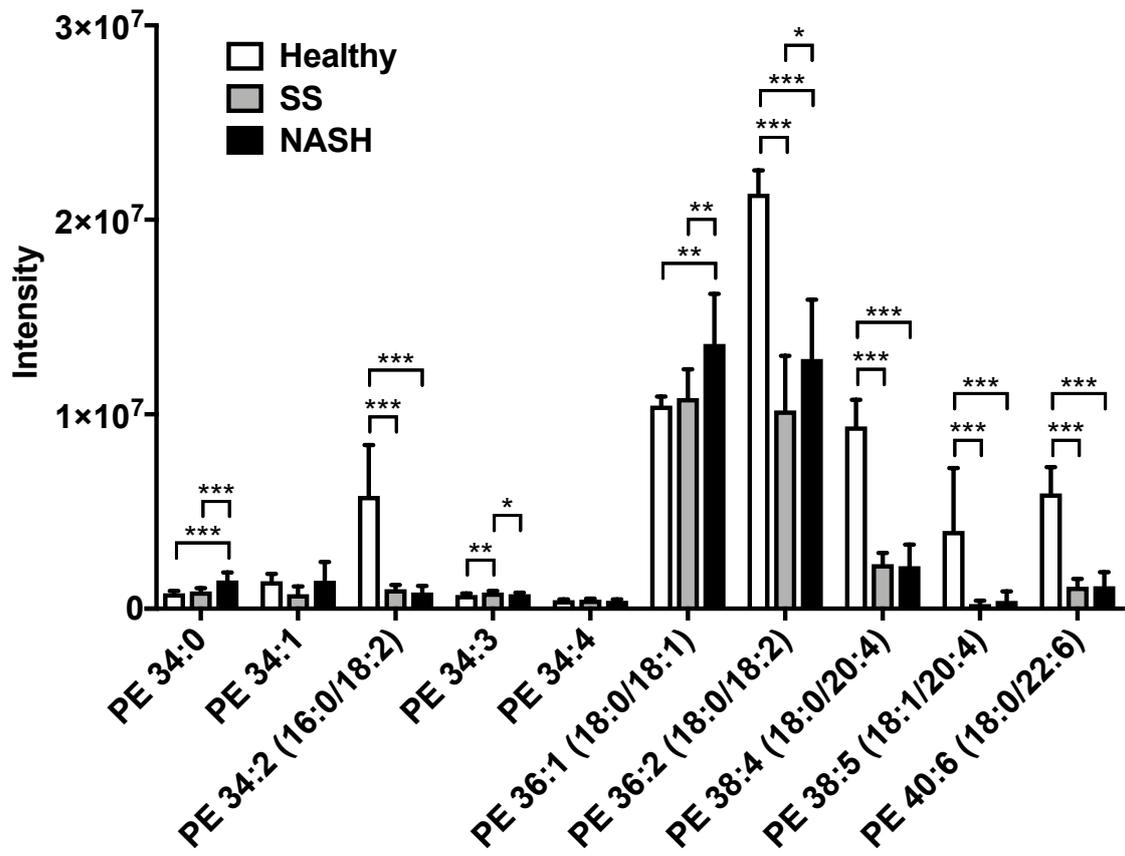


Fig. 3

**Table 1** Clinical parameters of serum samples. <sup>a</sup>Values are expressed as mean  $\pm$  SD, N. D. means not determined in this study, n. s. means not significant, SS: simple steatosis, NASH: non-alcoholic steatohepatitis, TC: total cholesterol, TG: triglyceride, PL: phospholipid, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density cholesterol, ApoAI: apoprotein AI, ApoB: apoprotein B, AST: aspartate aminotransferase, ALT: alanine aminotransferase,  $\gamma$ -GT: gamma-glutamyl transferas

	A: Healthy subjects (n = 8)	B: SS (n = 9)	C: NASH (n = 27)	A vs B	A vs C	B vs C
Age <sup>a</sup>	22.8 $\pm$ 1.7	42.8 $\pm$ 16.4	65.1 $\pm$ 14.0	$P < 0.01$	$P < 0.001$	$P < 0.001$
Female/Male	4/4	5/4	15/12	n. s.	n. s.	n. s.
TC (mg/dL) <sup>a</sup>	189.1 $\pm$ 27.7	208.7 $\pm$ 26.3	185.7 $\pm$ 30.9	n. s.	n. s.	n. s.
TG (mg/dL) <sup>a</sup>	55.6 $\pm$ 25.4	130.7 $\pm$ 80.7	166.8 $\pm$ 99.2	n. s.	$P < 0.01$	n. s.
PL (mg/dL) <sup>a</sup>	218.9 $\pm$ 18.0	225.3 $\pm$ 17.2	218.4 $\pm$ 29.9	n. s.	n. s.	n. s.
LDL-C (mg/dL) <sup>a</sup>	108.1 $\pm$ 23.4	130.1 $\pm$ 30.9	108.4 $\pm$ 29.3	n. s.	n. s.	n. s.
HDL-C (mg/dL) <sup>a</sup>	75.2 $\pm$ 4.4	62.7 $\pm$ 17.4	56.3 $\pm$ 11.5	n. s.	n. s.	n. s.
ApoAI (mg/dL) <sup>a</sup>	N. D.	150.9 $\pm$ 28.8	142.8 $\pm$ 26.4	-	-	n. s.
ApoB (mg/dL) <sup>a</sup>	N. D.	100.3 $\pm$ 19.4	88.0 $\pm$ 20.2	-	-	n. s.
AST (IU/L) <sup>a</sup>	N. D.	36.1 $\pm$ 40.9	34.8 $\pm$ 14.7	-	-	n. s.
ALT (IU/L) <sup>a</sup>	N. D.	60.9 $\pm$ 96.6	38.1 $\pm$ 23.5	-	-	n. s.
$\gamma$ -GT (IU/L) <sup>a</sup>	N. D.	64.6 $\pm$ 55.8	54.4 $\pm$ 46.2	-	-	n. s.

**Table 2** MS/MS conditions. Electrospray ionization (ESI) negative ion mode

Species	Parent ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Collision energy (V)	Tube lens (V)
LysoPE 15:0	438.3	241.2	23	87
LysoPE 16:0	452.3	255.3	28	114
LysoPE 18:0	480.3	283.3	28	142
LysoPE 18:1	478.3	281.3	28	113
LysoPE 18:2	476.3	279.3	28	106
LysoPE 20:4	500.3	303.5	23	124
LysoPE 20:5	498.3	301.2	21	147
LysoPE 22:6	524.3	327.2	21	120

**Table 3** Analysis of LOD and LOQ. <sup>a</sup>LOQ, limits of quantification, <sup>b</sup>LOD, limits of detection

Species	Equation for LysoPE measurement	R <sup>2</sup>	Range (nmol/mL)	LOQ <sup>a</sup> (nmol/mL)	LOD <sup>b</sup> (nmol/mL)
LysoPE 16:0	$y = 1.99187x - 0.179795$	0.9990	0.002-2.0	0.0010	0.0005
LysoPE 18:0	$y = 2.20326x - 0.355861$	0.9972	0.002-2.0	0.0020	0.0010
LysoPE 18:1	$y = 1.90653x - 0.0517308$	0.9997	0.002-2.0	0.0015	0.0010
LysoPE 18:2	$y = 1.3419x - 0.0332182$	0.9996	0.002-2.0	0.0020	0.0010
LysoPE 20:4	$y = 0.59105x + 0.0257216$	0.9997	0.010-2.0	0.0050	0.0020
LysoPE 20:5	$y = 0.098338x - 0.0120085$	0.9990	0.050-2.0	0.0050	0.0020
LysoPE 22:6	$y = 0.258826x - 0.0215138$	0.9993	0.010-2.0	0.0050	0.0033

**Table 4** Result of reproducibility and recovery test. <sup>a</sup>Values are expressed as mean  $\pm$  SD, <sup>b</sup>CV: coefficient of variation

Species	Reproducibility		Interday (n = 10, 3 days)		Recovery (n = 6)						
	Intraday (n = 10)		Average amount	Precision	Original amount	Expected amount	Measured amount	Precision	Recovery	Recovery range	
	Average amount (pmol) <sup>a</sup>	Precision CV <sup>b</sup> (%)	Average amount (pmol) <sup>a</sup>	Precision CV <sup>b</sup> (%)	Original amount (pmol)	Expected amount (pmol)	Measured amount (pmol) <sup>a</sup>	Precision CV <sup>b</sup> (%)	Recovery (%)	Recovery range (%)	
LysoPE 16:0	0.39 $\pm$ 0.05	12.6	0.37 $\pm$ 0.02	4.2	0.164	Low	0.254	0.255 $\pm$ 0.001	0.6	100.5	98.1-102.2
						Mid	0.344	0.341 $\pm$ 0.004	1.1	98.5	96.2-101.4
						High	0.524	0.537 $\pm$ 0.018	3.4	103.7	94.2-107.2
LysoPE 18:0	0.41 $\pm$ 0.08	8.9	0.37 $\pm$ 0.04	9.6	0.138	Low	0.228	0.222 $\pm$ 0.002	0.8	93.0	89.8-94.7
						Mid	0.318	0.324 $\pm$ 0.007	2.0	103.5	96.8-106.6
						High	0.498	0.486 $\pm$ 0.019	3.8	96.7	87.9-103.1
LysoPE 18:1	0.35 $\pm$ 0.04	12.3	0.33 $\pm$ 0.02	5.9	0.135	Low	0.225	0.225 $\pm$ 0.002	1.0	100.2	97.9-102.9
						Mid	0.315	0.309 $\pm$ 0.007	2.3	96.7	92.8-102.8
						High	0.495	0.480 $\pm$ 0.031	6.4	96.0	79.6-104.6
LysoPE 18:2	1.21 $\pm$ 0.08	6.8	1.17 $\pm$ 0.03	2.8	0.519	Low	0.869	0.846 $\pm$ 0.006	0.7	93.5	92.4-96.9
						Mid	1.219	1.254 $\pm$ 0.025	2.0	105.0	100.5-111.3
						High	1.919	1.826 $\pm$ 0.100	5.5	93.4	80.3-99.7
LysoPE 20:4	2.93 $\pm$ 0.20	6.8	2.77 $\pm$ 0.15	5.5	2.113	Low	3.363	3.389 $\pm$ 0.090	2.7	102.1	87.5-106.2
						Mid	4.613	4.568 $\pm$ 0.106	2.3	98.2	93.9-103.7
						High	7.113	7.485 $\pm$ 0.404	5.4	107.4	95.3-120.7
LysoPE 20:5	0.53 $\pm$ 0.05	10.2	0.54 $\pm$ 0.02	4.0	0.083	Low	0.173	0.175 $\pm$ 0.005	3.0	102.3	90.8-106.1
						Mid	0.263	0.272 $\pm$ 0.008	2.9	105.0	101.2-112.2
						High	0.443	0.400 $\pm$ 0.025	6.2	88.0	82.9-101.7
LysoPE 22:6	0.62 $\pm$ 0.05	7.4	0.67 $\pm$ 0.05	8.2	0.244	Low	0.394	0.374 $\pm$ 0.008	2.1	87.3	76.8-89.7
						Mid	0.544	0.577 $\pm$ 0.014	2.4	111.1	106.1-116.7
						High	0.844	0.871 $\pm$ 0.036	4.1	104.5	94.4-112.9