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Composition of plasmalogens in serum lipoproteins from patients with non-alcoholic steatohepatitis and their susceptibility to oxidation

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ABSTRACT

Background: Plasmalogens are ether phospholipids (PL) with an alkenyl group including vinyl ether bound at the *sn*-1 position and a polyunsaturated fatty acid bound at the *sn*-2 position, and are susceptible to oxidation. To date, there are no reports on the relationship between plasmalogen in serum lipoproteins and non-alcoholic steatohepatitis (NASH), caused by multiple factors including oxidative stress. Here, we have investigated the distribution of plasmalogens in serum lipoproteins isolated from NASH patients and healthy volunteers.
Methods: Serum lipoproteins were separated by gel-filtration chromatography, and analyzed for ethanolamine and choline plasmalogens using liquid chromatography-mass spectrometry.
Results: Both plasmalogen levels were higher in HDL than in VLDL or LDL. The plasmalogens/PL ratio was significantly lower in NASH than controls, for all lipoprotein fractions. Ethanolamine plasmalogens containing 20:4 and 22:6 at the *sn*-2 position and choline plasmalogens containing 16:0 at the *sn*-1 position were predominant in each group. In oxidation test using LDL from healthy serum, both types of plasmalogens were decreased during the early stages of oxidation.
Conclusion: Plasmalogens could be a potential biomarker for evaluating the early stages of oxidation in NASH.

1. Introduction

Plasmalogens consist of a characteristic structure with an alkenyl group including vinyl ether binding at the *sn*-1 position and a polyunsaturated fatty acid at the *sn*-2 position. Plasmalogens comprise two dominant head groups, namely ethanolamine and choline. The ethanolamine plasmalogens (PlsEtn) are rich in the brain, testes, and kidney, whereas choline plasmalogens (PlsCho) are rich in cardiac and skeletal muscle [1]. Previous reports suggest that plasmalogens are involved in vesicle formation and membrane fusion [2–4], ion transport [5–7], cholesterol efflux [8], and generation of secondary signal mediators [9,10]. Plasmalogens may also act as an endogenous lipidemic antioxidant, because the vinyl ether substituent at the *sn*-1 position of the glycerol backbone is highly sensitive to oxidative damage [11,12]. In clinical research, plasma plasmalogens concentrations decreased in patients with Alzheimer's disease [13] and severe coronary stenosis [14].

To show the association between plasma lipoproteins and plasmalogens, Maeba et al. demonstrated that plasma PlsCho, measured by high performance liquid chromatography (HPLC) using radioactive iodine, showed a positive correlation with high-density lipoprotein-cholesterol (HDL−C) [15]. Additionally, Wiesner et al. described the

Abbreviations: PlsEtn, ethanolamine plasmalogens; PlsCho, choline plasmalogens; HPLC, high performance liquid chromatography; HDL-C, high–density lipoproteins-cholesterol; LDL, low-density lipoproteins; LC/MS, liquid chromatography-mass spectrometry; VLDL, very low-density lipoproteins; TG, triglycerides; NASH, nonalcoholic steatohepatitis; TBARS, thiobarbituric acid reactive substances; TC, total cholesterol; PL, phospholipid; PBS, phosphate buffer saline; NaCl, Sodium Chloride; UPLC/ESI–MS/MS, ultra–performance liquid chromatography/electrospray ionization tandem mass spectrometry; $CuSO_4$, copper sulfate; ELISA, enzyme–linked immunosorbent assay; PPARα, Peroxisome proliferator-activated receptor-α; GNPAT, Glyceronephosphate O-acyltransferase.

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Table 1
Clinical parameters.

	Healthy subjects	NASH patients
n (M/F)	11 (7/4)	5 (1/4)
Age (y)	23.0 ± 1.1	61.6 ± 8.8*
PL (mmol/l)	2.7 ± 0.4	3.1 ± 0.4
TC (mmol/l)	4.8 ± 0.9	5.5 ± 0.8
TG (mmol/l)	0.9 ± 0.5	2.1 ± 1.5
HDL-C (mmol/l)	1.8 ± 0.5	1.3 ± 0.3*
LDL-C (mmol/l)	2.7 ± 0.8	3.6 ± 0.6*
Albumin (g/dl)	–	4.4 ± 0.4
ALT (IU/l)	–	65.6 ± 44.2

All values are presented as mean ± SD. *$P < .05$ vs. healthy subjects.

distribution of molecular species of PlsEtn measured by liquid chromatography-mass spectrometry (LC/MS) in lipoprotein fractions, namely very low-density lipoprotein (VLDL), LDL and HDL fractions [16]. However, the lipoprotein distribution of PlsCho, even in healthy subjects, remains unknown.

To date, there have been no reports on the distribution of plasmalogens of serum lipoproteins in patients with liver disorders. Previously, we reported that abnormal triglyceride (TG)-rich LDL appeared and the LDL was oxidized in patients with severe liver disease [17,18]. Here, we will focus on non-alcoholic steatohepatitis (NASH), which is caused by oxidative stress in addition to metabolic syndrome and can progress to end-stage liver disease with fibrosis, cirrhosis, and hepatoma. Plasma levels of thiobarbituric acid reactive substances (TBARS) were increased in patients with NASH [19]. Despite an increase in the number of patients worldwide, there are no established treatments or biomarkers for NASH. A previous report showed that plasma levels of total plasmalogens were significantly decreased in patients with NASH compared to healthy controls [20]. However, it remains unknown if the distribution of plasmalogens in each lipoprotein fraction changes in patients with NASH compared to healthy subjects with a normal liver.

In the present study, we analyzed both ethanolamine plasmalogen and choline plasmalogen of serum lipoproteins using LC-MS in healthy volunteers and in patients with NASH.

2. Materials and methods

2.1. Clinical samples

Fasting blood was drawn from healthy volunteers ($n = 11$, male:female = 7:4, mean age = 23 ± 1 y) and patients with NASH ($n = 5$, male:female = 1:4, mean age = 61.6 ± 8.8 y). Young healthy volunteers with no history of liver disease were recruited from the students with informed consent at the Hokkaido University. Diagnosis of NASH was made by histological examination of liver biopsy according to Brunt's classification [21] at Sapporo City General Hospital. Patients who met any of the following criteria were excluded in this study: alcohol abuse (≥20 g/day), hepatitis B and hepatitis C virus infection, autoimmune liver disease, primary biliary cholangitis and Wilson's disease. Sera were obtained by centrifugation (2000 × *g*, 10 min, room temperature). Serum lipid levels were measured by automated enzymatic methods using commercial kits (Sekisui Medical Co., Ltd.): Cholestest CHO for total cholesterol (TC), Cholestest TG for triglyceride, Pureauto S PL for phospholipid (PL), Qualigent LDL for LDL–cholesterol, and Qualigent HDL for HDL–cholesterol.

2.2. Gel filtration chromatography

Serum samples (0.3 ml each) were subjected to gel filtration chromatography on a Superose 6 column (GE Healthcare) in a liquid chromatography apparatus (Shimadzu), and 0.5 ml fractions were collected as described previously [17]. Phosphate buffered saline (PBS; 50 mmol/l; pH 7.4) containing 150 mmol/l NaCl was used as an elution buffer. Lipoprotein fractions were stored at −80 °C and analyzed for lipid content using automated enzymatic methods: Cholestest CHO for TC and Pureauto S PL for phospholipids (PL)(Sekisui Medical Co, Ltd).

2.3. Liquid chromatography mass spectrometry

The lipoprotein fractions separated by gel filtration chromatography were extracted using chloroform/methanol (1:1, *v*/v). The lipid extracts were analyzed for PlsEtn and PlsCho using ultra–performance liquid chromatography/electrospray ionization tandem mass spectrometry (UPLC/ESI–MS/MS) as described below, with some modifications [22]. Briefly, liquid chromatography was performed using an Accela U–HPLC system (Thermo Fisher Scientific) with an Acquity UPLCR BEH C8 column (1.7 µm, 2.1 mm × 100 mm I. D, Waters) at 60 °C and a flow rate of 450 µl/min. The auto sampler was kept at 4 °C. Sample injection volume was 10 µl. The mobile phase A was water containing 5 mmol/l ammonium formate, and the mobile phase B was acetonitrile. Separation was conducted according to the following gradient program: 0–1.0 min, 20% (mobile phase B) to 60% (B); 1.0–1.5 min, 60% (B) to 80% (B); 1.5–17.0 min, 80% (B) to 85% (B); 17.0–20.0 min, 85% (B) to 90% (B); 20.0–20.4 min, 90% (B) to 95% (B); 20.4–21.0 min, 95% (B); 21.0–21.1 min, 95% (B) to 20% (B); 21.1–21.5 min, 20% (B). Total turnaround time was 21.5 min. MS analysis was performed using a TSQ Quantum Access quadrupole tandem MS (Thermo Fisher Scientific) equipped with an ESI probe in positive ion mode.

For quantitation of plasmalogens, synthetic p18:0–20:4 plasmenylcholine (choline plasmalogen) and plasmenylethanolamine (ethanolamine plasmalogen) were used as standard compounds (Avanti

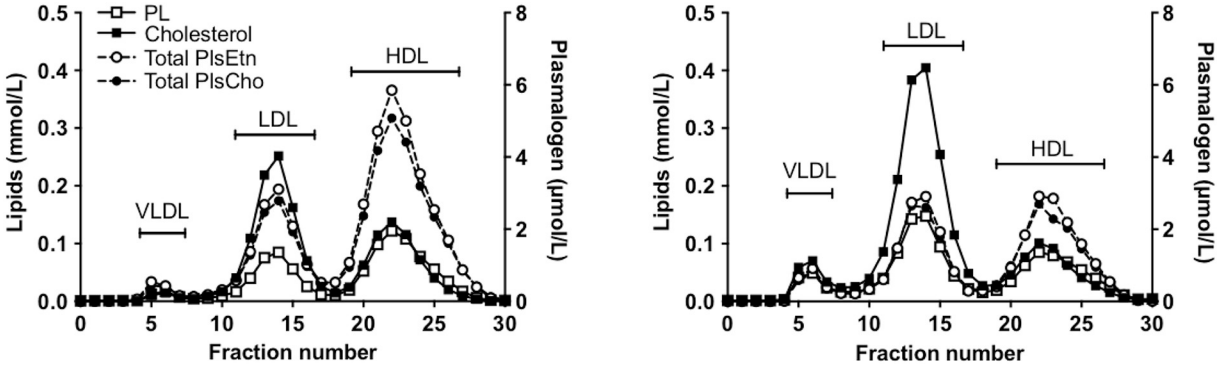

Fig. 1. Gel chromatography profile of human serum samples collected from healthy volunteers and patients with NASH. Phospholipid (open square, left scale); Cholesterol (closed square, left scale); Total ethanolamine plasmalogen (PlsEtn; open circle, right scale); Total choline plasmalogen (PlsCho; closed circle, right scale). VLDL eluted in fractions 4–8 and peaked in fractions 5–6; LDL eluted in fractions 9–17 and peaked in fractions 13–14; HDL-C eluted in fractions 18–29 and peaked in fractions 21–23.

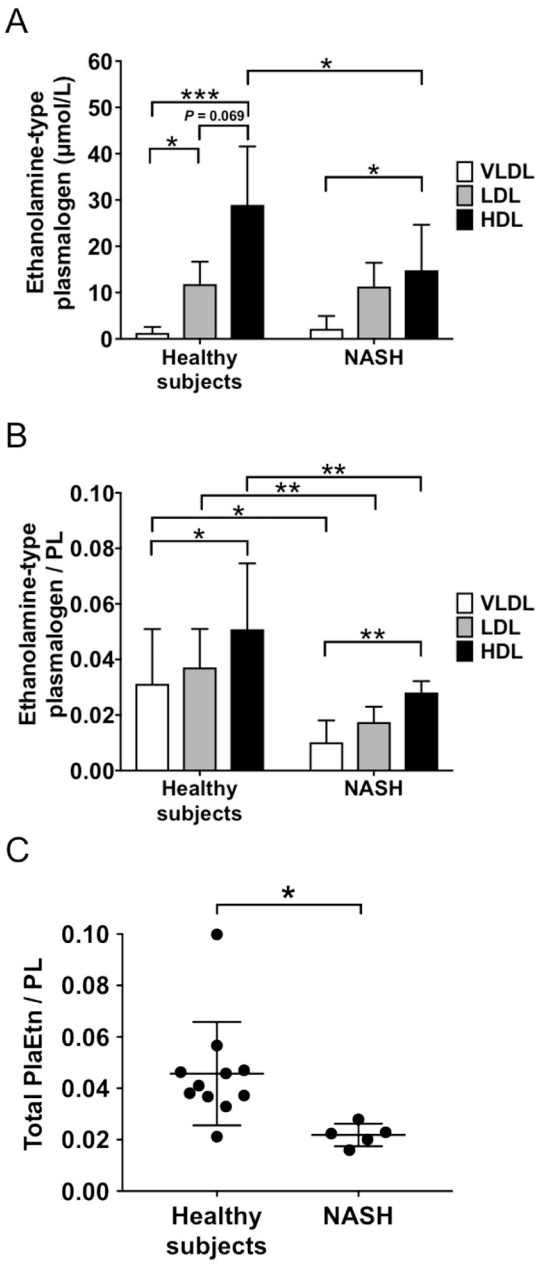

Fig. 2. Distribution of ethanolamine plasmalogen in serum lipoprotein fractions. A, Concentration of ethanolamine plasmalogen. B, Ratio of ethanolamine plasmalogen / phospholipids (mol/mol) in each lipoprotein fraction. C, Ratio of ethanolamine plasmalogen / phospholipids (mol/mol) in total lipoprotein fraction, which was calculated by sum of the ratio in VLDL, LDL and HDL fractions. All values presented as mean ± SD. * $P < .05$, ** $P < .01$, *** $P < .001$.

Polar Lipids, Inc.). Concentration of individual plasmalogen was calculated from the peak area on the chromatogram detected relative to the internal standard (1,2-dimyristoyl phosphatidylcholine for PlsCho and 1,2-dimyristoyl phosphatidylethanolamine for PlsEtn,). Capillary voltage was 3500 V, source temperature 80 °C, and desolvation temperature 400 °C. Cone voltage was 35 V. Collision energy was 20 eV for PlsEtn and 32 eV for PlsCho. Desolvation and cone gas flow were 800 and 50 l/h, respectively.

2.4. LDL oxidation

LDLs were purified from the serum of healthy volunteers by sequential ultracentrifugation in a near-vertical tube rotor (MLN-80; Beckman Coulter) in an Optima MAX ultracentrifuge (Beckman Coulter) [23]. To prepare copper-oxidized LDLs, aliquots of diluted LDLs (protein concentration: 0.3 mg/ml) were incubated in the presence of 3.3 μmol/l $CuSO_4$ at 37 °C. After various lengths of time (0–24 h), reaction mixtures were immediately analyzed for plasmalogen measurements, PL, TBARS and mildly oxidized LDL. PL was measured using automated enzymatic methods with Pureauto S PL. TBARS was measured using a commercial kit (Cayman Chemical Co.). Mildly oxidized LDL was measured using an in-house sandwich enzyme-linked immunosorbent assay (ELISA) [17]. Data from total PlsEtn, total PlsCho and PL are presented as a ratio to the baseline value (0 h) for observation of reduction rate. Data from mildly oxidized LDL and TBARS are indicated as absorbance and concentration, respectively.

2.5. Ethics

All individuals gave written informed consent to participate in this study. The study was approved by the ethics review board at the Faculty of Health Sciences, Hokkaido University.

2.6. Statistical analysis

Unless otherwise indicated, all values are presented as mean ± SD. The Student's *t*-test was done for comparisons of clinical parameters between healthy subjects and NASH patients. The chi-square test was used to determine sex differences. Comparison between three lipoprotein fractions in each group of patients was analyzed using the Kruskal Wallis test followed by Dunn's multiple-comparison test, with the use of GraphPad Prism Software. Comparison between the group in each lipoprotein fraction was analyzed using the Mann-Whitney *U* test. A $P < .05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics

The clinical data obtained from healthy subjects and NASH patients are shown in Table 1. There was a significant difference in age, HDL-C, and LDL-C between the groups ($P < .05$).

3.2. Distribution of plasmalogen in lipoprotein fractions

Lipid elution profiles are shown in Fig. 1. VLDL eluted in fractions 4–8 and peaked in fractions 5–6; LDL eluted in fractions 9–17 and peaked in fractions 13–14; HDL-C eluted in fractions 18–29 and peaked in fractions 21–23. The elution positions of total PlsEtn and total PlsCho are consistent with those of lipids. The 99.8% of PlsEtn was distributed into VLDL (3.1%), LDL (28.1%), and HDL fractions (68.6%). The 99.5% of PlsCho was distributed into VLDL (3.1%), LDL (28.6%), and HDL fractions (67.8%).

PlsEtn levels were highest in HDL (Fig. 2A). Despite the high levels of LDL-C in patients with NASH compared to healthy subjects, PlsEtn levels in LDL were similar between the groups, whereas the plasmalogen in HDL was significantly lower in patients with NASH than in healthy subjects (Fig. 2A). When the concentration of total PlsEtn was divided by that of PL in each lipoprotein fraction (VLDL, LDL and HDL), the ethanolamine plasmalogen/PL ratio was highest in HDL in both groups (Fig. 2B). Further, the ratio was significantly lower in patients with NASH than in healthy subjects in all lipoprotein fractions ($P < .05$)(Fig. 2C).

All molecular species of PlsEtn were rich in the HDL fraction both in healthy subjects (Fig. 3A) and in patients with NASH (Fig. 3B), and the distribution pattern was similar between the groups. In PlsEtn, the molecular species that contain 20:4 at the *sn*-2 position (16:0/20:4, 18:0/20:4, 18:1/20:4) and 22:6 at the *sn*-2 position (16:0/22:6, 18:0/

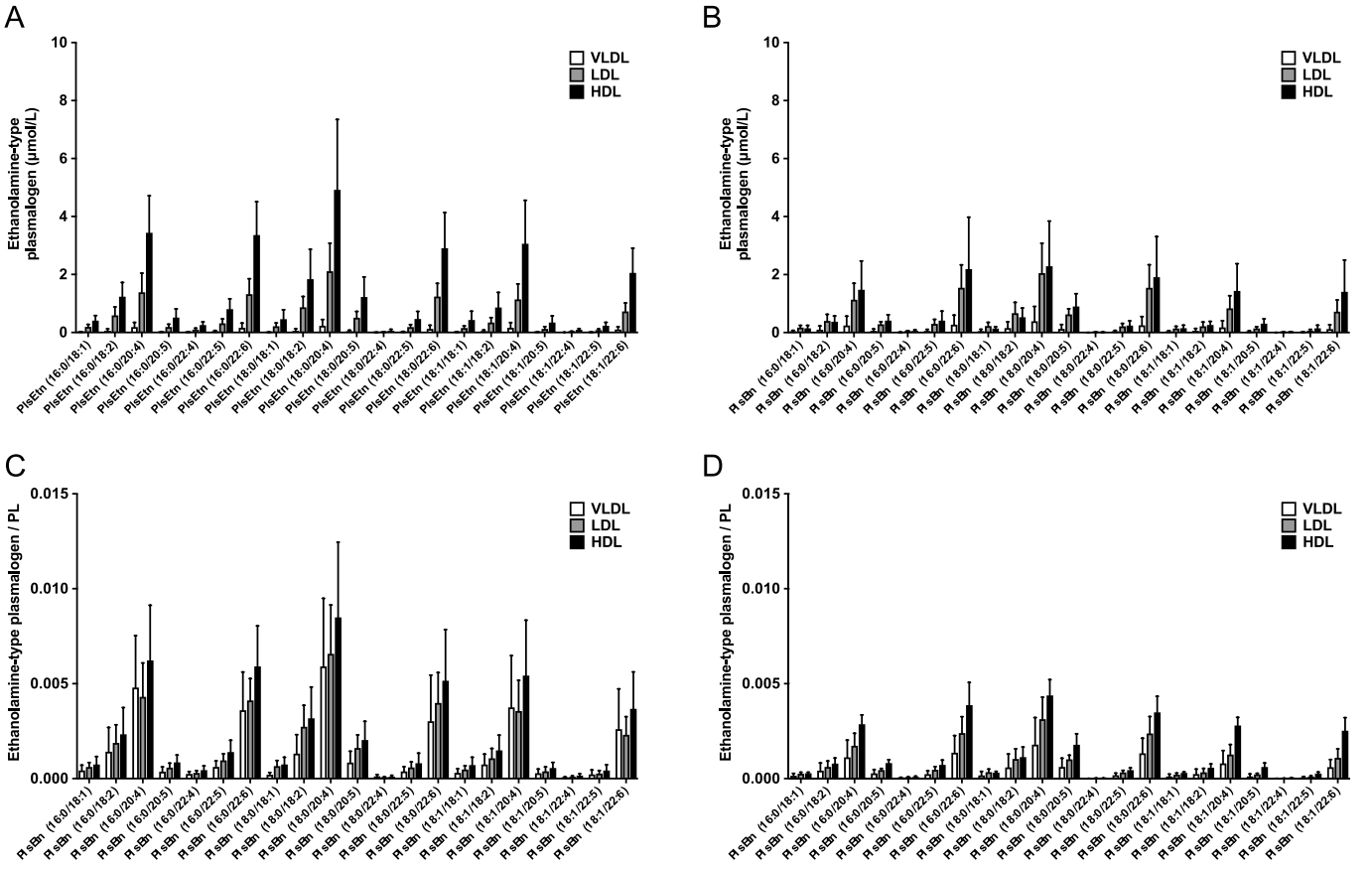

Fig. 3. Distribution of molecular species of ethanolamine plasmalogen in lipoprotein fractions. A and B, Concentration of ethanolamine plasmalogen in healthy subjects (A) and patients with NASH (B). C and D, Ratio of ethanolamine plasmalogen /phospholipids (mol/mol) in healthy subjects (C) and patients with NASH (D).

22:6, 18:1/22:6) were predominant in both groups (Fig. 3A and B). When the value of each molecular species of PlsEtn in VLDL, LDL and HDL was divided by that of total PL in each lipoprotein, the ethanolamine plasmalogen/PL ratio of the HDL fraction was higher than that of VLDL and LDL fractions both in healthy subjects (Fig. 3C) and in patients with NASH (Fig. 3D).

For PlsCho, the level and distribution pattern were very similar to those of PlsEtn (Fig. 4A–C). All molecular species of PlsCho were rich in the HDL fraction both in healthy subjects (Fig. 5A) and in patients with NASH (Fig. 5B), and the distribution pattern was similar between groups. In PlsCho, note that the molecular species that contain 16:0 at the *sn*-1 position (16:0/18:1, 16:0/18:2, 16:0/20:4, 16:0/20:5, 16:0/22:4, 16:0/22:5, 16:0/22:6) were predominant in both groups (Fig. 5A and B). Similar to PlsEtn, the choline plasmalogen/PL ratio of the HDL fraction was higher than that of VLDL and LDL fractions, both in healthy subjects (Fig. 5C) and in patients with NASH (Fig. 5D).

3.3. Changes in plasmalogens by LDL oxidation

Mildly oxidized LDL was increased instantly and linearly from the onset of copper oxidation, reached a maximum at 4 h, then declined to a basal level at 24 h (Fig. 6). TBARS also showed a prompt elevation, which peaked at 8 h, and then remained increased from 8 to 24 h. PL remained constant for 24 h. Both plasmalogens were decreased immediately and linearly from the onset of copper oxidation, then declined to 0% at 24 h. The reduction of PlsEtn occurred prior to that of PlsCho.

4. Discussion

The present study revealed that the elution positions of PlsCho and PlsEtn were consistent with that of PL and cholesterol, both in healthy subjects and in patients with NASH. To our knowledge, this is the first report of the distribution of PlsCho in serum lipoproteins. The distribution pattern of each molecular species of PlsEtn was consistent with previous reports on human plasma lipoproteins [16] and human circulating blood cells [24]. Furthermore, our study represents a new insight into the distribution of plasmalogen in serum lipoprotein in patients with NASH. It is worth noting that the plasmalogen/PL ratio of any serum lipoprotein in patients with NASH was significantly lower than in healthy subjects, which consistent with a previous study showing decreased total plasmalogens in plasma from patients with NASH compared with controls [20].

One possible mechanism for the lower plasmalogen levels of serum lipoproteins in patients with NASH is a reduction in plasmalogen biosynthesis. Plasmalogens are mainly synthesized in the liver, then secreted into the circulation as part of the lipoprotein component [25]. Plasmalogens are thought to be primarily synthesized in peroxisomes and act to prevent cellular damage caused by oxidative stress [26,27]. Several studies have shown that peroxisomal dysfunction as well as mitochondrial dysfunction could develop into hepatic steatosis [28–30]. A recent study using a NASH model mouse showed that these animals had lower levels of docosahexaenoic acid-containing plasmalogen and reduced expression of glyceronephosphate O-acyltransferase (*Gnpat*), which is located in the peroxisomal membrane and is the rate-limiting enzyme in plasmalogen biosynthesis, in the liver [31]. Therefore, the lower levels of plasmalogens on plasma lipoproteins in patients with NASH might be due to peroxisome dysfunction and/or impaired hepatic biosynthesis of plasmalogens.

Another potential mechanism is that the low levels of serum plasmalogen result from an endogenous response to oxidative stress in NASH. Previous in vitro studies demonstrated that plasmalogens with a

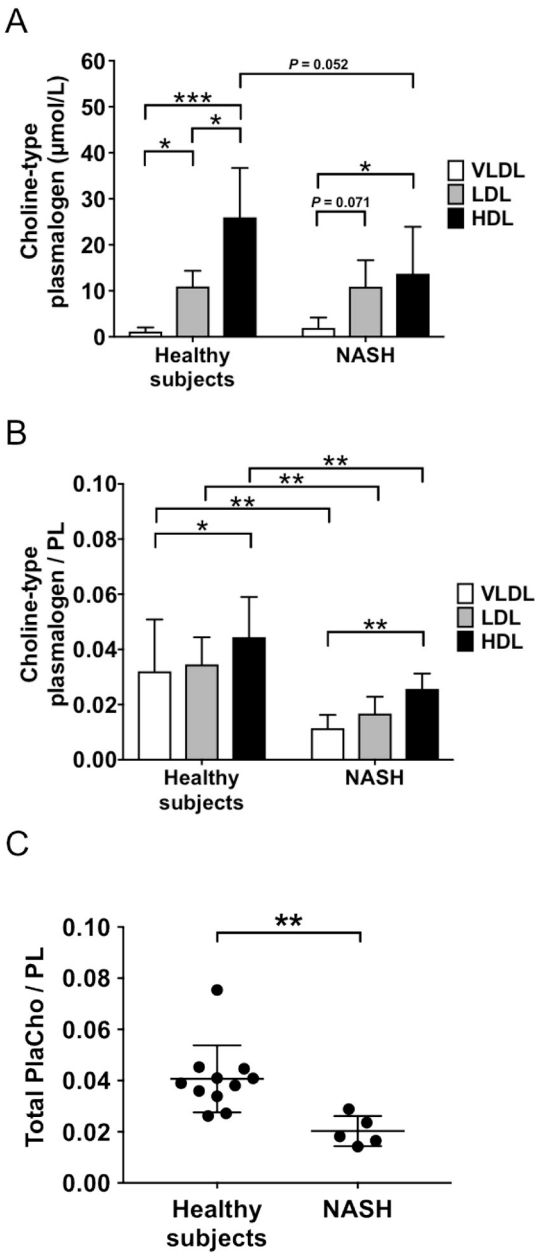

Fig. 4. Distribution of choline plasmalogen in serum lipoprotein fractions. A, Concentration of choline plasmalogen. B, Ratio of choline plasmalogen / phospholipids (mol/mol) in each lipoprotein fraction. C, Ratio of choline plasmalogen / phospholipids (mol/mol) in total lipoprotein fraction, which was calculated by sum of the ratio in VLDL, LDL and HDL fractions. All values presented as mean ± SD. * $P < .05$, ** $P < .01$, *** $P < .001$.

vinyl ether structure and polyunsaturated fatty acid are susceptible to oxidation using a lipid monolayer, such as serum lipoproteins and the lipid bilayer [12,32]. Regardless of the unchanged levels of plasma PL, low levels of plasmalogen and plasmalogen/PL ratio in all lipoprotein fractions were found in patients with NASH, which might suggest that the plasmalogens themselves were degenerated by oxidation. Thus, of the phospholipids in serum lipoproteins, plasmalogens could play an important role in counteracting oxidation.

In the present study, we used artificial oxidation of LDL to show that both types of plasmalogen, but not PL, were decreased by oxidation. This is consistent with a previous report showing that the vinyl-ether double band of plasmalogen is highly sensitive to oxidative agents in vitro [33]. Because the ELISA that we used to assay mildly oxidized LDL can detect oxidized LDL during the early process of oxidation, we propose that plasmalogen could be denatured/oxidized earlier following exposure to oxidation conditions. The artificial oxidation of LDL experiment also revealed that the reduction of PlsEtn occurred earlier than that of PlsCho. There were also differences in the species of fatty acid that binds with each plasmalogen-type. Polyunsaturated fatty acids (20:4 and 22:6 at the *sn*-2 position) were predominant in the molecular species of fatty acid in PlsEtn, suggesting that PlsEtn may be more susceptible to oxidation than PlsCho. Furthermore, oxidative stress may attack not only the vinyl ether bond but also polyunsaturated fatty acids of phospholipids [34]. In fact, plasmalogens with polyunsaturated fatty acids were decreased prior to those with 18:1 (data not shown). Thus, plasmalogen, and in particular PlsEtn, could be a potential biomarker for evaluating the earlier oxidative state in serum. Oxidative stress in patients with NASH could lead to the degeneration of plasmalogen in serum lipoproteins, possibly via oxidation.

There are some limitations in this study. First, the number of the samples, especially for NASH, was small. However, current data will be useful for a large-scale clinical study. Second, because of the age difference between study subjects and healthy controls, the effect of age on serum plasmalogen levels could not be determined as reported previously [15,22,35]. The reanalysis by our published data set [22] exhibited that there was no significant difference on PlsEtn/PL between young healthy subjects in their twenties and elderly subjects in their sixties, whereas PlsCho/PL in elderly subjects was significantly lower by 20% than that in young healthy subjects (unpublished data). In contrast, we observed relatively large decrease in both PlsEtn/PL and PlsCho/PL in NASH patients (approximately by 50%) (Fig. 2C and 4C). Thus, it is likely that the decline of plasmalogen/PL in elderly patients with NASH might be due to the disease process itself apart from aging. In the future, we would like to clarify the pathophysiological significance of plasmalogens in various diseases including NASH by increasing the number of cases and age-matched healthy control. Also, plasmalogens have been shown to play a role in antioxidant defense as a biological function [36–38], however this is controversial [39,40]. Plasmalogens can produce the highly reactive compounds α-hydroxyaldehydes after oxidation, which are likely to cause damage rather than protection in the human body [39]. The present study did not quantify the aldehyde compounds, therefore future studies will be required to measure the aldehydes and obtain a better understanding of the process.

5. Conclusions

We clarified the distribution of each molecular species of both ethanolamine plasmalogen and choline plasmalogen using LC-MS in serum lipoproteins isolated from sera of healthy volunteers and patients with NASH. The ethanolamine plasmalogen/PL and choline plasmalogen/PL was significantly lower in patients with NASH compared to healthy subjects, for all lipoprotein fractions. Thus, serum plasmalogens could be a potential biomarker for evaluating the earlier oxidative state in NASH.

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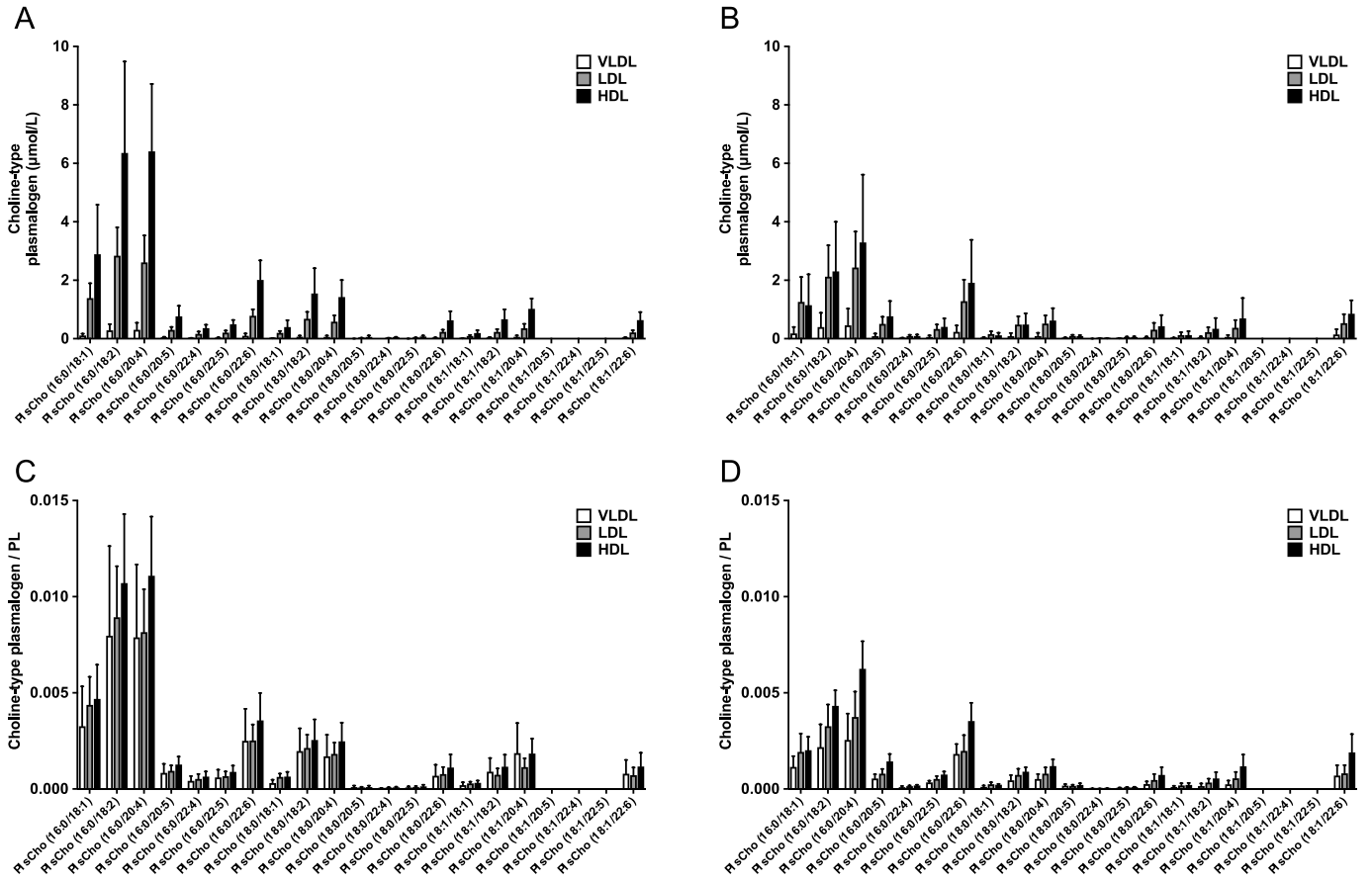

Fig. 5. Distribution of molecular species of choline plasmalogen in lipoprotein fractions. A and B, Concentration of choline plasmalogen in healthy subjects (A) and patients with NASH (B). C and D, Ratio of choline plasmalogen / phospholipids (mol/mol) in healthy subjects (C) and patients with NASH (D).

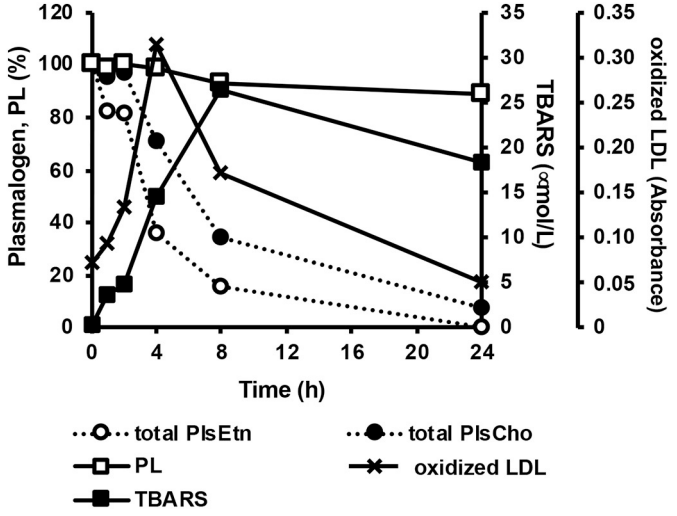

Fig. 6. Time course of copper-mediated LDL oxidation as determined by plasmalogens, phospholipids (PL), mildly oxidized LDL, and TBARS. Data from total ethanolamine plasmalogens (PlsEtn, open circle, left scale), total choline plasmalogens (PlsCho, closed circle, left scale) and PL (open square, left scale) presented as a ratio to baseline value (0 h) for observation of reduction rate. Data from mildly oxidized LDL (cross, second right axis) and TBARS (closed square, first right axis) are shown as absorbance and concentration, respectively.

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