

The Proportion of Nervonic Acid in Serum Lipids is Associated with Serum Plasmalogen Levels and Metabolic Syndrome

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Abstract: An increase in serum plasmalogens (1-*O*-alk-1-enyl-2-acyl glycerophospholipids), which are endogenous anti-oxidative phospholipids, can potentially prevent age-related diseases such as atherosclerosis and metabolic syndrome (MetS). Very long chain fatty acids (VLCFAs) in plasma may supply the materials for plasmalogen biosynthesis through peroxisomal beta-oxidation. On the other hand, elevated levels of saturated and monounsaturated VLCFAs in plasma appear to be associated with decreased peroxisomal function, and are a symptom of age-related diseases. To reconcile these contradictory findings, we attempted to investigate the relationship between the serum levels of saturated and monounsaturated VLCFAs, clinical and biochemical parameters, and serum levels of plasmalogens in subjects with MetS (n = 117), who were asymptomatic Japanese males over 40 years of age. Fatty acids in serum lipids were quantified using gas chromatography/mass spectrometry (GC/MS). Serum plasmalogen levels were determined by liquid chromatography using radioactive iodine (¹²⁵I-HPLC), and the molecular composition of serum plasmalogens was analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). We found that MetS subjects showed a significant reduction in the proportion of specific saturated and monounsaturated VLCFAs such as behenic acid (C22:0), lignoceric acid (C24:0), and nervonic acid (C24:1) in serum lipids compared to non-MetS subjects. These VLCFAs were positively associated with serum levels of high density lipoprotein cholesterol (HDL-C) as well as plasmalogen-related parameters, and inversely with serum levels of triglyceride (TG) and small dense low density lipoprotein cholesterol (sdLDL-C). In conclusion, the proportion of nervonic acid in serum lipids is associated with serum levels of plasmalogens and with MetS, and probably reflects the peroxisomal dysfunction and enhancement of endoplasmic reticulum (ER) stress seen in common age-related diseases.

Key words: very long chain fatty acid, nervonic acid, plasmalogen, metabolic syndrome, peroxisomal dysfunction

1 INTRODUCTION

Plasmalogens are a subclass of glycerophospholipids that are characterized by a vinyl-ether bond at the *sn*-1 position of the glycerol backbone. It has been suggested that serum plasmalogen levels are associated with diverse clinical manifestations¹. Recently, particular attention has been paid to the involvement of plasmalogens in metabolic diseases associated with oxidative stress and chronic inflammation²⁻⁶. Some studies have postulated that plasmalo-

gens serve as endogenous antioxidants and protect membrane lipids and lipoprotein particles from excessive oxidation by scavenging reactive oxygen species via the vinyl-ether moiety⁷⁻⁹. Therefore, an increase in serum plasmalogens may contribute to good health through the prevention of oxidative stress. Accordingly, it is important to identify the factor involved in the enhancement of plasmalogen biosynthesis.

The first two steps in plasmalogen biosynthesis take

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place exclusively in peroxisomes, with some patients with peroxisomal disorders exhibiting a systemic reduction in plasmalogen synthesis. It has recently been reported that plasmalogen synthesis is controlled by posttranslational regulation of fatty acyl-CoA reductase 1 (Far1), which converts a fatty acyl-CoA into a fatty alcohol in the peroxisome, subsequently introducing a fatty alcohol to the glycerol backbone at the *sn*-1 position as an alkenyl chain¹⁰. Although the origin of fatty acyl-CoA, which is a substrate for Far1, is still unknown, it is possibly derived from the synthesis of acetyl CoA from the peroxisomal beta-oxidation of VLCFAs such as lignoceric acid (C24:0)¹¹. If so, VLCFAs and peroxisome proliferator-activated receptor-alpha (PPAR α) agonists may contribute to increased serum plasmalogens by supplying materials for plasmalogen biosynthesis and activating peroxisomal beta-oxidation, respectively. Several studies suggest a link between peroxisomal beta-oxidation of VLCFAs and plasmalogen biosynthesis^{12,13}. In particular, nervonic acid (C24:1) is beta-oxidized preferentially in peroxisomes¹⁴, and is abundant in brain myelin, where high levels of plasmalogens are also found¹.

On the other hand, accumulation of saturated VLCFAs such as hexacosanoic acid (C26:0) in plasma, which probably results from dysfunctional peroxisomal beta-oxidation, is considered to be a predisposing factor and diagnostic marker for peroxisomal disorders¹⁵. Furthermore, the evidence suggests that peroxisomal dysfunction is associated not only with rare peroxisomal disorders, but also with more common age-related diseases including neurodegenerative disorders such as Alzheimer's disease¹⁶, as well as metabolic diseases related to oxidative stress and chronic inflammation^{17,18}. Thus, elevated plasma-saturated VLCFAs seem to be an indication of peroxisomal dysfunction as well as a symptom of age-related diseases^{19,20}.

These contradictory findings led us to investigate the relationship between the serum levels of saturated and monounsaturated VLCFAs, clinical and biochemical parameters, and serum levels of plasmalogens. The study was performed in Japanese asymptomatic males aged over 40 years with MetS ($n = 117$). Fatty acids in serum lipids were quantified using GC/MS. Serum plasmalogen levels were determined by liquid chromatography using radioactive iodine (¹²⁵I-HPLC), and the molecular composition of serum plasmalogen was analyzed by LC/MS/MS. We found that the proportions of saturated and monounsaturated VLCFAs such as nervonic acid in serum lipids were associated with serum levels of plasmalogens and with MetS, which suggests an involvement in the peroxisomal dysfunction and enhancement of ER stress seen in common age-related diseases.

2 MATERIALS & METHODS

2.1 Study subjects and design

Asymptomatic male subjects ($n = 117$; age, 40–66 years old) not on medication who were referred for routine health examinations 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 the blood collection tube containing coagulation accelerating agent by centrifugation at $1,500 \times g$ for 10 min, and were added with BHT (final concentration of 10 μ M) and EDTA (final concentration of 1 mM) as antioxidants, followed by immediate freezing at -80°C until analysis. Measurements of body weight, stature, waist circumference, and blood pressure were recorded.

2.2 Sample preparation for quantification of fatty acids in serum lipids

Lipid samples were prepared using a slightly modified direct transesterification method described by Lepage & Roy²¹. In brief, tridecanoic acid (C13:0) as an internal standard, dissolved in 1 mL of methanol/benzene (3:2, by vol), was added to an aliquot of 100 μ L of serum. One milliliter of freshly prepared acetyl chloride/methanol (5:100, by vol) was then added to each tube, which was capped and subjected to methanolysis at 100°C for 2 h. After cooling the tube to room temperature, 1 mL of hexane and 1 mL of water were added. The tubes were then shaken, centrifuged, and the hexane layer harvested. This procedure was repeated three times, and the hexane layer collected was stored at 4°C until analysis.

2.3 GC/MS analysis

Gas chromatography/mass spectrometry was conducted according to a slightly modified procedure described by Takemoto *et al.*²². In brief, methylated samples were subjected to a GC/MS system (GC; Agilent 7890A, MSD; Agilent 7975C, Tokyo, Japan) with a capillary INNOWAX column (30 m, 0.25 mm, Hewlett-Packard, Palo Alto, CA, USA). The initial oven temperature was 100°C , which was increased to 240°C ($15^{\circ}\text{C}/\text{min}$) and maintained for 15 min. One microliter of sample was injected into the GC/MS system in the splitless mode. Electron impact (EI) ionization was applied at 70 eV. Mass spectrometry acquisition of each substance was set to the selected-ion monitoring (SIM) mode. Intra-assay ($n = 10$) CV using C13:0, palmitic acid (C16:0), oleic acid (C18:1), arachidonic acid (C20:4), and C24:1 ranged from 1.2 to 6.1%.

2.4 Measurement of serum plasmalogen levels

Serum plasmalogen concentrations were determined by our original method using radioactive iodine (¹²⁵I) and high-performance liquid chromatography (¹²⁵I-HPLC)^{23,24}. In

brief, total lipids were extracted from serum using diethyl ether/ethanol (1:8, by vol). Lipid samples were mixed with iodine-125 reagent, prepared as described previously²³⁾, and stood overnight at 4°C to allow the specific and quantitative formation of iodine bound plasmalogens. Liquid chromatography separation was performed using a Prominence UFLC system (LC-20AD, Shimadzu, Kyoto, Japan) with a LiChrospher 100 Diol column (5 µm, 250 mm × 4 mm i.d.; Merck, Darmstadt, Germany) at 40°C and a flow rate of 1 mL/min. The sample was eluted with isocratic mobile phase A/B (65:35, by vol); A consisted of acetonitrile, and B consisted of acetonitrile/H₂O/acetic acid/NH₃ (80:19.7:0.2:0.1, by vol). The total elution time was 15 min. Peak areas were detected using a flow γ-counter (FC-3300, Bioscan Inc., NW, USA), and analyzed using SmartChrom software (KYA TECH Co., Tokyo, Japan).

2.5 Determination of molecular composition of serum plasmalogens

Total serum lipids were extracted using chloroform/methanol (1:2, by vol). Plasmalogens in the extracted lipids were analyzed by LC/MS/MS. Synthetic 18:0-18:1, 18:0-20:4, and 18:0-22:6 of each choline plasmalogen (PlsCho) and ethanolamine plasmalogen (PlsEtn; Avanti Polar Lipids, Inc., Alabaster, AL, USA) were used not only to generate a standard curve to quantify individual molecular species of plasmalogen, but also to assess the precision and accuracy of detection, as described previously²⁵⁾. The concentrations of individual molecular species of plasmalogens were determined using corresponding internal standards (1,2-dimyristoyl phosphatidylcholine; DMPC for PlsCho and 1,2-dimyristoyl phosphatidylethanolamine; DMPE for PlsEtn; Avanti Polar Lipids). The mean recovery of 18:0-18:1 and 18:0-20:4 of each PlsCho and PlsEtn, 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%. Twenty-nine molecular species were recorded for each class of plasmalogen, all of which were major molecules determined by LC/MS/MS.

2.6 LC/MS/MS analysis

Liquid chromatography-tandem mass spectrometry was conducted following a slightly modified method described previously²⁶⁾. 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, 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: 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 PlsCho, and 20 eV for PlsEtn. Data acquisition was performed with Xcalibur 1.3 software (Thermo Fisher Scientific Inc.). PlsEtn was quantified according to the procedure described by Zemski & Murphy²⁷⁾. 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. Choline plasmalogens 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 UPLC²⁸⁾. The presence of plasmalogens was further confirmed by the disappearance of the peak upon treatment with acid by exposing to HCL vapor for 10 min.

2.7 Biochemical analysis

Serum concentrations of triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (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 (sdLDL-C) 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).

2.8 Statistical analysis

Statistical analysis was performed using Excel (2010; Microsoft Corporation, Redmond, WA) with the add-in software Statcel 3 (OMS, Tokyo, Japan). All values are shown as mean ± SD. Results were analyzed by unpaired t-test (Fig. 1, Tables 1 and 3). Correlations among measurements were assessed with Pearson's product-moment correlation coefficient (Table 5).

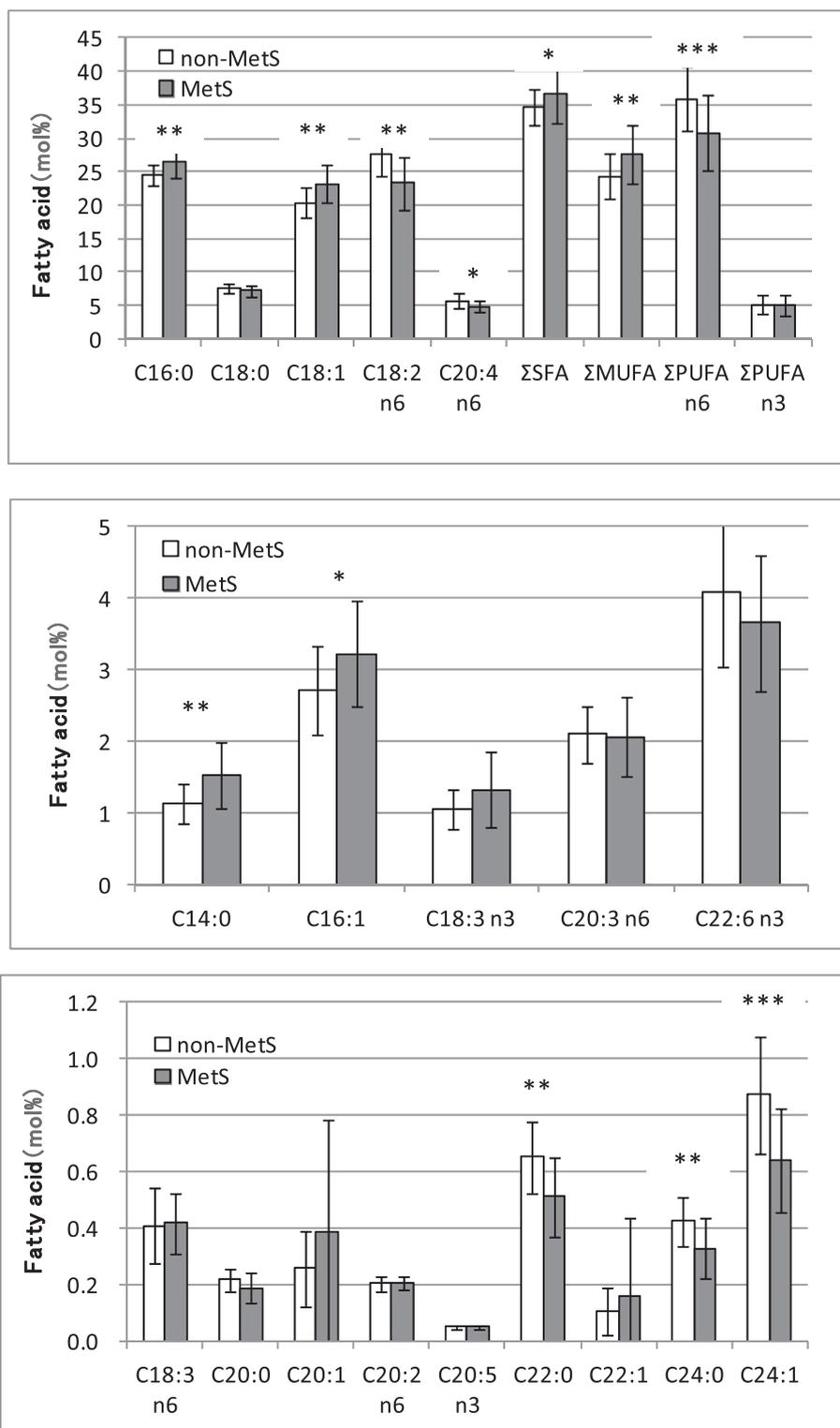


Fig. 1 Comparison of fatty acid composition (mol%) of serum lipids between non-MetS and MetS subjects. Bars (open; non-MetS, closed; MetS) represent the mean composition (%; non-MetS; n = 102, MetS; n = 15) of individual fatty acids in serum lipids. Error bars represent SD of mean values. The significance of differences between non-MetS and MetS subjects was evaluated by an unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Σ SFA; sum of all saturated fatty acids, Σ MUFA; sum of all monounsaturated fatty acids, Σ PUFA n6; sum of all n-6 series of polyunsaturated fatty acids, Σ PUFA n3; sum of all n-3 series of polyunsaturated fatty acids.

Table 1 Clinical parameters and biochemical measurements of the subjects.

	non-MetS n=102		MetS n=15	
Age	47.6 ±	6.3	49.9 ±	6.3
Height (cm)	169.6 ±	5.4	171.7 ±	6.1
Weight (kg)	65.2 ±	8.7	79.2 ±	6.7***
BMI (kg/m ²)	22.6 ±	2.6	26.9 ±	2.6***
Waist circumference (cm)	79.5 ±	6.4	91.8 ±	5.1***
s.p. (mmHg)	122 ±	12	131 ±	11**
d.p. (mmHg)	79 ±	9	86 ±	10*
Blood glucose (mg/dl)	92.9 ±	8.1	107.1 ±	11.2***
UA (mg/dl)	6.2 ±	1.5	7.0 ±	1.5
AST (U/l)	24.9 ±	10.1	29.0 ±	14.7
ALT (U/l)	27.0 ±	16.8	36.7 ±	29.2
γ-GTP (U/l)	54.4 ±	60.4	77.8 ±	35.8*
HDL-C (mg/dl)	68.2 ±	17.6	49.3 ±	11.9***
LDL-C (mg/dl)	122.5 ±	24.7	136.9 ±	21.9*
TG (mg/dl)	100.8 ±	58.1	260.4 ±	141.3***
sdLDL (mg/dl)	30.6 ±	11.8	46.0 ±	12.2***
hsCRP (μg/dl)	76.8 ±	75.0	126.8 ±	65.3*
Homocysteine (pmol/l)	11.2 ±	5.5	10.4 ±	2.0
Adiponectin (μg/ml)	7.0 ±	4.1	3.7 ±	1.6***

Values show means ± SD

Significance of difference between non-MetS and MetS males aged over 40 years was evaluated by unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3 RESULTS

3.1 Characteristics of study subjects

Clinical parameters and serum biochemical measurements of the subjects are shown in **Table 1** according to the presence or absence of MetS (non-MetS; $n = 102$, MetS; $n = 15$). The data indicate that there were significant differences in various clinical and biochemical parameters between non-MetS and MetS, including weight, body mass index (BMI), waist circumference, systolic (s.p.) and diastolic blood pressure (d.p.), blood glucose, serum levels of γ-GTP, HDL-C, LDL-C, sdLDL-C, TG, hsCRP, as well as adiponectin.

3.2 Fatty acids in serum lipids

The concentration (μM) and composition (mol%) of fatty acids in serum lipids for the study subjects (male, average age 47.9 ± 6.3 , $n = 117$) are shown in **Table 2**. A large fraction of the fatty acids were higher than the upper limit of the reference values provided (male; $n = 125$, aged 21-64 years; female; $n = 132$, aged 21-64 years; SRL Inc.). High values which exceeded reference data in the study may be

due to the influences of gender and age. Eicosapentaenoic acid (EPA, C20:5) on the other hand showed values 1/5-1/50 less than the reference value. The large difference in C20:5 values may be due to the low separation of C20:5 from C20:4 in gas chromatography (**Table 2**).

Significant differences in variable fatty acids were shown between non-MetS and MetS (**Fig. 1**). In addition to linoleic acid (C18:2) and C20:4 among the polyunsaturated fatty acids (PUFAs), the compositions (mol%) of particular saturated and monounsaturated VLCFAs such as C22:0, C24:0 and C24:1 were significantly reduced in MetS compared to non-MetS subjects (**Fig. 1**).

3.3 Serum plasmalogens

Serum concentrations of each class of plasmalogens, i.e., PlsCho and PlsEtn, as well as PL, along with the class ratio (PlsCho/PlsEtn) and their proportions (mol%) in total phospholipids (PlsCho/PL, PlsEtn/PL), are summarized for non-MetS and MetS subjects in **Table 3**. Compared to non-MetS subjects, MetS subjects showed significantly lower levels of PlsCho and higher levels of PL, with a resultant

Table 2 Concentration and composition of fatty acids in serum lipids.

Fatty acid	Serum concentration (μM)		Composition (mol%)
	measurement	references*	
C12:0	16.2 \pm 11.0	< 41.9	0.12 \pm 0.07
C14:0	163.3 \pm 86.4	25.4~ 173.8	1.19 \pm 0.33
C16:0	3327.3 \pm 1098.4	1097.8~3078.5	24.80 \pm 1.74
C18:0	988.7 \pm 258.1	367.3~ 891.5	7.49 \pm 0.66
C20:0	27.5 \pm 6.5	14.1~ 27.5	0.21 \pm 0.04
C22:0	81.8 \pm 14.8	28.5~ 55.2	0.64 \pm 0.13
C24:0	52.7 \pm 11.5	28.8~ 52.9	0.41 \pm 0.09
ΣSFA	4604.8		34.48
C16:1	381.5 \pm 178.3	61.7~ 310.5	2.79 \pm 0.66
C18:1	2787.3 \pm 1086.1	689.3~2712.6	20.53 \pm 2.46
C20:1	36.6 \pm 36.5	4.8~ 30.6	0.26 \pm 0.17
C22:1	15.0 \pm 22.5	< 6.5	0.11 \pm 0.11
C24:1	107.9 \pm 24.7	59.7~ 113.2	0.85 \pm 0.22
ΣMUSFA	2946.8		22.07
C18:2 n-6	3528.8 \pm 734.1	1423.1~3386.8	27.08 \pm 3.46
C18:3 n-6	54.4 \pm 23.7	6.5~ 73.6	0.41 \pm 0.13
C20:2 n-6	26.9 \pm 8.8	7.8~ 23.3	0.20 \pm 0.02
C20:3 n-6	277.9 \pm 84.0	35.6~ 153.0	2.11 \pm 0.41
C20:4 n-6	731.2 \pm 194.7	279.5~ 682.5	5.62 \pm 1.13
$\Sigma\text{PUSFA n-6}$	4619.2		34.59
C18:3 n-3	148.5 \pm 73.7	23.7~ 131.5	1.09 \pm 0.31
C20:5 n-3	6.5 \pm 1.9	38.4~ 354.4	0.05 \pm 0.01
C22:6 n-3	530.1 \pm 173.4	147.9~ 463.5	4.04 \pm 1.00
$\Sigma\text{PUSFA n-3}$	685.0		5.13
Total	13353.1 \pm 3667.2		100.00

Values show means \pm SD

* references data from SRL Inc.

decline in the PlsCho/PlsEtn, PlsCho/PL, and PlsEtn/PL ratios.

The molecular composition of each class of plasmalogens is shown in **Table 4**. In the long-chain fatty alcohol species in the *sn*-1 position of plasmalogens, hexadecanol (16:0) was the predominant fatty alcohol species in PlsCho, whereas three alcohol species were distributed almost equally in PlsEtn. Arachidonic acid, the major fatty acid in the *sn*-2 position, was common in both classes of plasmalogens. In addition, docosahexaenoic acid (DHA; C22:6) levels were high in PlsEtn, and C18:2 was specifically abundant in PlsCho.

3.4 Relationship between specific saturated and monounsaturated VLCFAs and biochemical and plasmalo-

gen-related parameters

We found that the proportions of specific saturated and monounsaturated VLCFAs such as C22:0, C24:0 and C24:1, as well as C20:4, in serum lipids were strongly associated with lipid-related risk factors for atherogenic status, i.e., HDL-C, TG and sdLDL-C (**Fig. 2**). These fatty acids correlated positively with serum levels of HDL-C, and inversely with serum levels of TG and sdLDL-C.

The interrelationship among these fatty acids and the relationship between fatty acids and plasmalogen-related parameters is shown in **Table 5**. In particular, these VLCFAs indicated strong associations, suggesting that they are metabolized through an identical pathway. In addition, these saturated and monounsaturated VLCFAs, especially C24:1, as well as C20:4, showed a significant positive corre-

Table 3 Comparison of serum plasmalogen-related parameters and total phospholipid concentration between non-MetS and MetS.

	non-MetS n=102	MetS n=15
PlsCho (μM)	64.2 ± 13.0	56.5 ± 8.9**
PlsEtn (μM)	74.3 ± 22.6	80.3 ± 15.2
Pls(Cho+Etn) (μM)	138.4 ± 33.2	136.7 ± 21.7
PlsCho/PlsEtn ratio	0.90 ± 0.18	0.72 ± 0.11***
PL (mM)	3.11 ± 0.51	3.48 ± 0.49*
PlsCho/PL (%)	2.10 ± 0.44	1.65 ± 0.32***
PlsEtn/PL (%)	2.41 ± 0.70	2.33 ± 0.43***
Pls(Cho+Etn)/PL (%)	4.51 ± 1.05	3.98 ± 0.68***

Values show means ± SD

Significance of difference between non-MetS and MetS males aged over 40 years was evaluated by unpaired t-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

lation with plasmalogen-related parameters, i.e., the serum levels of PlsCho and PlsEtn, and the proportions of PlsCho (PlsCho/PL) or total plasmalogens in total phospholipids ((PlsCho + PlsEtn)/PL) (Table 5).

4 DISCUSSION

The proportion of subjects with MetS in the present study (12.8%) was unexpectedly low when compared with that of the Japanese middle-aged male population (20-25%), and was probably due to the exclusion of subjects taking medications such as hypoglycemic, hypotensive, or hypolipidemic drugs.

We found that MetS subjects showed a significant reduction in the proportions of specific saturated and monounsaturated VLCFAs such as C22:0, C24:0 and C24:1, as well as C18:2 and C20:4 in serum lipids compared to non-MetS subjects (Fig. 1). These VLCFAs as well as C20:4 were positively associated with serum levels of HDL-C, and inversely with serum levels of TG and sdLDL-C (Fig. 2). The results suggest that these VLCFAs are a potential indicator of MetS, because the dyslipidemia of MetS is characterized by the low level of HDL-C and high levels of TG and sdLDL²⁹⁾. However, previous studies have reported that an increase in saturated VLCFAs such as C26:0 in red blood cells was associated with atherosclerosis and MetS^{19, 20)}. In the present study, although we could not quantify C26:0 in serum lipids of apparently healthy subjects as the concentrations were too low to detect, other saturated and monounsaturated VLCFAs showed different results to previous studies.

Elevated plasma and tissue levels of both saturated

Table 4 Molecular composition of serum plasmalogens.

		PlsCho	PlsEtn	
<i>sn</i> -1	<i>sn</i> -2	mol %	mol %	
16:0	16:0	4.77 ± 0.69	0.02 ± 0.02	
	18:1	8.24 ± 1.12	1.08 ± 0.31	
	18:2	19.71 ± 2.60	3.26 ± 0.98	
	18:3	0.32 ± 0.08	0.01 ± 0.02	
	20:4	22.81 ± 3.07	11.21 ± 2.23	
	20:5	4.83 ± 1.91	3.95 ± 1.72	
	22:4	0.98 ± 0.37	0.35 ± 0.20	
	22:5	2.62 ± 0.48	2.44 ± 0.48	
	22:6	7.78 ± 1.47	12.61 ± 2.02	
	18:0	16:0	n.d.	0.04 ± 0.05
		18:0	n.d.	0.03 ± 0.03
		18:1	0.67 ± 0.17	1.35 ± 0.43
		18:2	3.29 ± 1.03	5.09 ± 1.60
		18:3	n.d.	0.04 ± 0.05
20:4		3.63 ± 0.78	15.38 ± 2.93	
20:5		2.36 ± 0.59	7.63 ± 2.97	
22:4		0.03 ± 0.02	0.11 ± 0.10	
22:5		0.22 ± 0.13	1.43 ± 0.37	
22:6		1.97 ± 0.46	10.56 ± 1.52	
18:1		16:0	n.d.	0.29 ± 0.15
		18:0	n.d.	0.02 ± 0.03
		18:1	0.45 ± 0.17	0.99 ± 0.34
		18:2	5.56 ± 1.99	2.54 ± 0.72
	18:3	n.d.	0.02 ± 0.04	
	20:4	4.14 ± 0.83	8.82 ± 1.84	
	20:5	0.96 ± 0.36	2.84 ± 1.20	
	22:4	n.d.	0.10 ± 0.08	
	22:5	n.d.	0.86 ± 0.27	
	22:6	1.98 ± 0.49	6.90 ± 1.14	
	<i>sn</i> -2	16:0	4.77 ± 0.69	0.35 ± 0.16
		18:0	2.67 ± 0.53	0.09 ± 0.07
		18:1	9.36 ± 1.22	3.42 ± 0.85
		18:2	28.56 ± 3.64	10.89 ± 2.96
18:3		0.32 ± 0.08	0.07 ± 0.07	
20:4		30.58 ± 4.02	35.41 ± 5.99	
20:5		8.16 ± 2.68	14.42 ± 5.48	
22:4		1.01 ± 0.37	0.56 ± 0.26	
22:5		2.84 ± 0.52	4.73 ± 0.81	
22:6		11.73 ± 2.09	30.06 ± 3.59	
<i>sn</i> -1		16:0	74.74 ± 2.75	34.95 ± 3.17
		18:0	12.17 ± 1.71	41.67 ± 3.25
		18:1	13.10 ± 2.08	23.38 ± 2.14

Values show means ± SD, n.d.; not detected

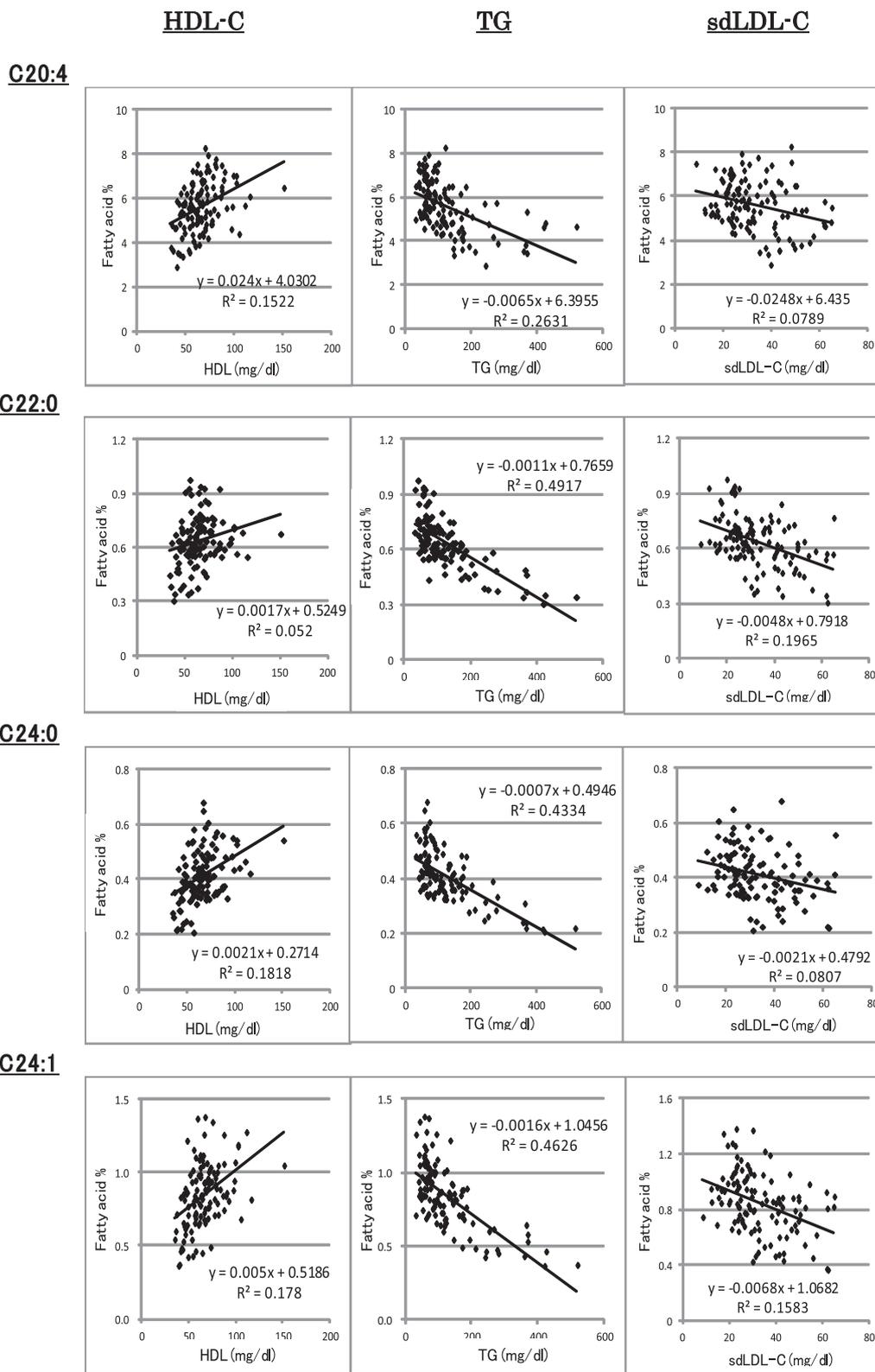


Fig. 2 Relationship between target fatty acids and HDL-C, TG, or sdLDL-C in all subjects (n = 117).

(C24:0, C26:0) and monounsaturated (C26:1) VLCFAs are typically observed in X-linked adrenoleukodystrophy (X-

ALD), the most common peroxisomal disorder. The elevation of these VLCFAs in X-ALD is thought to result from

Table 5 Correlations between serum levels of fatty acids and plasmalogen-related parameters.

Fatty acid & Pls parameter	C20:4	C22:0	C24:0	C24:1
C20:4				
C22:0	0.264**			
C24:0	0.299**	0.753***		
C24:1	0.415***	0.595***	0.635***	
PlsCho	0.484***		0.279**	0.352**
PlsEtn	0.281**		0.226*	
PlsCho/PL	0.473***	0.383***	0.285**	0.449***
Pls (Cho+Etn)/PL	0.406***	0.290**	0.210*	0.407***

Values show Pearson's product-moment correlation coefficient

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

increased elongation of chain length in fatty acids, in addition to reduced peroxisomal beta-oxidation activity³⁰). The treatment of X-ALD patients with "Lorenzo's oil"³¹, which consists of supplements of C18:1 and erucic acid (C22:1), resulted not only in normalization of C26:0 levels, but also a significant increase in C24:1³⁰, probably by competition for the microsomal elongation system³². Gene expression of Elov13, a microsomal enzyme involved in VLCFA elongation, may be under the control of PPAR α ³³, which is well known to regulate peroxisomal beta-oxidation. In patients with a deficiency in acyl-CoA oxidase (Acox1), the first and rate-limiting enzyme of the peroxisomal beta-oxidation pathway, plasma VLCFA is markedly elevated¹⁵. Interestingly, the restoration of ACOX1 expression in ACOX1-deficient mice strongly increased C24:1 levels³⁴. These findings suggest that the peroxisome and ER act in concert to regulate plasma VLCFAs. Age-related diseases such as atherosclerosis and MetS lead to a decline in peroxisomal activity and enhance ER stress^{35, 36}, causing a severe imbalance in the synthesis and degradation of VLCFAs, which may eventually result in increased C26:0 and decreased C24:1 in plasma, although the mechanism is unknown.

Similarly to C22:0, C24:0 and C24:1, serum levels of PlsCho and the resultant PlsCho/PlsEtn and PlsCho/PL ratios of MetS subjects were significantly reduced compared to non-MetS subjects (Table 3). This result is in agreement with our previous study, in which hyperlipidemic subjects with MetS showed a considerable reduction in serum levels of both classes of plasmalogens compared to those without MetS³⁷. In addition, these VLCFAs as well as C20:4 (% in serum lipids) showed a significant positive correlation, particularly with PlsCho-related parameters (Table 5). As both classes of plasmalogens are rich in C20:4 (Table 4), it may be reasonable to suppose that there is a strong association between C20:4 and plasmalogen-related parameters. It is interesting to note that these

VLCFAs, especially C24:1, are positively associated with plasmalogen-related parameters, despite the fact that plasmalogens are free from these VLCFAs (Table 4). In serum, these VLCFAs are largely contained in sphingolipids, the elevation of which is deemed to be a risk parameter for atherosclerosis³⁸. However, these VLCFAs are minor components of sphingolipids. Interestingly, ingestion of edible oils which contain massive monounsaturated VLCFAs such as C22:1 and C24:1 resulted in significantly increased serum plasmalogens in rats³⁹. However, until now, there have been no reports of direct evidence suggesting a link between plasmalogen biosynthesis and C24:1. Therefore the significant positive correlation of C24:1 in serum lipids with serum levels of plasmalogens may stem from decreased peroxisomal function and augmentation of ER stress, as plasmalogen biosynthesis involves both organelles⁴⁰.

5 CONCLUSION

The proportion of nervonic acid (C24:1) in serum lipids was associated with MetS and with levels of serum plasmalogens, especially PlsCho. This is probably a reflection of peroxisomal dysfunction and the enhancement of ER stress seen in common age-related diseases.

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References

- 1) Braverman, N. E.; Moser, A. B. Functions of plasmalogen lipids in health and disease. *Biochem. Biophys. Acta* **1822**, 1442-1452 (2012).
- 2) Wallner, S.; Schmitz, G. Plasmalogens the neglected regulatory and scavenging lipid species. *Chem. Phys. Lipids* **164**, 573-589 (2011).
- 3) Colas, R.; Sassolas, A.; Guichardant, M.; Cugnet-Anceau, C.; Moret, M.; Moulin, P.; Lagarde, M.; Calzada, C. LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets. *Diabetologia* **54**, 2931-2940 (2011).
- 4) Stenvinkel, P.; Diczfalusy, U.; Lindholm, B.; Heimbürger, O. Phospholipid plasmalogen, a surrogate marker of oxidative stress, is associated with increased cardiovascular mortality in patients on renal replacement therapy. *Nephrol. Dial. Transplant.* **19**, 972-976 (2004).
- 5) Graessler, J.; Schwudke, D.; Schwarz, P. E.; Herzog, R.; Shevchenko, A.; Bornstein, S. R. Top-down lipidomics reveals ether lipid deficiency in blood plasma of hypertensive patients. *PLoS One* **4**, e6261 (2009).
- 6) Maeba, R.; Maeda, T.; Kinoshita, M.; Takao, K.; Takenaka, H.; Kusano, J.; Yoshimura, N.; Takeoka, Y.; Yasuda, D.; Okazaki, T.; Teramoto, T. Plasmalogens in human serum positively correlate with high-density lipoprotein and decrease with aging. *J. Atheroscler. Thromb.* **14**, 12-18 (2007).
- 7) Zoeller, R. A.; Lake, A. C.; Nagan, N.; Gaposchkin, D. P.; Legner, M. A.; Lieberthal, W. Plasmalogens as endogenous antioxidants: somatic cell mutants reveal the importance of vinyl ether. *Biochem. J.* **338**, 769-776 (1999).
- 8) Hahnel, D.; Thiery, J.; Brosche, T.; Engelmann, B. Role of plasmalogens in the enhanced resistance of LDL to copper-induced oxidation after LDL apheresis. *Arterioscler. Thromb. Vasc. Biol.* **19**, 2431-2438 (1999).
- 9) Maeba, R.; Ueta, N. Ethanolamine plasmalogens prevent the oxidation of cholesterol by reducing the oxidizability of cholesterol in phospholipid bilayers. *J. Lipid Res.* **44**, 164-171 (2003).
- 10) Honscho, M.; Asaoku, S.; Fujiki, Y. Posttranslational regulation of fatty acyl-CoA reductase 1, Far1, controls ether glycerophospholipid synthesis. *J. Biol. Chem.* **285**, 8537-8542 (2010).
- 11) Hayashi, H.; Hara, M. 1-Alkenyl group of ethanolamine plasmalogen derives mainly from de novo-synthesized fatty alcohol within peroxisomes, but not extraperoxisomal fatty alcohol or fatty acid. *J. Biochem.* **121**, 978-983 (1997).
- 12) Wei, H.; Kemp, S.; McGuinness M. C.; Moser, A. B.; Smith, K.D. Pharmacological induction of peroxisomes in peroxisome biogenesis disorders. *Ann. Neurol.* **47**, 286-296 (2000).
- 13) Schedin, S.; Sindelar, P. J.; Pentchev, P.; Brunk, U.; Dallner, G. Peroxisomal impairment in Niemann-Pick type C disease. *J. Biol. Chem.* **272**, 6245-6251 (1996).
- 14) Sandhir, R.; Khan, M.; Chahal, A.; Singh, I. Localization of nervonic acid β -oxidation in human and rodent peroxisomes: impaired oxidation in Zellweger syndrome and X-linked adrenoleukodystrophy. *J. Lipid Res.* **39**, 2161-2171 (1998).
- 15) Wanders, R. J. A.; Waterham, H. R. Peroxisomal disorders: The single peroxisomal enzyme deficiencies. *Biochem. Biophys. Acta* **1763**, 1717-1720 (2006).
- 16) Kou, J.; Kovacs, G. G.; Hoftberger, R.; Kulik, W.; Brodde, A.; Forss-Petter, S.; Honigschnabl, S.; Gleiss, A.; Brugger, B.; Wanders, R.; Just, W.; Budka, H.; Jungwirth, S.; Fischer, P.; Berger, J. Peroxisomal alterations in Alzheimer's disease. *Acta Neuropathol.* **122**, 271-283 (2011).
- 17) Nordgren, M.; Fransen, M. Peroxisomal metabolism and oxidative stress. *Biochimie*, in press.
- 18) Perichon, R.; Bourre, J. M.; Kelly, J. F.; Roth, G. S. The role of peroxisomes in aging. *Cell. Mol. Life Sci.* **54**, 641-652 (1988).
- 19) Antoku, Y.; Tsukamoto, K.; Miyoshi, Y.; Nagino, H.; Anzaki, M.; Suwa, K.; Narabe, Y. Correlations of elevated levels of hexacosanoate in erythrocyte membranes with risk factors for atherosclerosis. *Atherosclerosis* **153**, 169-173 (2000).
- 20) Kume, A.; Miyazaki, T.; Kitamura, Y.; Oshida, K.; Yanagisawa, N.; Takizawa, H.; Fujii, K.; Kiyanagi, T.; Sumiyoshi, K.; Ohmura, H.; Mokuno, H.; Shimada, K.; Daida, H. High levels of saturated very long-chain fatty acid (hexacosanoic acid; C26:0) in whole blood are associated with metabolic syndrome in Japanese men. *Diabetes Res. Clin. Pract.* **80**, 259-264 (2008).
- 21) Lepage, G.; Roy, C. C. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *J. Lipid Res.* **25**, 1391-1396 (1984).
- 22) Takemoto, Y.; Suzuki, Y.; Horibe, R.; Shimozaawa, N.; Wanders, R. J. A.; Kondo, N. Gas chromatography/mass spectrometry analysis of very long chain fatty acids, docosahexaenoic acid, phytanic acid and plasmalogen for the screening of peroxisomal disorders. *Brain Dev-Jpn.* **25**, 481-487 (2003).
- 23) Maeba, R.; Ueta, N. Determination of choline and ethanolamine plasmalogens in human plasma by HPLC using radioactive triiodide (1-) ion ($^{125}\text{I}_3^-$). *Anal. Biochem.* **331**, 169-176 (2004).
- 24) Maeba, R.; Yamazaki, Y.; Nezu, T.; Okazaki, T. Improvement and validation of 125I-high-performance liquid chromatography method for determination of total human serum choline and ethanolamine plasmalogens. *Ann. Clin. Biochem.* **49**, 86-93 (2012).
- 25) Hui, S. P.; Chiba, H.; Kurosawa, T. Liquid chromatogra-

- phy-mass spectrometric determination of plasmalogens in human plasma. *Anal. Bioanal. Chem.* **400**, 1923-1931 (2011).
- 26) Nishimukai, M.; Yamashita, M.; Watanabe, Y.; Yamazaki, Y.; Nezu, T.; Maeba, R.; Hara, H. Lymphatic absorption of choline plasmalogen is much higher than that of ethanolamine plasmalogen in rats. *Eur. J. Nutr.* **50**, 427-436 (2011).
- 27) Zemski Berry, K. A.; Murphy, R. C. Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *J. Am. Soc. Mass Spectrom.* **15**, 1499-1508 (2004).
- 28) Norris, C.; Fong, B.; MacGibbon, A.; McJarrow, P. Analysis of phospholipids in rat brain using liquid chromatography-mass spectrometry. *Lipids* **44**, 1047-1054 (2009).
- 29) Nikolic, D.; Katsiki, N.; Montalto, G.; Isenovic, E. R.; Mikhaelidis, D. P.; Rizzo, M. Lipoprotein subfractions in metabolic syndrome and obesity: clinical significance and therapeutic approaches. *Nutrients* **5**, 928-948 (2013).
- 30) Kemp, S.; Valianpour, F.; Denis, S.; Ofman, R.; Sanders, R. J.; Mooyer, P.; Barth, P. G.; Wanders, R. J. A. Elongation of very long-chain fatty acids is enhanced in X-linked adrenoleukodystrophy. *Mol. Genet. Metab.* **84**, 144-151 (2005).
- 31) Moser, H. W.; Moser, A. B.; Hollandsworth, K.; Brereton, N. H.; Raymond, G. V. "Lorenzo's oil" therapy for X-linked adrenoleukodystrophy: rationale and current assessment of efficacy. *J. Mol. Neurosci.* **33**, 105-113 (2007).
- 32) Koike, R.; Tsuji, S.; Ohno, T.; Suzuki, Y.; Orii, T.; Miyatake, T. Physiological significance of fatty acid elongation system in adrenoleukodystrophy. *J. Neurol. Sci.* **103**, 188-194 (1991).
- 33) Jakobsson, A.; Jörgensen, J. A.; Jakobsson, A. Differential regulation of fatty acid elongation enzymes in brown adipocytes implies a unique role for Elovl3 during increased fatty acid oxidation. *Am. J. Physiol. Endocrinol. Metab.* **289**, E517-526 (2005).
- 34) Vluggens, A.; Andreoletti, P.; Viswakarma, N.; Jia, Y.; Matsumoto, K.; Kulik, W.; Khan, M.; Huang, J.; Guo, D.; Yu, S.; Sarkar, J.; Singh, I.; Rao, M. S.; Wanders, R. J.; Reddy, J. K.; Cherkaoui-Malki, M. Functional significance of the two ACOX1 isoforms and their crosstalks with PPAR α and RXR α . *Lab. Invest.* **90**, 696-708 (2010).
- 35) Faust, P. L.; Kovacs, W. J. Cholesterol biosynthesis and ER stress in peroxisome deficiency. *Biochimie pii: S0300-9084(13)00390-8*. Doi (2013).
- 36) Hotamisligil, G. S. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell* **140**, 900-917 (2010).
- 37) Maeba, R.; Hara, H.; Ishikawa, H.; Hayashi, S.; Yoshimura, N.; Kusano, J.; Takeoka, Y.; Yasuda, D.; Okazaki, T.; Kinoshita, M.; Teramoto, T. Myo-inositol treatment increases serum plasmalogens and decreases small dense LDL, particularly in hyperlipidemic subjects with metabolic syndrome. *J. Nutr. Sci. Vitaminol.* **54**, 196-202 (2008).
- 38) Bismuth, J.; Lin, P.; Yao, Q.; Chen, C. Ceramide: a common pathway for atherosclerosis? *Atherosclerosis* **96**, 497-504 (2008).
- 39) Yamazaki, Y.; Nezu, T.; Maeba, R.; Nishimukai, M.; Hara, H. *Jpn Pat.*, 2009-62364 (2008).
- 40) Wanders, R. J. Peroxisomes, lipid metabolism, and peroxisomal disorders. *Mol. Genet. Metab.* **83**, 16-27 (2004).