Serum choline plasmalogens, particularly those with oleic acid in sn-2, are associated with proatherogenic state

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Running title: Serum PlsCho as antiatherogenic biomarker

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Abbreviations:

PL, phospholipids; Pls, plasmalogen(s); Pak, alkylphospholipids; PlsCho, choline plasmalogen; PlsEtn, ethanolamine plasmalogen; PakCho,
1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine; PakEtn,
1-O-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine; PtdCho, 1,2-diacyl phosphatidylcholine; PtdEtn, 1,2-diacylphosphatidylethanolamine
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ABSTRACT

Serum plasmalogens (1-\(\text{O-}
\text{alk-1-}
\text{enyl-2-acyl}
\text{glycerophospholipids; Pls})\) are of particular interest for studies on metabolic disorders associated with oxidative stress and chronic inflammation. Serum levels of Pls are known to correlate positively with HDL-cholesterol (HDL-C); however, few studies have examined serum Pls molecular species in association with pathophysiological conditions and their clinical significance. To clarify these, we determined serum levels of individual ether glycerophospholipids in Japanese asymptomatic cohorts (\(n = 428\); 362 male and 66 female subjects) by LC/MS/MS, and examined their correlations with clinical parameters. We found that the proportion of choline plasmalogens (PlsCho) among total serum phospholipids was significantly lower in the male group over 40 years old and was associated with multiple risk parameters more strongly than HDL-C. The abundance of serum PlsCho with oleic acid (18:1) in \(sn-2\) exhibited the strongest positive correlation with serum concentrations of adiponectin and HDL-C, while being inversely associated with waist circumference and the serum levels of triglycerides (TG) and small dense LDL-cholesterol (sdLDL-C). The characterization of serum ether glycerophospholipids verified the specificity of PlsCho, particularly the ones with 18:1 in \(sn-2\) as a sensitive
biomarker for the atherogenic state.

**Supplementary Keywords:**

ether glycerophospholipid, HDL-cholesterol, atherosclerosis, serum biomarker
INTRODUCTION

Plasmalogens (Pls) are a subclass of glycerophospholipids; they are characterized by the presence of a vinyl-ether bond and an ester bond at the sn-1 and sn-2 positions, respectively, of the glycerol backbone. On the basis of their polar head groups at the sn-3 position, Pls are mainly classified into either PlsCho or ethanolamine plasmalogens (PlsEtn); the former are localized in a few tissues such as cardiac muscle and blood plasma, whereas the latter belong to a predominant class distributed in a wide variety of cells and tissues (1). Serum Pls levels are associated with diverse clinical manifestations (2). For instance, some patients with peroxisomal disorders were found to exhibit the systemic reduction of Pls, which was explained by the fact that the first two steps of Pls biosynthesis occur exclusively in peroxisomes (3). In addition, since Pls are abundant in the brain and play essential roles in neuronal functions and myelin formation, defects in Pls contents represent a pathological factor for a number of neurodegenerative disorders such as Alzheimer’s disease (AD) (4). Goodenowe et al. also reported that the serum level of PlsEtn containing docosahexaenoic acid (DHA; 22:6) could be a useful biomarker for the early detection and long-term follow-up of AD (5).
Recently, particular attention has been paid to the involvement of PIs in metabolic diseases associated with oxidative stress and chronic inflammation (6). Plasmalogens may be involved in the etiology of both type 1 and type 2 diabetes mellitus (T1D and T2D, respectively) through oxidative stress. Patients with T1D exhibit absolute insulin deficiency due to autoimmune destruction of insulin producing pancreatic β-cells. In a longitudinal study, children who develop T1D later have been found to have reduced serum levels of ether type choline glycerophospholipids, including PlsCho at birth and consistently throughout medical follow-ups (7).

Plasmalogen deficiency may cause enhanced susceptibility of pancreatic β cells to oxidative damage and trigger progression to diabetes. Oxidative stress may also be associated with T2D and related cardiovascular diseases. Notable decreased PlsEtn levels and increased lipid peroxidation were found in LDL particles from obese patients with metabolic syndrome (MetS) and patients with T2D (8), which may be a risk factor for atherosclerosis. Plasma PIs may be a good marker for inferring level of oxidative stress and predicting cardiovascular mortality in patients with end-stage renal disease (9). Plasma concentration of PIs also decreases with hypertension (10) as well as age and chronic inflammatory-related diseases such as atherosclerosis and AD (11, 12). Plasmalogens function as reservoirs for precursor fatty acids, such as
arachidonic acid and DHA, which generate bioactive lipid mediators. Eicosanoids derived from arachidonic acid play an important role in the regulation of vascular tone and possibly link PIs with hypertension via blood pressure regulation. Studies have postulated that PIs serve as endogenous antioxidants and protect membrane lipids and lipoprotein particles from excessive oxidation by scavenging reactive oxygen species via the vinyl-ether moiety (13-15).

To date, however, available data regarding the relationship between serum PIs and clinical disease entities are still limited, and the association of PIs molecular species with preclinical pathophysiological conditions is yet to be clarified. Previously, we performed an advanced comprehensive analysis of serum ether glycerophospholipids, including PIs, by LC/MS/MS, which has allowed the accurate quantification of each molecular species (16, 17). The present study provides the reference data from 428 Japanese asymptomatic subjects on their serum levels of individual ether glycerophospholipid species. By analyzing the correlation of each molecular species with clinical parameters or biochemical measurements, we found that PIsCho with 18:1 in the sn-2 position strongly correlated with a wide range of risk factors for atherosclerosis and MetS. Our finding suggests that serum PIsCho, like HDL-C, may serve as a sensitive antiatherogenic biomarker.
METHODS

Study subjects and design

Asymptomatic subjects (n = 428; 362 male and 66 female subjects; age, 22–66 years old) who were referred for a routine health examination were enrolled in the study. Written informed consent was obtained from all participants, and the studies were approved by the ethics committees of Hokkaido University, Teikyo University School of Medicine, and ADEKA Co., Ltd. Sera were separated from fasting blood in a blood collection tube containing coagulation accelerating agent by centrifugation at 1,500 × g for 10 min, and then an antioxidant was added (dibutylhydroxytoluene; BHT, final concentration of 10 µM) and metal chelator (ethylenediaminetetraacetic acid; EDTA, final concentration of 1 mM), followed by immediate freezing at –80°C until analysis. Measurements of body weight, stature, waist circumference, and blood pressure were recorded. Participants were asked to complete health questionnaires consisting of 24 questions with regard to their medical history, as well as their dietary, smoking, drinking, and exercise habits.
Measurement of ether glycerophospholipids

Serum lipids were extracted by chloroform/methanol (1:2, v/v) after the serum was freeze-dried. Briefly, 1 mL of methanol, including internal standards (1,2-dimyristoyl-phosphatidylcholine; DMPC and phosphatidylethanolamine; DMPE) and BHT, was added to the freeze-dried serum (100 μL) and shaken for 30 sec. Then, 0.5 mL of chloroform was added to each tube and shaken for 30 sec. After letting the tube stand at room temperature for 30 min, the tubes were shaken, centrifuged, and the chloroform/methanol layer was harvested. This procedure was repeated three times, and the collected chloroform/methanol layer was evaporated until dry. The dried samples, including extracted lipids, were dissolved with methanol. Ether glycerophospholipids, including Pls, in extracted lipids were analyzed by LC/MS/MS. Synthetic 18:0-18:1, 18:0-20:4, and 18:0-22:6 of each PlsCho and PlsEtn, as well as 16:0-18:1 and 16:0-20:4 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (PakCho) (Avanti Polar Lipids, Inc., Alabaster, AL, USA), were used not only to generate a standard curve for quantifying individual molecular species of ether glycerophospholipids but also to assess the precision and accuracy of the detection, as described previously (17). The mean recovery of 18:0-18:1 and 18:0-20:4 for
PlsCho and PlsEtn, respectively, as well as DMPC, was 91.8%. Intra-assay (n = 10) coefficient of variation percentage (CV) of these phospholipids ranged from 5.1 to 8.5%.

Synthetic 16:0-18:1 1-O-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine (PakEtn) synthesized enzymatically from the corresponding PakCho by exchanging choline to ethanolamine was used to generate a standard curve for quantifying PakEtn molecular species. The concentrations of individual molecular species of ether glycerophospholipids were determined using corresponding internal standards (DMPC for PlsCho and PakCho, and DMPE for PlsEtn and PakEtn, Avanti Polar Lipids). The numbers of molecular species recorded were 24 and 30 for each class of alkylphospholipids (Pak) and Pls, respectively, all of which were major molecules determined by LC/MS/MS.

**Condition of LC/MS/MS**

Liquid chromatography-tandem mass spectrometry was conducted following a slightly modified method described previously (16). Liquid chromatography separation was performed using an Accela UPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a BEH C8 column (1.7 μm, 100 mm × 2.1 mm i.d.;
Waters Corp., Milford, MA, USA) at 60°C and a flow rate of 450 µL/min. Mobile phase A consisted of water containing 5 mM ammonium formate, while mobile phase B consisted of acetonitrile. Mobile phase A was set at 80% at 0 min, decreased linearly to 20% at 1.5 min, to 10% at 18 min, and then to 5% at 20 min, and maintained at 5% for 1 min before being returned to starting conditions. The MS analysis was performed using a TSQ Quantum Access Max (Thermo Fisher Scientific Inc.) equipped with an HESI probe in positive ion mode. The MS operating conditions were optimized as follows: the spray voltage of 3,000 V; and the capillary and vaporizer temperatures, 250°C and 500°C, respectively. Nitrogen was used as both the sheath and auxiliary gas. Argon was used as the collision gas at a pressure of approximately 1.0 mTorr. The collision energy was 32 eV for PakCho and PlsCho, 20 eV for PlsEtn, and 18 eV for PakEtn. Data acquisition was performed with Xcalibur 1.3 software (Thermo Fisher Scientific Inc.). Mass fragments used for identifying each class of Pls and Pak molecular species are summarized in supplemental Tables I and II, respectively. PlsEtn was quantified according to the procedure described by Zemski and Murphy (18). In brief, fragment ions at m/z 364, 391, and 392 were used for the identification of the sn-1 position of PlsEtn containing hexadecanol (16:0), octadecanol (18:0), and octadecenol (18:1), respectively. PakCho and PlsCho identified by three characteristic
fragments (precursor 184 derived from phosphocholine, as well as sn-1 origin and sn-2 origin fragments) were quantified using a fragment ion at m/z 184, following the separation of each molecule species by UPLC. PakEtn identified by 3 characteristic fragments (neutral loss 141 derived from phosphoethanolamine, sn-1 origin fragments, and fatty acids derived from the sn-2 position) was quantified using a fragment ion at m/z 141, following the separation of each molecule species by UPLC (19). The presence of Pls was further confirmed by the disappearance of the peak upon treatment with acid.

**Biochemical analysis**

Serum concentrations of TG, HDL-C, LDL-C, total phospholipids (PL), blood glucose, uric acid (UA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (γ-GTP) were determined enzymatically with an AutoAnalyzer (JCA-BM8060; JEOL, Ltd, Tokyo, Japan) and reagent kits (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Small dense low-density lipoprotein cholesterol was measured using the sdLDL-EX “Seiken” Kit (Denka Seiken, Tokyo, Japan). High-sensitivity C-reactive protein (hsCRP) was quantified by a latex photometric immunoassay (*LPIA ACE CRP-H II*; Mitsubishi Chemical Medience Corp).
Homocysteine (Hcy) was measured enzymatically using the Alfresa Auto Hcy Kit (Alfresa Pharma Corp., Osaka, Japan). Total adiponectin was determined using a sandwich ELISA system (Adiponectin ELISA Kit; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Excel (2010; Microsoft Corporation, Redmond, WA) with the add-in software Statcel 3 (OMS, Tokyo, Japan). Data are presented as mean ± SD or median and interquartile range. Results were analyzed by Tukey-Kramer post-hoc tests for parametric data (Supplemental Table III), and Steel-Dwass (Table 1, Supplemental Tables III, IV, and V) as well as Mann-Whitney U tests (Table 1, Supplemental Tables III, IV, V, and VI) for non-parametric data. Correlations among measurements were assessed with Spearman’s rank tests (Figures 3, 4, and 6, Tables 2 and 3).

RESULTS
Background of asymptomatic subjects

Clinical parameters and serum biochemical measurements of the subjects were classified by age (under 39 or over 40 years old) and gender (Supplemental Table III). The data indicate that male subjects over 40 years old (≥40) exhibited various abnormalities in clinical and biochemical parameters, including waist circumference, blood systolic and diastolic pressures (s.p. and d.p.), blood glucose, the serum levels of UA, AST, ALT, γ-GTP, and TG, as well as the atherogenic index of plasma (AIP, calculated as log (TG/HDL-C)). To confirm that these ≥40 year old subjects belonged to the population at risk, serum concentrations of sdLDL, hsCRP, Hcy, and adiponectin were determined. The data of certain male subjects ≥40 indicated that they were near the upper or lower limit of a normal serum range, suggesting that male subjects over 40 years old are associated with proatherogenic status (Supplemental Table III).

Characteristics of serum ether glycerophospholipids

The serum concentrations of each class of Pls, i.e., PlsCho and PlsEtn, as well as Pak, i.e., PakCho and PakEtn, along with their class ratios (PlsCho/PlsEtn and PakCho/PakEtn) and their proportions (mol%) in total phospholipids (PlsCho/PL,
PlsEtn/PL, Total Pls/PL, PakCho/PL, PakEtn/PL, and Total Pak/PL), were summarized according to gender and age (Table 1). The results show that while the serum levels of PlsCho and PlsEtn were almost equal, the serum concentrations of PakEtn were much lower than those of PakCho. In addition, the PlsCho/PL ratio for the ≥40 male group was significantly lower than those for other groups, whereas the PlsCho/PlsEtn ratio and Total Pls/PL (mol%) were higher among the under 39 year old (≤39) female and ≥40 female groups, respectively (Table 1). The statistical comparisons of age groups were adjusted by confounding factors such as diet, smoking, drinking, and exercise. Although the significant differences in some of these confounding factors were observed among age groups, these confounding factors only had significant effects on PL but not other Pls-related parameters. Heavy drinkers (more than 3 times per week) showed significantly higher serum concentrations of PL, but there were no significant differences related to the frequency of drinking among groups (data not shown). The proportion of female subjects in the study population (15.4%) was exceedingly low, so the gender comparisons in this study might be limited.

We also determined the serum levels of each molecular species of ether glycerophospholipids (Supplemental Tables IV, V, and VI) and examined their molecular profiles (Figs. 1 and 2). Long-chain fatty alcohol species, i.e., hexadecanol
(16:0), octadecanol (18:0), and octadecenol (18:1), were common in the sn-1 position of all ether glycerophospholipids. Hexadecanol (16:0) was the predominant fatty alcohol species among the choline class of both Pak and Pls, whereas among the ethanolamine class, three alcohol species were distributed almost equal. Arachidonic acid (20:4), the major fatty acid in the sn-2 position, was common in ether glycerophospholipids. In addition, DHA was rich among the ethanolamine class, and linoleic acid (18:2) was specifically rich in PlsCho. The interrelationship among serum ether glycerophospholipids is summarized in Fig. 3. A strong positive correlation was observed between the serum concentrations of PakCho and PakEtn \((r = 0.714, P < 0.01)\), presumably because they share a common biosynthetic precursor, 1-alkyl-2-acyl-glycerol. The synthetic pathway of PlsEtn from PakEtn appears to be supported by both the strong positive correlation between their serum levels \((r = 0.630, P < 0.01)\) and the marked similarity between their molecular profiles (Fig. 1). However, the biosynthetic route of PlsCho is considered to be derived from PlsEtn, but not from PakCho (20, 21). As such, the correlation between the serum levels of PlsCho and PlsEtn \((r = 0.611, P < 0.01)\) was higher than that between PlsCho and PakCho \((r = 0.521, P < 0.01)\). The molecular profiles of PlsCho and PlsEtn were remarkably different from each other (Fig. 1), suggesting that a part of serum PlsCho may be
different origins of other serum ether glycerophospholipids.

Relationship between ether glycerophospholipids and risk factors for the atherogenic state

We found that the PlsCho/PL (mol%) was significantly associated with multiple risk factors for the atherogenic status and that these correlations were at similar, or even higher, levels than those of HDL-C (Table 2). In addition, we found that among PlsCho species, PlsCho with 18:1 and 18:2 in sn-2 correlated positively with the serum adiponectin concentration ($r = 0.465$ and 0.340, respectively), but inversely with waist circumference ($r = -0.420$ and $-0.371$, respectively), the serum concentrations of TG ($r = -0.528$ and $-0.479$, respectively), sdLDL-C ($r = -0.311$ and $-0.261$, respectively), and AIP ($r = -0.656$ and $-0.574$, respectively; Fig. 4). In contrast, neither any species of Pak nor PlsEtn showed significant associations with these parameters (data not shown). The correlations with these parameters were compared among versatile classes or subclasses of glycerophospholipids containing 18:1 in sn-2 (Table 3). The results indicate that among the corresponding glycerophospholipids, only PlsCho showed significant correlations with all of these parameters. In contrast, diacyl choline (PtdCho) and diacyl ethanolamine
glycerophospholipids (PtdEtn) with 18:1 in \textit{sn}-2, as well as total 18:1 lipids in sera, correlated positively with TG, sdLDL-C, and AIP (Table 3).

In addition, we noticed that the proportion of PlsCho with 18:2 in total serum phospholipids (PlsCho18:2/PL) correlated negatively with age (Fig. 5). Interestingly, our results also suggest that both classes of Pls with eicosapentaenoic acid (20:5) in \textit{sn}-2 are associated with the frequency of fish intake (PlsCho, \( r = 0.373, P < 0.05 \); PlsEtn, \( r = 0.273, P < 0.05 \)), whereas total 20:5 lipids in sera exhibited no significant correlation (data not shown).

Of note, the serum levels of both classes of Pls molecular species were found to highly correlate with the serum HDL-C concentration, as described previously (22). The comparison of correlation coefficients among various molecular species in the \( \geq 40 \) male subjects (\( n = 186 \)) demonstrates that the amount of serum PlsCho with 18:1 in the \textit{sn}-2 position correlated most strongly with the HDL-C concentration (\( r = 0.741, P < 0.001 \); Fig. 6). Notably, the levels of both classes of Pls with docosapentaenoic acid (22:5) or 22:6 in \textit{sn}-2 were also found to be associated with the HDL-C levels.
DISCUSSION

Here, we report for the first time that the PlsCho/PL (mol%) not only was significantly lower in the ≥40 male group (Table 1) but also showed stronger correlations with multiple antiatherogenic parameters than HDL-C (Table 2). In addition, serum PlsCho with 18:1 or 18:2 in sn-2 also showed strong inverse correlations with a wide range of risk factors, such as age (Figure 4 and 5). Given the high susceptibility of their vinyl-ether moiety to oxidation (23), serum Pls may represent systemic markers for oxidative stress. However, only specific molecular species, i.e., PlsCho with 18:1 or 18:2 in sn-2, can be antiatherogenic indicators, despite the shared possession of vinyl-ether linkage in all Pls molecules. It is likely that besides serving as an oxidative index, PlsCho with 18:1 or 18:2 in sn-2 plays additional roles; it may be associated with different origins of other serum ether glycerophospholipids, which is evidenced by their specific molecular profiles (Figs. 1–3).

Plasma Pls are primarily synthesized in the liver and intestine in addition to dietary source of Pls, and are secreted in the circulating blood as lipoprotein components. Because the liver contains only low amounts of Pls, a large part of the Pls synthesized by the liver seem to be preferentially transported to the blood stream
In particular, PlsCho are predominantly localized in lipoproteins in blood milieu, suggesting their involvement in lipoprotein metabolism and/or function. Choline plasmalogen represent 3–5% of serum choline glycerophospholipids (ChoGpl). Choline glycerophospholipids are synthesized in the liver via two major pathways, i.e., the CDP-choline and phosphatidylethanolamine (PE) methylation pathways, and the latter is catalyzed by the hepatocyte-specific enzyme phosphatidylethanolamine N-methyltransferase (PEMT) through three steps during reaction with S-adenosylmethionine.

The CDP-choline pathway is the dominant route for ChoGpl synthesis in all mammalian tissues, while the PEMT methylation pathway is responsible for ~30% of ChoGpl generated in the liver. Given that PlsCho are principally synthesized via PlsEtn, a part of plasma PlsCho may be generated through hepatic PEMT methylation pathway (26). Interestingly, a polymorphism of the human PEMT gene is associated with diminished activity and may confer susceptibility to non-alcoholic fatty liver disease (NAFLD) (27, 28). NAFLD shares common clinical manifestation with MetS, such as abdominal obesity, T2D, dyslipidemia, and insulin resistance (29). Indeed, it has been demonstrated that NAFLD is associated with peroxisomal dysfunction and decreased plasma Pls, as observed in a comprehensive analysis of plasma lipids in
NAFLD patients (30).

Moreover, malnutrition of methyl donors such as methionine or choline may cause the suppression of PEMT methylation, resulting in decreased PlsCho and elevated homocysteine levels, which is a risk factor for cardiovascular disease. These considerations suggest that an insufficient PEMT methylation process is, at least in part, associated with MetS and atherosclerosis. Our previous intervention study on hyperlipidemic subjects has shown that increase in plasma PlsCho by ingestion of myo-inositol, which is a known nutrient to improve fatty liver disease, is accompanied by reduction in atherogenic sdLDL (31). This finding may provide direct evidence for the validity of plasma PlsCho as a biomarker for MetS and atherosclerosis.

Alternatively, the enhancement of enzymatic degradation of PlsCho may be considered the cause of decreased plasma PlsCho in a proatherogenic state. Exposure of hypoxia as well as thrombin stimulation to endothelial cells induce the activation of membrane-associated, calcium-independent phospholipase A2, which selectively hydrolyzes membrane PIs and preferentially PlsCho with arachidonic acid in sn-2 (32).

PlsCho with 18:1 in sn-2 occupies only one-tenth of the whole PlsCho population. The comparison of the correlations of clinical and biochemical parameters...
with the class or subclass of glycerophospholipids containing 18:1 in sn-2 reveals the specificity of PlsCho with 18:1 (Table 3). These results verify the prominence of PlsCho with 18:1 as a biomarker for the atherogenic state. Interestingly, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0-18:1 PtdCho) was previously identified as an endogenous ligand for the peroxisome proliferator-activated receptor alpha (PPARα) in the liver (33). We speculate that 16:0-18:1 PlsCho, the main species among PlsCho with 18:1 in sn-2, may have a similar function. Activation of PPARα exhibits pleiotropic beneficial effects on plasma lipid profile and vascular wall inflammation (34). For example, its activation promotes cholesterol efflux from macrophages and enhances reverse cholesterol transport by strongly inducing expression of genes that encode the cholesterol transporter ATP-binding cassette transporter A1 (ABCA1) (35). Moreover, PPARα activation facilitates the biosynthesis of Pls (36) as well as apolipoprotein A-I, as seen by the resultant increased plasma HDL and Pls levels.

Our study reveals that a wide range of Pls molecular species correlate positively with HDL-C in a class- or molecular species-dependent manner. Plasmalogens are relatively abundant in HDL fractions compared to other lipoprotein fractions (37). However, this cannot fully explain the observation that the correlation
between the serum levels of PlsCho and HDL-C ($r = 0.699, P < 0.01$) was considerably higher than that between PlsEtn and HDL-C ($r = 0.411, P < 0.01$; Fig. 6), because the Pls class distribution is not significantly different among various lipoprotein fractions (37). The reason for the highly positive correlation between the serum levels of Pls and HDL-C remains elusive. A recent lipidomic analysis demonstrated that individuals with low HDL-C displayed changes in the quality of HDL particles, along with decreased Pls contents (38). In addition, phospholipids have also been suggested to play important roles in HDL-mediated reverse cholesterol transport (RCT) (39-42). Interestingly, Pls-deficient cells have been shown to exhibit reduced cholesterol efflux by HDL (43), which may be associated with impaired cellular processing of cholesterol (44, 45). These notions suggest the relevance of serum Pls to the physiologically important functions of HDL, including RCT. Further studies are needed to elucidate the involvement of Pls in HDL metabolism and functions.

In conclusion, our detailed characterization of serum ether glycerophospholipids suggests that PlsCho with 18:1 or 18:2 in the sn-2 position may serve as a sensitive biomarker for the atherogenic state.
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Disclosures

None
REFERENCES


FIGURE LEGENDS

Fig. 1. Molecular profiles of individual ether glycerophospholipids.

Columns represent the median of the serum concentrations of individual ether glycerophospholipid molecular species of over 40 year old subjects (n = 206; 186 male and 20 female subjects).

Fig. 2. Distribution of molecular species in the sn-1 and sn-2 positions of ether glycerophospholipids.

Columns represent the median of the serum concentrations of fatty alcohols in sn-1 (inside Figure) and fatty acids in sn-2 of individual ether glycerophospholipids of over 40 year old subjects (n = 206; 186 male and 20 female subjects).

Fig. 3. Overview of serum ether glycerophospholipids.

Values (r) represent results from a Spearman’s correlation coefficient by rank test between serum levels of each ether glycerophospholipids in all subjects (n = 428).

PakCho: 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine

PakEtn: 1-O-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine
PleCho: 1-O-alkenyl-2-acyl-sn-glycero-3-phosphocholine (choline plasmalogen)

**Fig. 4.** Correlations between the serum levels of choline plasmalogen molecular species and various parameters.

Columns represent results from a Spearman’s correlation coefficient by rank test for over 40 year old male subjects ($n = 186$). Dashed lines indicate the $r$ value of 0.18, which is taken to be statistically significant with $P < 0.01$.

**Fig. 5.** Relationship between the proportion of PleCho with 18:2 in total phospholipids (PleCho18:2/PL) and age in all subjects ($n = 428$).

**Fig. 6.** Correlations between the serum levels of plasmalogen molecular species and HDL-C.

Columns represent results from a Spearman’s correlation coefficient by rank test for over 40 year old male subjects ($n = 186$). Dashed lines indicate the $r$ value of 0.18, which is taken to be statistically significant with $P < 0.01$. 


Table 1. Serum ether glycerophospholipid-related parameters of the subjects, classified by age and gender

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>PlsCho (µM)</th>
<th>PlsEtn (µM)</th>
<th>Total Pls (µM)</th>
<th>PlsCho/PlsEtn ratio</th>
<th>PlsCho/PL (mol%)</th>
<th>PlsEtn/PL (mol%)</th>
<th>Total Pls/PL (mol%)</th>
<th>PL (mM)</th>
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<td><strong>Male ≤39</strong></td>
<td>176</td>
<td>62.9 [ 55.3 - 70.1 ]</td>
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<td>131.2 [ 119.6 - 150.1 ]</td>
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<td>b 2.23 [ 2.00 - 2.51 ] a</td>
<td>2.48 [ 2.22 - 2.83 ] a</td>
<td>4.70 [ 4.28 - 5.17 ]</td>
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<td>≥40</td>
<td>186</td>
<td>59.7 [ 53.2 - 69.1 ]</td>
<td>71.3 [ 59.0 - 84.6 ]</td>
<td>130.8 [ 115.3 - 151.6 ]</td>
<td>0.84 [ 0.73 - 0.99 ]</td>
<td>b 1.94 [ 1.66 - 2.30 ] b</td>
<td>2.30 [ 1.95 - 2.72 ] b</td>
<td>4.29 [ 3.70 - 4.90 ]</td>
<td>b 3.09 [ 2.80 - 3.42 ]</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>362</td>
<td>61.3 [ 54.0 - 70.1 ]</td>
<td>70.8 [ 60.2 - 82.6 ]</td>
<td>130.8 [ 116.7 - 150.9 ]</td>
<td>0.86 [ 0.75 - 1.00 ]</td>
<td>a 2.08 [ 1.83 - 2.41 ] b</td>
<td>2.41 [ 2.16 - 2.79 ] b</td>
<td>4.52 [ 3.98 - 5.10 ]</td>
<td>2.91 [ 2.63 - 3.26 ]</td>
</tr>
<tr>
<td><strong>Female ≤39</strong></td>
<td>46</td>
<td>66.8 [ 59.3 - 74.8 ]</td>
<td>62.3 [ 51.0 - 82.1 ]</td>
<td>133.3 [ 111.5 - 147.3 ]</td>
<td>1.05 [ 0.90 - 1.20 ]</td>
<td>a 2.36 [ 2.13 - 2.67 ] a</td>
<td>2.24 [ 1.76 - 2.78 ] ab</td>
<td>4.64 [ 3.94 - 5.29 ]</td>
<td>b 2.90 [ 2.67 - 3.11 ]</td>
</tr>
<tr>
<td>≥40</td>
<td>20</td>
<td>72.3 [ 64.0 - 80.1 ]</td>
<td>78.5 [ 71.0 - 89.0 ]</td>
<td>157.0 [ 132.2 - 163.7 ]</td>
<td>0.87 [ 0.79 - 1.00 ]</td>
<td>b 2.37 [ 2.06 - 2.76 ] a</td>
<td>2.66 [ 2.15 - 2.97 ] ab</td>
<td>4.95 [ 4.34 - 5.67 ]</td>
<td>a 3.06 [ 2.76 - 3.21 ] ab</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>66</td>
<td>68.7 [ 59.5 - 78.1 ]</td>
<td>70.2 [ 56.2 - 97.2 ]</td>
<td>135.9 [ 118.3 - 161.7 ]</td>
<td>1.02 [ 0.84 - 1.17 ]</td>
<td>b 2.36 [ 2.09 - 2.71 ] b</td>
<td>2.39 [ 1.95 - 2.83 ] b</td>
<td>4.81 [ 4.01 - 5.49 ]</td>
<td>b 2.95 [ 2.70 - 3.17 ]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>PakCho (µM)</th>
<th>PakEtn (µM)</th>
<th>Total Pak (µM)</th>
<th>PakCho/PakEtn ratio</th>
<th>PakCho/PL (mol%)</th>
<th>PakEtn/PL (mol%)</th>
<th>Total Pak/PL (mol%)</th>
<th>PakEtn (µM)</th>
<th>PakEtn (µM)</th>
<th>Total Pak (µM)</th>
<th>PakCho/PakEtn ratio</th>
<th>PakCho/PL (mol%)</th>
<th>PakEtn/PL (mol%)</th>
<th>Total Pak/PL (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>≥40</td>
<td>186</td>
<td>42.5 [ 35.1 - 48.6 ]</td>
<td>5.1 [ 3.8 - 6.2 ]</td>
<td>47.5 [ 39.8 - 54.3 ]</td>
<td>8.55 [ 7.27 - 9.88 ]</td>
<td>1.34 [ 1.12 - 1.57 ]</td>
<td>0.16 [ 0.12 - 0.21 ]</td>
<td>1.51 [ 1.28 - 1.77 ]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>≥40</td>
<td>20</td>
<td>53.4 [ 38.5 - 66.9 ]</td>
<td>6.7 [ 4.7 - 9.8 ]</td>
<td>62.7 [ 43.2 - 75.9 ]</td>
<td>7.81 [ 5.88 - 8.31 ]</td>
<td>1.85 [ 1.24 - 2.18 ]</td>
<td>0.21 [ 0.15 - 0.37 ]</td>
<td>2.10 [ 1.40 - 2.61 ]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are median and interquartile range [25%-75%].


Significance of difference among groups was evaluated by Steel-Dwass test.

Differences with $P < 0.05$ were taken to be statistically significant, and values not sharing a common superscript are significantly different (uppercase).

Significance of difference between male and female was evaluated by Mann-Whitney’s U test, *$P < 0.05$ (lowercase).
Table 2. Comparisons of the correlations with various parameters among PlsCho, PlsCho/PL and HDL-C.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.293</td>
<td>-0.333</td>
<td>-0.084</td>
<td>-0.091</td>
<td>-0.174</td>
<td>-0.135</td>
<td>-0.171</td>
<td>-0.057</td>
<td>-0.213</td>
</tr>
<tr>
<td>PlsCho/PL</td>
<td>-0.408</td>
<td>-0.460</td>
<td>-0.269</td>
<td>-0.314</td>
<td>-0.272</td>
<td>-0.337</td>
<td>-0.440</td>
<td>-0.237</td>
<td>-0.398</td>
</tr>
<tr>
<td>HDL-C</td>
<td>-0.453</td>
<td>-0.457</td>
<td>-0.106</td>
<td>-0.102</td>
<td>-0.218</td>
<td>-0.196</td>
<td>-0.265</td>
<td>-0.134</td>
<td>-0.327</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HDL-C</th>
<th>LDL-C</th>
<th>TG</th>
<th>AIP</th>
<th>sdLDL #</th>
<th>hsCRP #</th>
<th>Hcy#</th>
<th>Adiponectin #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PlsCho</td>
<td>0.663</td>
<td>0.025</td>
<td>-0.363</td>
<td>-0.508</td>
<td>-0.253</td>
<td>-0.256</td>
<td>-0.196</td>
</tr>
<tr>
<td>PlsCho/PL</td>
<td>0.472</td>
<td>0.342</td>
<td>-0.648</td>
<td>-0.654</td>
<td>-0.455</td>
<td>-0.266</td>
<td>-0.174</td>
</tr>
<tr>
<td>HDL-C</td>
<td>-0.241</td>
<td>-0.545</td>
<td>-0.794</td>
<td>-0.404</td>
<td>-0.329</td>
<td>-0.202</td>
<td>0.401</td>
</tr>
</tbody>
</table>

Values represent Spearman’s correlation coefficient by rank test.

# indicates data from over 40 years old (n=206).

* p < 0.05 ** p < 0.01 *** p < 0.001
Table 3. Comparisons of the correlations with various parameters for over 40-year-old male subjects among individual class or subclass of glycerophospholipids containing oleic acid in sn-2

<table>
<thead>
<tr>
<th>Molecular species</th>
<th>Waist circumference</th>
<th>HDL-C</th>
<th>TG</th>
<th>ATP</th>
<th>sdLDL</th>
<th>Adiponectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmalogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cho16:0-18:1</td>
<td>-0.418</td>
<td>0.730</td>
<td>-0.513</td>
<td>-0.640</td>
<td>-0.294</td>
<td>0.458</td>
</tr>
<tr>
<td>Cho18:0-18:1</td>
<td>-0.378</td>
<td>0.607</td>
<td>-0.504</td>
<td>-0.598</td>
<td>-0.347</td>
<td>0.403</td>
</tr>
<tr>
<td>Cho18:1-18:1</td>
<td>-0.201</td>
<td>0.415</td>
<td>-0.253</td>
<td>-0.338</td>
<td>-0.152</td>
<td>0.265</td>
</tr>
<tr>
<td>Cho sn-2 18:1</td>
<td>-0.421</td>
<td>0.742</td>
<td>-0.528</td>
<td>-0.656</td>
<td>-0.312</td>
<td>0.465</td>
</tr>
<tr>
<td>Alkylphospholipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cho16:0-18:1</td>
<td>-0.266</td>
<td>0.391</td>
<td>-0.260</td>
<td>-0.326</td>
<td>-0.126</td>
<td>0.190</td>
</tr>
<tr>
<td>Cho18:0-18:1</td>
<td>-0.262</td>
<td>0.361</td>
<td>-0.305</td>
<td>-0.348</td>
<td>-0.170</td>
<td>0.191</td>
</tr>
<tr>
<td>Cho18:1-18:1</td>
<td>-0.292</td>
<td>0.318</td>
<td>-0.239</td>
<td>-0.282</td>
<td>-0.148</td>
<td>0.167</td>
</tr>
<tr>
<td>Cho sn-2 18:1</td>
<td>-0.281</td>
<td>0.381</td>
<td>-0.270</td>
<td>-0.329</td>
<td>-0.144</td>
<td>0.186</td>
</tr>
<tr>
<td>Diacylphospholipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cho16:0-18:1</td>
<td>0.122</td>
<td>0.077</td>
<td>0.392</td>
<td>0.269</td>
<td>0.317</td>
<td>-0.143</td>
</tr>
<tr>
<td>Cho18:0-18:1</td>
<td>0.088</td>
<td>0.077</td>
<td>0.316</td>
<td>0.213</td>
<td>0.271</td>
<td>-0.121</td>
</tr>
<tr>
<td>Cho18:1-18:1</td>
<td>-0.012</td>
<td>0.055</td>
<td>0.235</td>
<td>0.160</td>
<td>0.198</td>
<td>-0.108</td>
</tr>
<tr>
<td>Cho sn-2 18:1</td>
<td>0.066</td>
<td>0.076</td>
<td>0.347</td>
<td>0.236</td>
<td>0.296</td>
<td>-0.134</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td></td>
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<tr>
<td>Plasmalogen</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Etn16:0-18:1</td>
<td>-0.171</td>
<td>0.348</td>
<td>-0.204</td>
<td>-0.255</td>
<td>-0.067</td>
<td>0.109</td>
</tr>
<tr>
<td>Etn18:0-18:1</td>
<td>-0.061</td>
<td>0.278</td>
<td>-0.129</td>
<td>-0.175</td>
<td>-0.056</td>
<td>0.125</td>
</tr>
<tr>
<td>Etn18:1-18:1</td>
<td>-0.210</td>
<td>0.322</td>
<td>-0.259</td>
<td>-0.295</td>
<td>-0.120</td>
<td>0.103</td>
</tr>
<tr>
<td>Etn sn-2 18:1</td>
<td>-0.131</td>
<td>0.361</td>
<td>-0.221</td>
<td>-0.273</td>
<td>-0.095</td>
<td>0.135</td>
</tr>
<tr>
<td>Alkylphospholipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etn16:0-18:1</td>
<td>-0.180</td>
<td>0.301</td>
<td>-0.091</td>
<td>-0.160</td>
<td>0.069</td>
<td>0.114</td>
</tr>
<tr>
<td>Etn18:0-18:1</td>
<td>-0.150</td>
<td>0.156</td>
<td>-0.054</td>
<td>-0.084</td>
<td>-0.057</td>
<td>0.087</td>
</tr>
<tr>
<td>Etn18:1-18:1</td>
<td>-0.140</td>
<td>0.249</td>
<td>-0.076</td>
<td>-0.134</td>
<td>0.018</td>
<td>0.072</td>
</tr>
<tr>
<td>Etn sn-2 18:1</td>
<td>-0.179</td>
<td>0.300</td>
<td>-0.090</td>
<td>-0.160</td>
<td>0.043</td>
<td>0.118</td>
</tr>
<tr>
<td>Diacylphospholipid</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etn16:0-18:1</td>
<td>0.243</td>
<td>-0.209</td>
<td>0.606</td>
<td>0.533</td>
<td>0.327</td>
<td>-0.219</td>
</tr>
<tr>
<td>Etn18:0-18:1</td>
<td>0.287</td>
<td>-0.253</td>
<td>0.596</td>
<td>0.540</td>
<td>0.343</td>
<td>-0.219</td>
</tr>
<tr>
<td>Etn18:1-18:1</td>
<td>0.212</td>
<td>-0.307</td>
<td>0.659</td>
<td>0.603</td>
<td>0.376</td>
<td>-0.221</td>
</tr>
<tr>
<td>Etn sn-2 18:1</td>
<td>0.239</td>
<td>-0.289</td>
<td>0.656</td>
<td>0.596</td>
<td>0.373</td>
<td>-0.223</td>
</tr>
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<td>Total 18:0 in</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>serum lipids</td>
<td>0.330</td>
<td>-0.406</td>
<td>0.761</td>
<td>0.711</td>
<td>0.514</td>
<td>-0.274</td>
</tr>
</tbody>
</table>

Values represent Spearman’s correlation coefficient by rank test, and the values over 0.18 are taken to be statistically significant with P < 0.01.

Identification and quantification of individual ether glycerophospholipid molecular species were performed with the method as described in the Materials and Methods.

Diacylglycerophospholipid molecular species were identified using the mass fragments same as used for identification of alkylphospholipid molecular species. Synthetic 16:0-18:1 of each PtdCho and PtdEtn were used for generating a standard curve to quantify individual diacylphospholipids molecular species.
Fig. 1. Molecular profiles of individual ether glycerophospholipids
**Fig. 2.** Distribution of molecular species in the *sn*-1 and *sn*-2 positions of ether glycerophospholipids
Fig. 3. Overview of serum ether glycerophospholipids
Fig. 4. Correlations between the serum levels of choline plasmalogen molecular species and various parameters.
Fig. 5. Relationship between the proportion of PlsCho with 18:2 in total phospholipids (PlsCho18:2/PL) and age in all subjects (n = 428).
Fig. 6. Correlations between the serum levels of plasmalogen molecular species and HDL-C.