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Determination of plasma lysophosphatidylethanolamines (lyso-PE) by LC-MS/MS revealed a possible relation between obesity and lyso-PE in Japanese preadolescent children: the Hokkaido study

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Declarations

Conflicts of interests

The authors declare that there is no conflict of interest.

Ethics approval

This study was approved by the ethics review board of the Faculty of Health Sciences, Hokkaido University (approval number: 22–33) and the Center for Environmental and Health Sciences, Hokkaido University (approval number: 21–136).

Source of biological materials

Non-fasting plasma samples from healthy preadolescent children were provided by the participants of the Hokkaido Study on Environment and Children's Health, the Hokkaido Cohort.

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44 **Author contributions**

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53 **Data availability statement**

54 The raw data for this study are available from the corresponding author (Prof. Shu-Ping Hui,
55 keino@hs.hokudai.ac.jp) upon request.

56

57 **Supplemental Information**

58 This article contains supplemental data.

59

60 **Abbreviations**

61 CV: Coefficient of variation; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LOD:
62 Limits of detection; LOQ: Limits of quantification; Lyso-PC: lysophosphatidylcholine; Lyso-PE:
63 Lysophosphatidylethanolamine; Lyso-PL: Lysophospholipid; NAFLD: Non-alcoholic fatty liver
64 disease; NASH: Non-alcoholic steatohepatitis; MeOH: Methanol; POW: Percentage of overweight.

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Abstract (242 words)

Background: Lysophosphatidylethanolamines (lyso-PEs) are the partial hydrolysis products of phosphatidylethanolamine. Although lyso-PEs are important biomarkers in various diseases, their determination is limited by the lack of simple and efficient quantification methods. This study aims to develop an improved quantitative method for the determination of lyso-PEs and its application to an epidemiological study.

Methods: Single reaction monitoring channels by collision-induced dissociation for seven lyso-PEs were established using liquid chromatography-tandem mass spectrometry. Plasma lyso-PEs were extracted with a single-phase method using an isotopically labeled internal standard for quantification. The proposed method was adopted to define lyso-PEs in plasma samples of children aged 9–12 years living in Sapporo, Japan.

Results: The limit of detection and limit of quantification for each lyso-PE ranged between 0.001–0.015 and 0.002–0.031 pmol/ μ L, respectively. Recoveries were found to be > 91% for all the species. The analysis results of children's plasma showed that the total lyso-PE concentrations in boys (n = 181) and girls (n = 161) were 11.53 and 11.00 pmol/ μ L (median), respectively. Participants were further classified by the percentage of overweight and subgrouped as underweight (n = 12), normal range (n = 292), or overweight (n = 38). Interestingly, the reduction of lyso-PE 16:0 and increased

lyso-PE 22:6 were observed in overweight children compared with normal range (Fold change: 0.909 and 1.174, respectively).

Conclusions: This study successfully established a simple quantitative method to determine lyso-PE concentrations. Furthermore, our method revealed the possible relation between plasma lyso-PEs and overweight status.

Keywords

Childhood obesity, Lysophosphatidylethanolamine, Lysophospholipid, plasma, Liquid chromatography, mass spectrometry, Single-phase extraction.

1. Introduction

Lysophospholipids (lyso-PLs) are partially deacylated products of phospholipids produced by phospholipases A1 and A2¹. There are several types of lyso-PLs, such as lysophosphatidic acid, lysophosphatidylcholine (lyso-PC), lysophosphatidylethanolamine (lyso-PE), lysophosphatidylglycerol, lysophosphatidylinositol, and lysophosphatidylserine based on their head groups^{2, 3}. Lyso-PLs are widely distributed in the human body and mediate various physiological functions, including cell signaling, proliferation, and migration^{4, 5}. Lyso-PE is the predominant type of lyso-PL following lyso-PC⁶. Lyso-PE has been reported to have bioactivities, such as stimulation of intracellular Ca²⁺ concentrations⁷, neurite outgrowth stimulation⁸, activation of mitogen-activated protein kinase signaling⁹, and anti-inflammatory effects by inhibiting M1 macrophage polarization^{10, 11}. Moreover, our previous study suggested that lyso-PE was associated with metabolic dysfunction in the liver¹². There are several reports on the relation between lyso-PEs and various diseases. Lyso-PE concentrations were decreased in the serum of patients with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) than in healthy individuals^{13,14}. In addition, several lyso-PE species were decreased and increased in the serum of patients with type 2 diabetes (T2D)^{15,16}. Lyso-PLs, such as lyso-PC and lyso-PE, seem to be associated with glucose-mediated insulin secretion in diabetes targeting tissues¹⁷. Higher concentrations of these lyso-PLs may induce

insulin resistance and islet dysfunction¹⁸. The relation between the alternation of lyso-PEs and diseases has been controversial. However, these findings suggest that lyso-PE is a potential biomarker for lifestyle-related diseases.

To date, semi-quantitative methods have been used for the quantification of lyso-PEs owing to the lack of authentic standards^{19, 20}. In a previous study, we developed an absolute quantitative method for the determination of lyso-PE species using liquid chromatography-tandem mass spectrometry (LC-MS/MS), enabling the detection of small amounts of lyso-PE species in clinical samples¹⁴. However, this method is time-consuming for largescale studies due to its complex extraction process, and the odd-chain internal standard used for quantification may overlap with endogenous species. Herein, we developed an improved quantitative method for seven major lyso-PE species (lyso-PE 16:0, 18:0, 18:1, 18:2, 20:4, 20:5, and 22:6) using LC-MS/MS with modifications to our previous method¹⁴. The isotopically labeled internal standard, lyso-PE 18:1 (d7), was used for quantification.

Dysregulated lipid metabolism in childhood obesity is known to cause various lifestyle-related diseases, such as T2D and NAFLD²¹⁻²³. The prevention and treatment of childhood obesity are essential for reducing the risk of various diseases in adults. This requires a whole-system approach based on the mechanisms underlying childhood obesity development²⁴. Moreover, its elucidation

needs to reveal the lipid profiles in healthy pediatric plasma. However, the absolute concentrations of lipids, such as lyso-PE, in pediatric plasma have not been explored. Hence, after validation, the proposed method was applied to plasma samples from healthy preadolescent children living in Sapporo, Hokkaido, Japan.

2. Material and Methods

2.1. Materials

LC-MS-grade methanol (MeOH) and acetonitrile were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). LC-MS-grade isopropanol and CHCl_3 were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. Ammonium acetate (1 mol/L solution) and ethylenediaminetetraacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,6-di-tert-butyl-*p*-cresol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Deuterated lyso-PE (lyso-PE 18:1[d7]), used as the internal standard, was purchased from Avanti Polar Lipids, Inc. (AL, USA). The lyso-PE standards analyzed in this study included 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 16:0), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 18:0), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 18:1), 1-linoleoyl-2-hydroxy-*sn*-glycero-3-

phosphoethanolamine (lyso-PE 18:2), 1-arachidonyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 20:4), 1-eicosapentaenoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 20:5), and 1-docosahexaenoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 22:6). Lyso-PE 16:0, 18:0, and 18:1 were purchased from Avanti Polar Lipids, Inc. (AL, USA), while other lyso-PE species were synthesized in-house¹⁴.

2.2. Lyso-PE extraction

150 μ L of MeOH and 50 μ L of internal standard (lyso-PE 18:1[d7], 0.42 μ mol/L) in MeOH were added to 10 μ L of plasma in a 1.5 mL Eppendorf. Subsequently, 100 μ L of CHCl₃ and 20 μ L of Milli-Q (ultrapure) were added to the mixture and vortexed at 3,500 rpm for 10 min (MSV-3500, Funakoshi Co., Ltd., Tokyo, Japan). After centrifugation at 15,000 rpm at 4°C for 10 min (Tabletop Micro Refrigerated Centrifuge Model 3520, KUBOTA Co., Ltd., Tokyo, Japan), the single-phase centrifuge was transferred to a new vial and evaporated under vacuum at 4°C with a centrifugal concentrator (CVE-3000, EYELA Co., Ltd., Tokyo, Japan). The dried lipid extracts were redissolved in 100 μ L of MeOH and vortexed for 1 min. Following that, the extracts were centrifuged at 15,000 rpm at 4°C for 10 min to remove any insoluble compounds and then transferred to LC-MS vials. All MeOH used for the extraction contained 0.001% 2,6-di-tert-butyl-*p*-cresol to prevent lipid oxidation.

2.3. Analysis of lyso-PE species by targeted LC-MS/MS

The LC-MS/MS analysis was performed using a modified version of our previously described method¹⁴. Single reaction monitoring channels by collision-induced dissociation for each lyso-PE species were established using a TSQ Quantum Access mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in the electrospray ionization negative ion mode. The optimized ion source parameters were set as follows: spray voltage: 3,000 V; vaporizer temperature: 300°C; capillary temperature: 200°C; sheath gas (nitrogen) pressure: 30 psi; ion sweep gas (nitrogen) pressure: 4 psi; auxiliary gas (nitrogen) pressure: 35 psi. The single reaction monitoring channels obtained for each lyso-PE species are presented in Supplemental Information Table S1.

The high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) with a Hypersil GOLD column (50 × 2.1 mm, 5.0 μm, Thermo Fisher Scientific Inc., MA, USA) was used for lyso-PE separation. The oven temperature was maintained at 45°C, and the sample tray was kept at 4°C. Briefly, 5 μL of the sample was injected into the equipment. Chromatographic separation was performed using a mobile phase consisting of 60% Milli-Q, 20% MeOH, and 20% acetonitrile with 5 mmol/L aqueous ammonium acetate containing 500 nmol/L EDTA (A), and isopropanol (B). The gradient at a flow rate of 0.4 mL/min was set as follows: 0–1 min (80% A, 20% B); 1–3 min (gradually

changed to 40% A, 60% B); 3–5 min (40% A, 60% B); 5–6 min (gradually changed to 0% A, 100% B); 6–7 min (0% A, 100% B); 7–10 min (80% A, 20% B).

2.4. Method validation

To evaluate the linearity of this method, a series of diluted lyso-PE standard solutions were prepared at final concentrations of 0.001, 0.002, 0.004, 0.007, 0.015, 0.031, 0.063, 0.125, 0.25, 0.5, 1, and 2 pmol/ μ L with a constant amount of internal standard (lyso-PE 18:1[d7]; final concentration: 0.21 μ mol/L). The limits of detection (LOD) and limits of quantification (LOQ) were evaluated at signal-to-noise ratios of 3 and 10, respectively. The recovery and matrix effect of the method were confirmed by spiking the known concentration of lyso-PE standards (Low: 0.5 pmol/ μ L; Medium: 1.0 pmol/ μ L; High: 2.0 pmol/ μ L) into 10 μ L human plasma (n = 6 replicates). Recovery was calculated as the ratio of the area of (spiked sample) to the area of (spiked extract). The matrix effect was calculated as the ratio of the area of (spiked extract) to the area of (standard).

2.5. Field validation

Plasma samples from non-fasting individuals were provided by the participants of the Hokkaido Study on Environment and Children's Health, the Hokkaido Cohort²⁵⁻²⁸ and stored at -80°C.

Selection of the participants to collect samples was written elsewhere^{29, 30}. The participants were healthy preadolescent children aged 9–12 years (n = 342 volunteers, boys = 181, girls = 161) living in Sapporo City in Hokkaido, Japan. The height and weight of the children were measured at pediatric clinics on the same day of blood collection. The research objectives and methods were explained to the participants, and written informed consent was obtained from all parents, as well as an informed assent from all children.

GraphPad Prism 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA) was used as the software for all statistical analysis. Nonparametric Mann–Whitney U-test was used for the comprehensive analysis of lyso-PE concentration between boys and girls. For comparison involving three or more groups, the nonparametric Kruskal-Wallis test was used. Statistical significance was set at $p < 0.05$. The results of lyso-PE concentrations were expressed as median (interquartile range).

2.6. Definitions

Body mass index (BMI) was calculated as weight (kg) / height squared (m^2). Under BMI, underweight, normal range, and overweight were defined (according to the cut-off points) as less than 18.5 kg/m^2 , 18.5–25.0 kg/m^2 , and more than 25.0 kg/m^2 , respectively³¹. Percentage of overweight (POW) is based on age- and sex-specific standard body weight for height and is used to define

childhood obesity in Japan³². POW was calculated as [measured weight (kg) – standard weight (kg) / standard weight (kg)] × 100³³. According to POW, children with POW < -20% and children with POW ≥ +20% were classified as underweight and overweight, respectively. Children with POW between these cutoff points were classified as a normal range^{33,34}. In this study, obesity was included in overweight.

3. Results

3.1. Method validation (linearity, sensitivity, separation, and extraction of lyso-PEs)

Under optimized conditions, the linearity, LOD, and LOQ of each lyso-PE species were evaluated (Supplemental Information Table S2). For all lyso-PE standards, the R^2 was > 0.9936, indicating good linearity. The LOD and LOQ for lyso-PEs ranged from 0.001 to 0.015 pmol/μL and 0.002 to 0.031 pmol/μL, respectively.

Lyso-PEs in plasma samples were extracted using a single-phase extraction method, as shown in Figure 1a. Separation of the lyso-PE species was achieved in approximately 4 min under optimized conditions. Symmetrical peaks were obtained for each lyso-PE species in both standard and plasma samples (Figure 1b). All lyso-PE standards were spiked into the plasma, and the recovery, matrix effects, and reproducibility were evaluated (Supplemental Information Table S3). The

recoveries of all lyso-PE species ranged from 91.2% to 120.3%, with coefficients of variation (CVs) within 10%, except for lyso-PE 20:5 (approximately 16.9%). The matrix effect for all lyso-PE species ranged from 61.6% to 96.5%, with CVs within 15%. The CVs of intra-day and inter-day assays for most lyso-PE species at different concentrations were within 15%.

3.2. Determination of plasma lyso-PE concentrations in preadolescent children

After validating the method, it was applied to determine the plasma lyso-PE concentrations in preadolescent children. The participants' characteristics are shown in Table 1. The mean height, weight, BMI, and POW were depicted according to sex and age. The mean BMI of boys and girls ranged from 17.4 ± 2.5 to 19.4 ± 3.3 kg/m² and from 16.2 ± 2.2 to 18.4 ± 2.6 kg/m², respectively. The mean POW of boys and girls ranged from 1.40 ± 13.56 to $5.12 \pm 17.81\%$ and from -6.83 ± 10.05 to $0.07 \pm 14.04\%$, respectively. Regarding boys, both the BMI and POW increased with age. The number of children categorized according to BMI and POW is shown in Table 2. The number of overweight children classified by BMI was small in both boys and girls (boys, n = 6; girls, n = 2), whereas the number of underweight children was the largest among the three categories (boys, n = 119; girls, n = 120). In contrast, the number of children classified as normal range by POW was large for both boys and girls (boys: n = 150; girls: n = 142).

The proposed method detected seven lyso-PE species in children's plasma samples except for lyso-PE 20:5. Regarding lyso-PE 20:5, it was not detected in 48 out of 342 samples (detection percentage above LOD: 86.0%). The plasma lyso-PE concentrations were compared between boys and girls (Table 3). The concentrations of lyso-PE species in boys and girls ranged from 0.91 to 3.64 pmol/ μ L and from 0.82 to 3.24 pmol/ μ L, respectively. The total lyso-PE represents the combined lyso-PE concentrations of the seven species. The total lyso-PE concentrations for boys and girls were 11.53 pmol/ μ L and 11.00 pmol/ μ L, respectively. The concentrations of lyso-PE 20:4, 20:5, and 22:6 were significantly higher in boys than in girls.

Differences in lyso-PE concentrations according to age were also evaluated (Figure 2). The total lyso-PE concentrations in all children of each age (9, 10, 11, 12 years old) were 11.05 (8.60–13.48) pmol/ μ L, 11.69 (9.38–13.32) pmol/ μ L, 11.59 (9.88–13.75) pmol/ μ L, and 10.47 (8.40–13.15) pmol/ μ L, respectively. Results are expressed as median (interquartile range). There were no significant differences in the concentrations of any of the lyso-PE species according to age.

The relative abundance of lyso-PE species in plasma samples from children was confirmed (Figure 3). Lyso-PE 18:2 was the most abundant species in plasma samples from children. Lyso-PE 18:2 accounted for $30.1 \pm 5.5\%$, followed by lyso-PE 20:4 of $14.5 \pm 2.9\%$, lyso-PE 22:6 of $13.6 \pm 3.7\%$, lyso-PE 18:1 of $12.5 \pm 2.9\%$, lyso-PE 16:0 of $11.3 \pm 2.2\%$, lyso-PE 18:0 of $10.9 \pm 2.1\%$, and

lyso-PE 20:5 of $7.2 \pm 5.3\%$ (mean $\pm$ standard deviation).

To explore the relation between plasma lyso-PE concentrations and childhood obesity, the differences in lyso-PE concentrations among the subgroups (underweight, normal range, and overweight) according to POW were confirmed. The total lyso-PE concentration in the plasma samples was found to be 11.26 (9.44–13.27) pmol/ μ L in underweight, 11.30 (8.97–13.67) pmol/ μ L in normal range, 10.69 (8.29–12.71) pmol/ μ L in overweight children (Figure 4). There were no significant differences in almost all lyso-PE concentrations, including total lyso-PE concentrations, among the groups. In addition, no significant correlation was observed between lyso-PE concentrations and POW (data not shown). However, the plasma lyso-PE 16:0 concentrations were significantly lower in overweight children than in normal range children ($p = 0.048$). The concentrations of lyso-PE 16:0 in underweight, normal range, and overweight children were 1.40 (1.07–1.62) pmol/ μ L, 1.22 (1.02–1.48) pmol/ μ L, and 1.11 (0.90–1.30) pmol/ μ L, respectively. Moreover, lyso-PE 22:6 was increased in overweight children compared with normal range children ($p = 0.034$). The concentration of lyso-PE 22:6 in underweight, normal range, and overweight children were 1.60 (1.09–1.69) pmol/ μ L, 1.45 (1.12–1.78) pmol/ μ L, 1.70 (1.33–1.92) pmol/ μ L, respectively.

4. Discussion

283 In this study, we developed a simple and sensitive method for the quantitative determination
284 of lyso-PE species by modifying a previously reported method¹⁴. We improved the recovery and
285 sensitivity of our method by modifying the extraction method and changing the internal standard. In
286 our previous study, odd-chain lyso-PE (lyso-PE 15:0) was used as an internal standard for
287 quantification¹⁴. However, odd-chain fatty acids can exist endogenously in human plasma at low
288 concentrations and may significantly interfere with analysis³⁵. In this method, deuterated lyso-PE
289 (lyso-PE 18:1[d7]) was used as an internal standard to quantitate the analytes with higher accuracy
290 and precision. For lipid extraction, we modified the previous method to a simpler one using a single-
291 phase method. In this method, the extraction efficiency was improved by introducing a single-phase
292 extraction technique and reducing the extraction time by approximately half compared with our
293 previous method¹⁴. The recovery rate for all lyso-PE species was greater than 90% with low CVs, and
294 the matrix effect ranged from 61.6% to 96.5% (Supplemental Information Table S3), suggesting that
295 our methodology is suitable for minimizing the ion suppression and enhancement by matrix effects.
296 On the other hand, our previous method with the more complex pretreatment shows that the recoveries
297 for all the lyso-PE range from 87.3 to 111.1%, of CVs within 6.4%¹⁴. It is suggested that our new
298 extraction method is non-inferior to the previous one regardless of a simple single-phase method. The
299 CVs of the method were less than 15% when evaluated with authentic standards. We also monitored

LC-MS system conditions by injecting lyso-PE standards before and after injecting plasma samples. However, between the sample batches, the quality controls were not injected, which is one of the limitations of this study. The data of LOD and LOQ (0.001–0.015 pmol/ μ L, 0.002–0.031 pmol/ μ L, respectively) showed that the proposed method had high sensitivity enough to detect a small amount of lyso-PE species in human plasma samples. There are several reports on the alternation of serum/plasma lyso-PE concentrations in several diseases using LC-MS¹³⁻¹⁶. However, semi-quantitative analysis was utilized in most of them except for our present and previous reports¹⁴ (Table 4). Our proposed quantitative method for lyso-PE species could be useful to elucidate the relation between lyso-PE concentrations and diseases.

We established LC-MS/MS conditions to separate the lyso-PE species but not the regioisomers. Lyso-PEs have one acyl chain attached to either the *sn*-1 or *sn*-2 position of glycerol. Research on the fatty acid distribution of lyso-PLs revealed that saturated fatty acids were found mainly at the *sn*-1 position, and polyunsaturated fatty acids were detected mainly at the *sn*-2 position³⁶. However, both saturated fatty acid-containing lyso-PEs and polyunsaturated fatty acid-containing lyso-PEs have an acyl chain at the *sn*-1 position in the plasma and serum, because *sn*-2 types of lyso-PEs are quickly converted to more stable *sn*-1 types of lyso-PEs in an aqueous environment^{6, 36}. Therefore, we established a targeted LC-MS/MS method using *sn*-1 lyso-PE standards that did not

distinguish *sn*-1 from *sn*-2 in the samples. Under LC-MS/MS conditions, a single sharp peak was observed for each lyso-PE species in both the standard and plasma samples (Figure 1b), showing clean separation. However, it is necessary for the quantification to consider the natural isotopes because the retention time was similar among the species whose *m/z* were close, such as lyso-PE 18:0, 18:1, and 18:2. Theoretically, one lyso-PE species has several natural isotopes because it can be replaced by a rare stable isotope. Our method with the triple quadrupole mass spectrometer can reduce the interference of a natural isotope with the detection of another species, but cannot eliminate it. However, our results indicated that the proposed method has a high sensitivity for each lyso-PE species in clinical samples in which there is a less natural isotope.

We revealed the profiling of plasma lyso-PE concentrations in preadolescent children using a newly established method. First, we confirmed the difference in lyso-PE concentrations between boy and girl participants. The concentrations of several lyso-PE species were significantly higher in boys than in girls (Table 3), indicating that plasma lyso-PE concentrations are associated with sex. To date, there have been no reports comparing plasma lyso-PE concentrations in boys and girls. To the best of our knowledge, this is the first study to reveal sex-related differences in lyso-PE concentrations. The association between the bioactivities of lyso-PEs and sex differences needs to be investigated in the future.

Second, we confirmed differences in the concentrations and composition of lyso-PEs according to age. As shown in Figure 2, there was no significant difference in lyso-PE concentrations among all age groups. In a previous study, we determined the composition of serum lyso-PEs in adults³⁷. The relative abundance of lyso-PE in plasma samples from children was evaluated and compared with that in adults (Figure 3). Our previous research revealed that lyso-PE 18:2 accounted for $24.1 \pm 5.2\%$, followed by lyso-PE 20:4, lyso-PE 18:0, lyso-PE 22:6, lyso-PE 16:0, lyso-PE 18:1, and lyso-PE 20:5³⁷. Compared with that in adults, the concentration of lyso-PE 18:2 was slightly higher in children ($30.1 \pm 5.5\%$). Lyso-PE 18:2 and lyso-PE 20:4 were the dominant species, and lyso-PE 20:5 was the least abundant among all the lyso-PE species in both children and adults. These results suggest that the concentrations and composition of lyso-PE species in children are similar to those in adults. However, the data about the lyso-PE concentration in adults and children was obtained from different methods. Further research on plasma lyso-PE in adults using the new method are needed to reveal the appropriate difference between adults and children.

Finally, we explored the association between lyso-PEs and obesity. The children were classified by two cut-offs, BMI and POW, and the concentrations of lyso-PEs in the plasma were compared among the subgroups (underweight, normal range, and overweight). In the previous research, lyso-PE concentrations were inversely associated with BMI in the plasma of adult

participants³⁸. However, in this study, no significant correlation was observed between the lyso-PE concentrations and BMI in preadolescent children (data not shown). It was difficult to compare the three groups using statistical analysis because of the small sample size of overweight children categorized by BMI. We evaluated the difference in lyso-PE concentration according to age within the same BMI groups, but there was no significant difference (Supplemental Information Table S4). To date, there is no gold standard for evaluating childhood obesity³⁹. Although BMI is used for the definition of childhood obesity²⁴, it has not reached a consensus. In Japan, POW is used to define overweight children³². Several reports suggest that POW seems to be more appropriate than BMI for the evaluation of childhood obesity because BMI dynamically changes in childhood^{32, 40}. In addition, it is suggested that POW is associated with biochemical parameters related to childhood obesity, such as hypertension⁴¹ and the reduced high-density lipoprotein-cholesterol concentration⁴². Therefore, we also confirmed the relation between the lyso-PE concentrations and POW. As shown in Figure 4, lyso-PE 16:0 was significantly lower in overweight children than in normal range (Fold change: 0.909), whereas lyso-PE 22:6 was significantly higher in overweight compared with normal range (Fold change: 1.174). Reports have revealed that serum lyso-PE concentrations are lower in patients with NAFLD and NASH than in healthy individuals^{13, 14}. We also found that lyso-PE concentrations were decreased in the livers of NASH mice⁴³. Furthermore, lyso-PE 18:2 concentration was decreased in

patients with T2D¹⁵, whereas lyso-PE 16:0, 18:0, and 20:1 were increased in patients with T2D and diabetes nephropathy¹⁶. Lyso-PEs have also been reported to be potential biomarkers of other diseases^{44, 45} (Table 4). Both NAFLD and T2D are often accompanied by metabolic syndromes, including obesity⁴⁶. Overweight in children, especially in adolescence, leads to metabolic disorders, such as increased fasting glucose, impaired glucose tolerance, T2D, and NAFLD²¹⁻²⁴ when they become adults. Our findings suggest that reduced plasma lyso-PE 16:0 and increased lyso-PE 22:6 concentrations in overweight children may be associated with lifestyle-related diseases, such as T2D and NAFLD, in adults. So far, the study of plasma lipid profiling in children has been limited because it is difficult to collect the samples from healthy children. In the previous research, several lyso-PE concentrations positively correlated with age in Alström syndrome/Bardot-Biedl syndrome patients⁴⁷. However, significant correlations were not observed between lyso-PE concentrations and age in healthy preadolescent children. Further research on plasma lyso-PE concentrations in different ages, such as infants and geriatric participants, is required to reveal the correlations between lyso-PEs and age. The understanding of the changes associated with growth and senescence in lyso-PE concentrations could contribute to finding new insight into the bioactivities of lyso-PEs. Our research on the absolute plasma lyso-PE concentrations in preadolescent children may lead to the prediction of lifestyle-related disease onset in adults and new treatment approaches in the future.

This study had some limitations. Although we have improved our sample pre-treatment procedure, we have not performed a comparative investigation with other complex pretreatment procedures but only compared the previous pre-treatment method. The LC-MS system conditions were monitored by injecting but lyso-PE standards not the quality controls during the measurement. The interference of the natural isotope of lyso-PEs with the triple quadrupole mass spectrometer could not be eliminated. The association between plasma lyso-PE concentrations and overweight status was evaluated without adjustment for confounding factors, including age, and sex. We measured the concentrations of lyso-PE species in non-fasting plasma samples. Moreover, the number of overweight children was small in this study. Further investigation is required to confirm the relation between lyso-PE species and food intake using fasting plasma samples with more sample number of overweight participants.

5. Conclusions

We developed a more robust, simple, and sensitive quantitative method for the determination of lyso-PE species using LC-MS/MS than that used in our previous method. The proposed method was applied to plasma samples from healthy preadolescent children and it revealed that the concentrations of several lyso-PE species were significantly higher in boys than in girls. In

402 addition, our findings suggest that plasma lyso-PE concentrations are associated with overweight
403 status. A further investigation targeting a larger number of overweight children is required to
404 determine the association between lyso-PEs and obesity. To the best of our knowledge, this is the first
405 study to reveal the profile of plasma lyso-PE concentrations in Japanese preadolescent children. This
406 study may contribute to the elucidation of the relation between lyso-PEs and childhood obesity.

6. References

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Table 1. Characteristics of participants.

	Age (years old)	Height (cm)	Weight (kg)	^a BMI (kg/m ²)	^b POW (%)
Boys	9 (n = 68)	134.9 ± 5.0	31.8 ± 6.1	17.4 ± 2.5	1.40 ± 13.56
(n = 181)	10 (n = 63)	139.0 ± 6.4	35.0 ± 8.3	17.9 ± 3.3	2.31 ± 16.90
	11 (n = 20)	148.3 ± 6.5	41.8 ± 8.1	19.0 ± 3.3	2.69 ± 18.54
	12 (n = 30)	150.6 ± 9.4	44.2 ± 9.8	19.4 ± 3.3	5.12 ± 17.81
Girls	9 (n = 46)	136.1 ± 5.7	30.2 ± 5.9	16.2 ± 2.2	-5.18 ± 11.72
(n = 161)	10 (n = 70)	140.4 ± 7.0	34.6 ± 8.1	17.4 ± 2.8	0.07 ± 14.04
	11 (n = 16)	151.0 ± 5.0	39.5 ± 5.8	17.3 ± 2.0	-6.83 ± 10.05
	12 (n = 29)	151.5 ± 6.5	42.6 ± 8.4	18.4 ± 2.6	-2.75 ± 12.69

Results are expressed as mean ± standard deviation. ^aBMI, body mass index; ^bPOW, percentage of overweight.

Table 2. The number of children categorized by BMI and POW.

Boys (n = 181)

Age (years old)	^a BMI			^b POW		
	Underweight	Normal Range	Overweight	Underweight	Normal Range	Overweight
9	49	19	0	2	57	9
10	45	15	3	0	53	10
11	11	8	1	0	16	4
12	14	14	2	1	24	5

Girls (n = 161)

Age (years old)	BMI			POW		
	Underweight	Normal Range	Overweight	Underweight	Normal Range	Overweight
9	41	5	0	3	42	1
10	52	17	1	3	60	7
11	12	4	0	2	14	0
12	15	13	1	1	26	2

555 ^aBMI, body mass index; ^bPOW, percentage of overweight. Underweight: Children with POW < -20%;
556 Normal range: Children with POW between -20% and 20%; Overweight: Children with POW $\geq$
557 +20%.

Table 3. Comparison of lyso-PE concentrations between boys and girls.

Species	Boys (pmol/ μ L)	Girls (pmol/ μ L)	P value
Lyso-PE 16:0	1.21 (1.05–1.49)	1.19 (0.96–1.43)	n.s.
Lyso-PE 18:0	1.19 (1.00–1.41)	1.18 (0.98–1.36)	n.s.
Lyso-PE 18:1	1.45 (1.05–1.98)	1.32 (0.90–1.83)	n.s.
Lyso-PE 18:2	3.64 (2.60–4.55)	3.24 (2.43–4.35)	n.s.
Lyso-PE 20:4	1.61 (1.32–2.03)	1.50 (1.25–1.84)	0.027
Lyso-PE 20:5	0.91 (0.68–1.24)	0.82 (0.00–1.10)	0.014
Lyso-PE 22:6	1.54 (1.24–1.89)	1.38 (1.03–1.70)	0.002
Total lyso-PE	11.53 (9.46–14.15)	11.00 (8.56–12.59)	0.010

Results are expressed as median (interquartile range). Nonparametric Mann–Whitney U-test was used for data comparisons. n.s. not significant.

Table 4. Alteration of serum/plasma ^alyso-PE concentrations in several diseases measured by LC-MS.

Authors (reference)	Disease	Age (years old)	n	Calibration	Specified lyso-PE	Fold change (Disease/Control)
Yamamoto et al., 2021 ¹⁴	^b NASH ^c SS	Control: 22.8 ± 1.7	8	Lyso-PE 15:0	Lyso-PE 16:0	Total lyso-PE
		NASH: 65.1 ± 14.0	27	Internal standard	Lyso-PE 18:0	NASH: 0.305
		SS: 42.8 ± 16.4	9		Lyso-PE 18:1	SS: 0.270
					Lyso-PE 18:2	
					Lyso-PE 20:4	
		Lyso-PE 20:5				
		Lyso-PE 22:6				
Tiwari-Heckler et al., 2018 ¹³	^d NAFL NASH	Control; 39.4 ± 2.6	28	^h Lyso-PC 17:0	not specified	Total lyso-PE
		NAFL: 46.4 ± 2.9	25	ⁱ PC 14:0/14:0		NAFL: down
		NASH: 43.7 ± 2.2	42	^j PE 12:0/12:0		(p < 0.05)
				Ceramide 17:0		NASH: down
				Internal standard	(p < 0.05)	
García-Fontana et al., 2016 ¹⁵	^e T2D	Control: 57 ± 5	10	-	not specified	Lyso-PE 18:2
		T2D+ ^g CVD: 58 ± 4	10			T2D+CVD: 0.505
		T2D-CVD: 55 ± 8	10			T2D-CVD: 0.664
Xu et al., 2021 ¹⁶	T2D	Control: 34.47 ± 9.2	59	-	not specified	Lyso-PE 16:0: 1.580
		T2D: 56.65 ± 10.9	60			Lyso-PE 18:0: 2.006
						Lyso-PE 20:1: 2.927
Wawrzyniak et al., 2023 ³⁸	^f PAH	Control: 44.0 ± 16.4	36	1-(4- fluorobenzyl)-5- oxoproline Internal standard	not specified	Lyso-PE 22:6: 0.29
		PAH: 46 ± 18	43			Lyso-PE 18:2: 0.06
						Lyso-PE 18:0: 0.19
						Lyso-PE 20:4: 0.07
						Lyso-PE 20:1: 0.08
						Lyso-PE 20:0: 0.08
Kierath et al., 2023 ³⁹	Childhood burn injury	Control (boys): 6.1 ± 0.9	8	lyso-PE	not specified	Lyso-PE 18:1: 1.704
		Control (girls): 6.0 ± 1.0	13	18:1(d7)		Lyso-PE 18:0: 1.455
		Burn (boys): 6.2 ± 1.0	15	Internal standard		
		Burn (girls): 5.7 ± 0.9	18			

562 ^aLyso-PE, lysophosphatidylethanolamine; ^bNASH, non-alcoholic steatohepatitis; ^cSS, simple
563 steatosis; ^dNAFL, non-alcoholic fatty liver; ^eT2D, type 2 diabetes; ^fPAH, pulmonary arterial
564 hypertension; ^gCVD, cardiovascular disease; ^hLyso-PC: lysophosphatidylcholine; ⁱPC:
565 phosphatidylcholine; ^jPE: phosphatidylethanolamine. Results are expressed as mean ± standard
566 deviation.

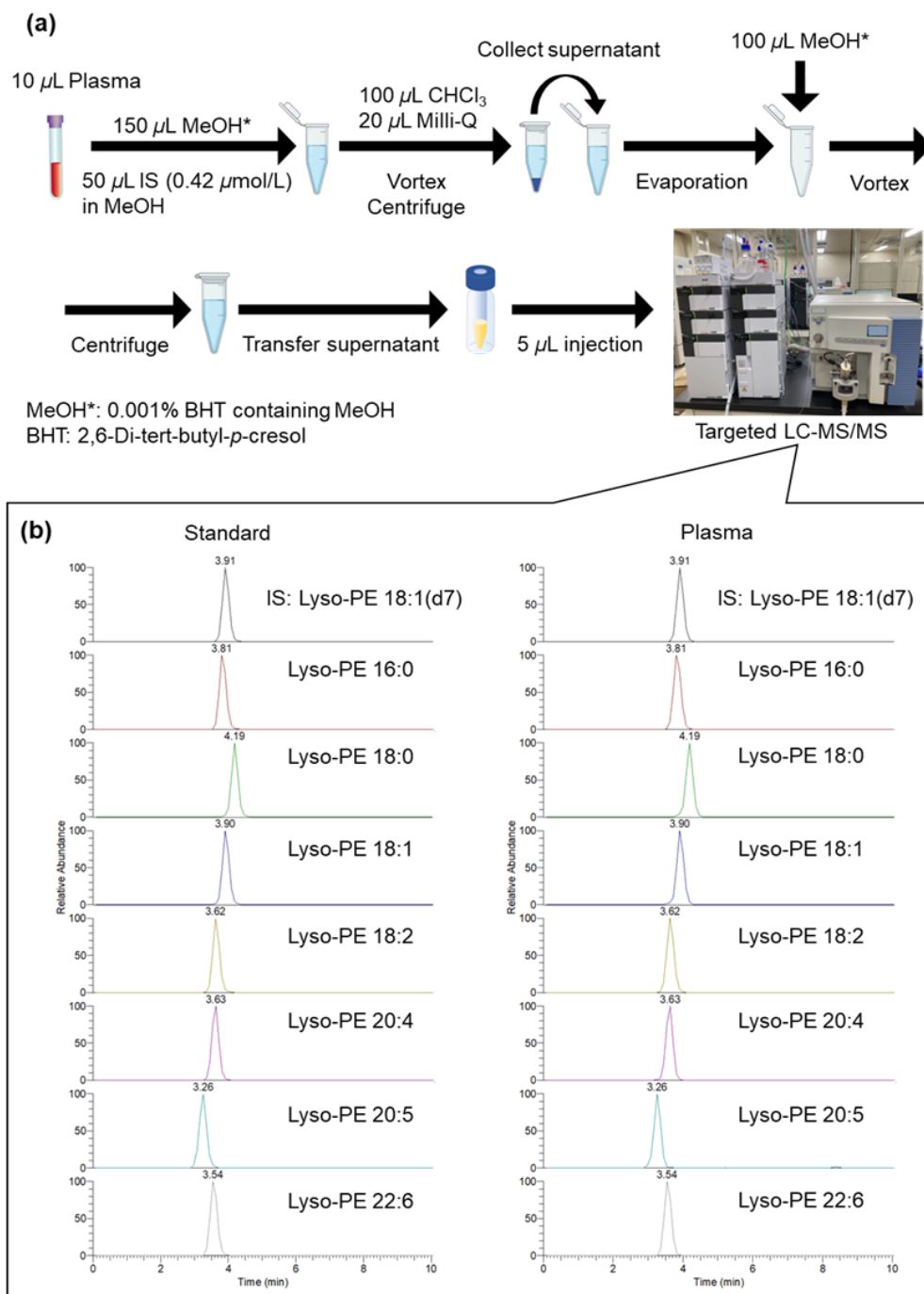


Figure 1. Workflow of the proposed method from sample preparations to liquid chromatography-tandem mass spectrometry analysis. (a) Lipid extraction by single-phase method. (b) Single reaction monitoring chromatograms of internal standard (IS) and lysophosphatidylethanolamine (lyso-PE) standards in a standard and plasma sample.

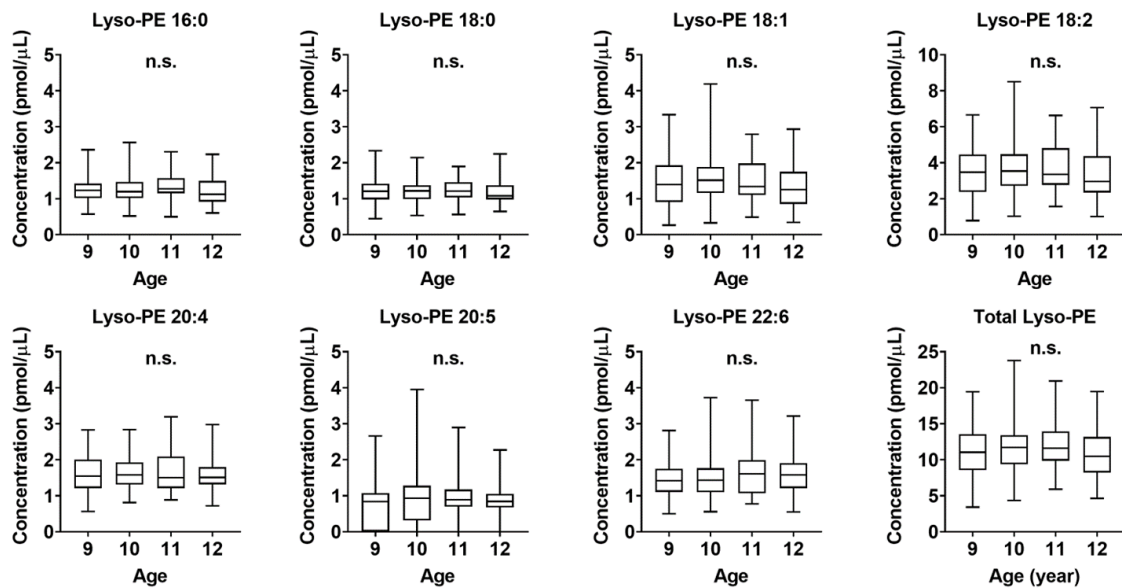
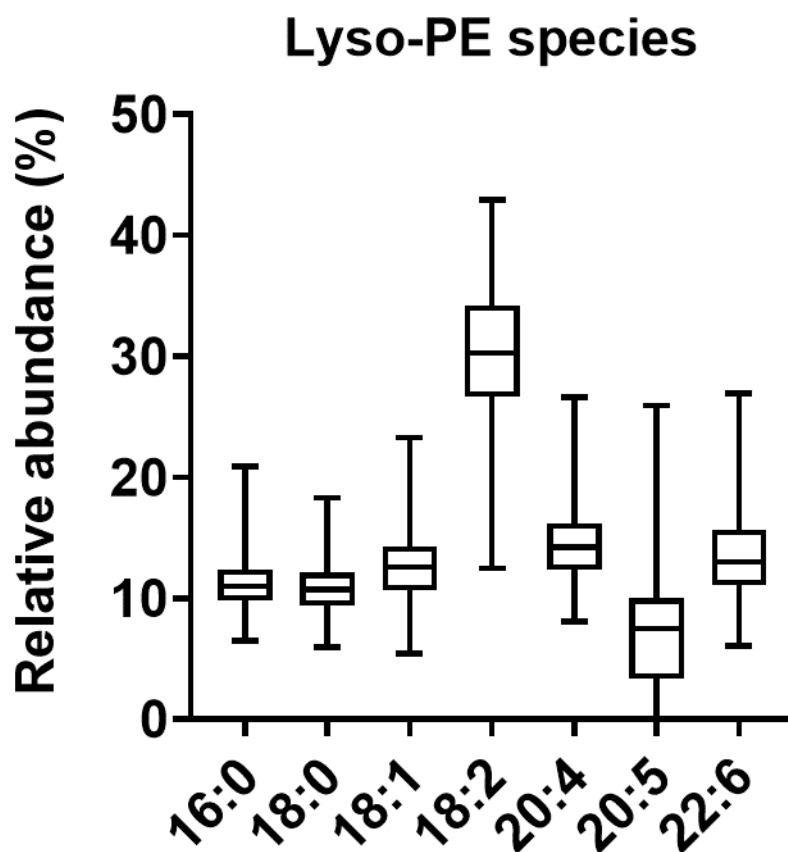


Figure 2. Box plots of each lysophosphatidylethanolamine concentration in the plasma of preadolescent children aged from 9 to 12 years old. n.s., not significant; Lyso-PE, Lysophosphatidylethanolamine.



575 **Figure 3.** Relative abundance of lysophosphatidylethanolamine species in plasma samples of
 576 preadolescent children (n = 342). Lyso-PE, Lysophosphatidylethanolamine.

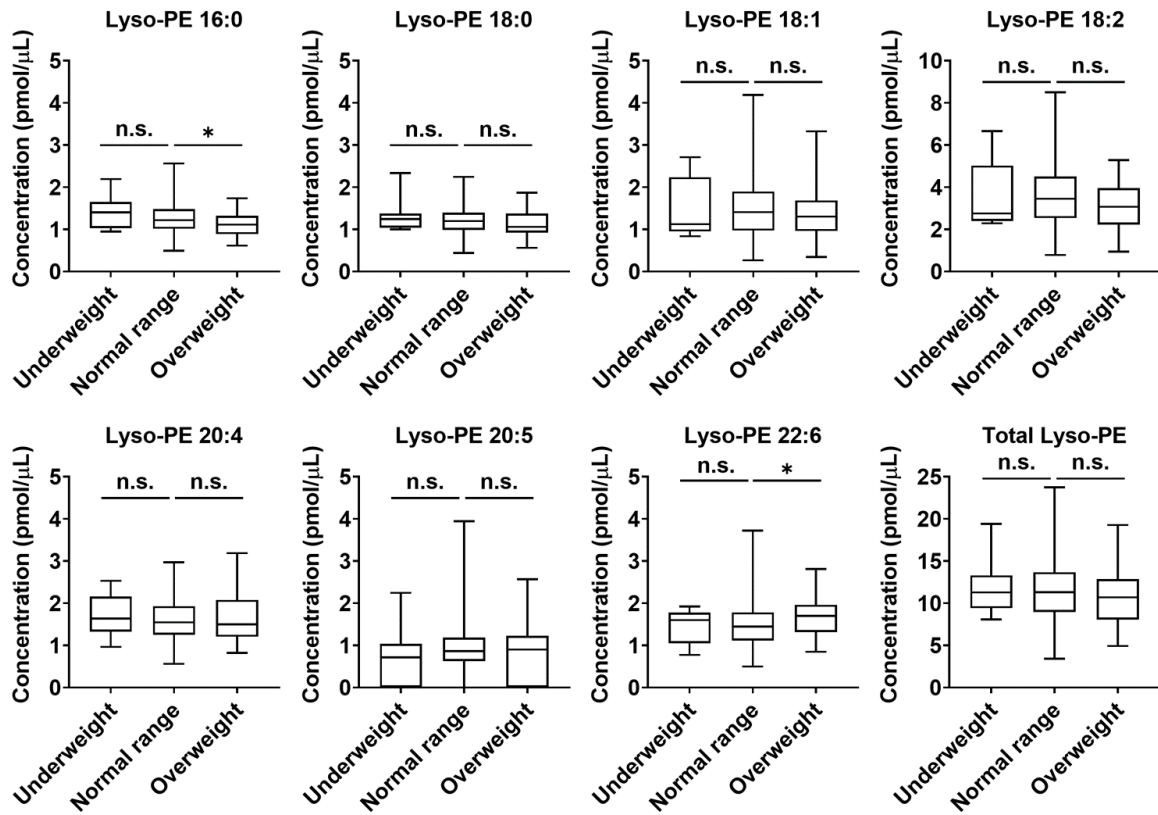


Figure 4. Box plots of each lysophosphatidylethanolamine concentration in the plasma of preadolescent children according to percentage of overweight (POW). Underweight: Children with POW < -20%; Normal range: Children with POW between -20% and 20%; Overweight: Children with POW ≥ +20%. Nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test, * p < 0.05, n.s. not significant. Lyso-PE, Lysophosphatidylethanolamine.