12

Serum Choline Plasmalogen is a Reliable Biomarker for Atherogenic Status

Ryouta Maeba[1] and Hiroshi Hara[2]
[1]*Department of Biochemistry, Teikyo University School of Medicine*
[2]*Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University*
Japan

1. Introduction

Plasmalogens (Pls) belong to a subclass of glycerophospholipids, and widely distributed in human and animal tissues (Nagan & Zoeller, 2001). Although the clinical significance of these phospholipids is recognized in relation to peroxisomal disorders (Wanders & Waterham, 2006), the physiopathological roles of Pls are not fully understood. Recently, serum (or plasma) Pls have gained interest in several clinical symptoms of life-style related disease possibly because of their antioxidant properties (Brites et al., 2004). We have developed a highly sensitive and simple method to determine the serum concentrations of choline (PlsCho) and ethanolamine plasmalogen (PlsEtn) separately, using a radioactive iodine and high-performance liquid chromatography (^{125}I-HPLC method) (Maeba & Ueta, 2004; Maeba et al, 2012). The method has improved as auto-analytical system by introducing online detection with flow γ-counter. We have applied the system to the determination of serum Pls from normal subjects and CAD patients, and investigated the clinical significance of serum Pls as a biomarker for metabolic syndrome and atherosclerosis. This chapter briefly describes Pls structure, distribution, functions, and biosynthesis, and describes in detail the methods for measurement of Pls and clinical significance of serum Pls.

2. Plasmalogens (Pls)

Pls are one of the three subclasses of glycerophospholipids, and characterized by a vinyl-ether bond (-O-CH=CH-) in *sn*-1 and an ester bond in *sn*-2 position of the glycerol backbone (**Fig.1**).

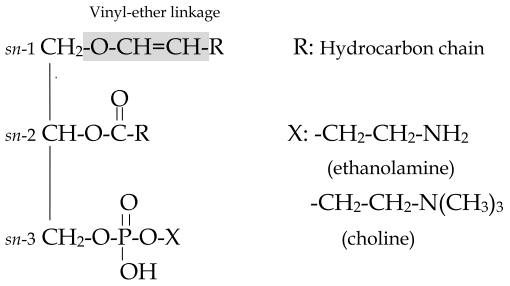


Fig. 1. Chemical structure of Pls

Pls constitute 18% of the total phospholipids mass in humans, mostly as membrane structure component. The polar head group of Pls mainly consists of choline or ethanolamine. Ethanolamine plasmalogens (PlsEtn) are predominantly distributed in almost organs, tissues and cells except heart and blood plasma, in which PlsEtn and choline plasmalogens (PlsCho) are equally contained. Pls are abundantly distributed in brain, heart, kidney, lung, testis, skeletal muscle, erythrocyte, macrophage, and lymphocytes in human body. These organs, tissues, and cells are characterized by the frequent occurrence of cell membrane fusion or oxidative stress.

2.1 Functions and biosynthesis of Pls

Major functions of Pls are shown in **Fig.2**. 1) Modulating membrane fluidity is essential for regulating membrane protein functions, as well as cell fusion, exocytosis, and endocytosis. This function is derived from high propensity of PlsEtn to form locally inverse hexagonal phase in phospholipid bilayer membranes (Glaser & Gross, 1994). 2) Endogenous antioxidant function of Pls is derived from the presence of vinyl ether double bond, which is capable of scavenging free radicals (Zoeller et al., 1988). In addition, we have recently found a novel antioxidant function of PlsEtn to lower the susceptibility of cholesterol in phospholipid bilayer and membranes to oxidation, probably through modulating the physical property of membranes (Maeba & Ueta, 2003a, 2003b). 3) The function of storage of bioactive lipid source comes from the fact that the lipid mediator producing fatty acids such as arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are preferentially located at *sn*-2 position of Pls.

1. Membrane modulator

membrane fluidity, membrane fusion

2. Endogenous antioxidant

radical scavenger

lower susceptibility of cholesterol and membranes to oxidation

3. Storage of bioactive lipid source

arachidonic acid, DHA, EPA

Fig. 2. Major functions of Pls

Next, dihydroxyacetone phosphate (DHAP) is a starting material for biosynthesis of Pls. DHAP-acyltransferase (DHAP-AT) and alkyl DHAP synthase (ADHAP-S) are responsible for the 1st and 2nd steps of Pls biosynthesis, respectively, and are localized in peroxisome (**Fig.3**). Therefore, peroxisomal disorders often show Pls deficiency (Wanders & Waterham, 2006). Mental retardation represents a phenotype of peroxisomal disorders, suggesting the essential role of Pls in the normal function of central nervous system (CNS). This is supported by Pls-deficient mouse, which revealed severe phenotypic alterations, including defects in CNS myelination (Gorgas et al., 2006). In addition to peroxisomal disorders, decreased brain Pls content has been reported in a number of neurological disorders such as spinal cord injury, Alzheimer's disease, Down syndrome, and multiple sclerosis (Lessig & Fuchs, 2009). The final step of PlsEtn biosynthesis is the desaturation of 1-alkyl-2-acyl-GPE via the action of Δ1 desaturase, whereas PlsCho is primarily formed from PlsEtn via polar-head group modification, by which mechanism remains still obscure (Lee, 1998). Facilitation

of PlsCho biosynthesis is presumably important to prevent from atherogenic status, as described later, therefore elucidation of the regulatory mechanism of transfer from PlsEtn to PlsCho must become important.

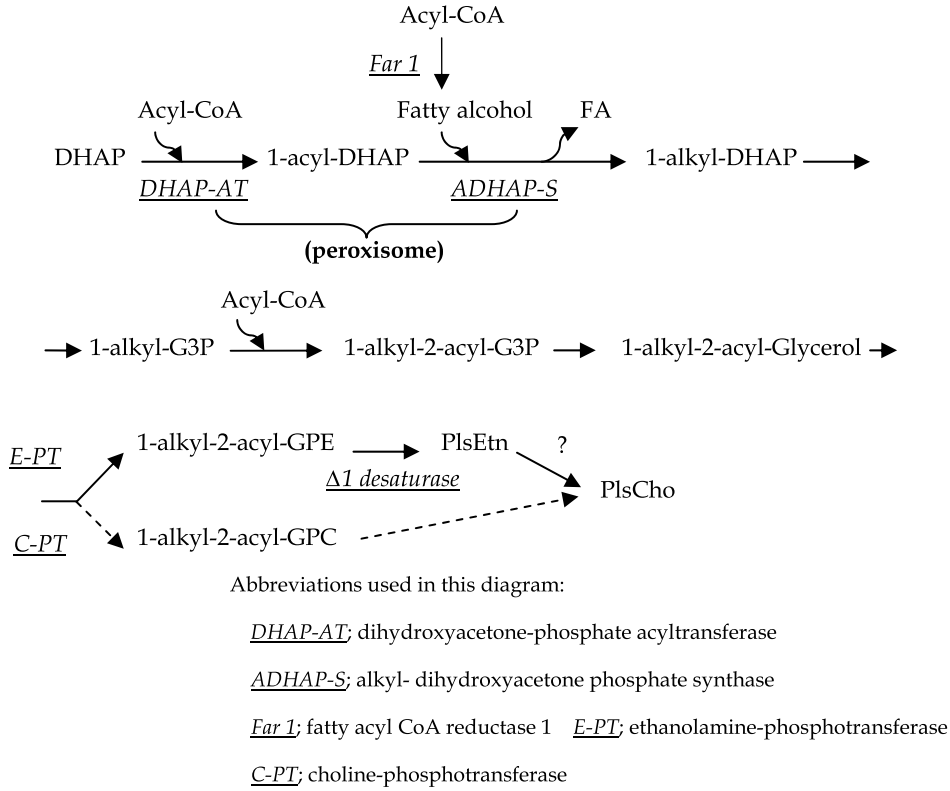


Fig. 3. Biosynthetic pathway of Pls

2.2 Quantification of Pls

Analytical methods for Pls have been developed principally based on acid labile property of the vinyl-ether linkage of Pls, which leads to the sequential decomposition into fatty aldehydes and 1-lysophospholipids. The resultant fatty aldehydes are usually measured as dimethyl acetal derivatives using gas chromatography/mass spectrometry (GC/MS) (Ingrand et al., 2000), and 1-lysophospholipids are measured by two-dimensional thin layer chromatography (TLC) (Horrocks, 1968). However, these methods are laborious and insufficient to accurately quantify Pls in a small amount of serum (or plasma).

2.2.1 LC/MS analysis of Pls

Recently, LC/MS has been applied to the analysis of Pls at the molecular species levels (Zemski & Murphy, 2004). We also have established the analytical method for Pls molecular species with UPLC-MS/MS (**Fig.4**).

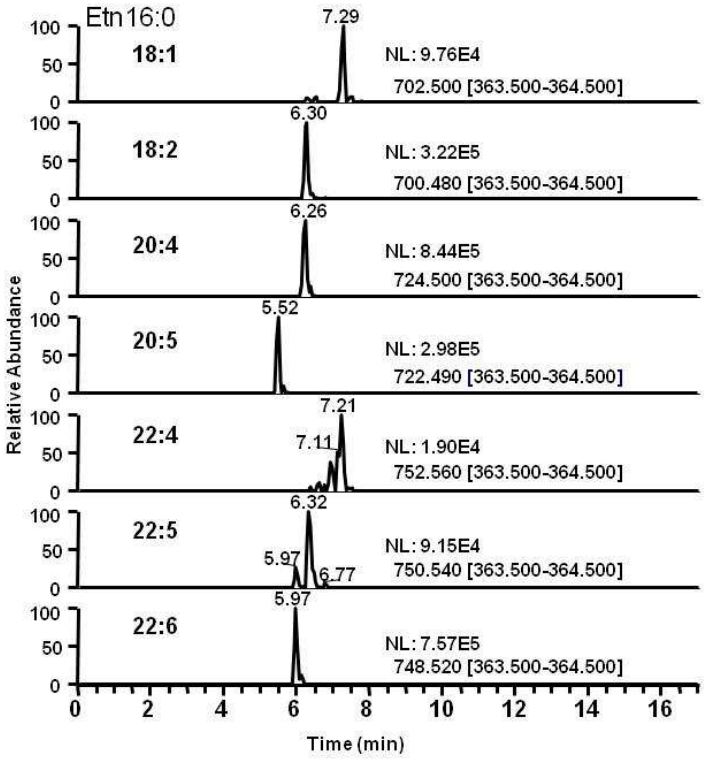


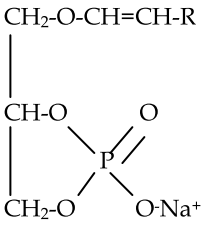
Fig. 4. UPLC-MS/MS analysis of PlsEtn 16:0 molecular species

The long chain fatty alcohol in *sn*-1 position consists almost exclusively of 16:0, 18:0, and 18:1 alkenyl groups, while *sn*-2 position is esterified predominantly with n-6 or n-3 series polyunsaturated fatty acids. LC/MS analysis is powerful method to provide substantial useful information, but the excess data is unlikely suitable for a routine diagnostic test, except for specific diagnostic molecular species for particular disorders such as Alzheimer's disease (Goodenowe et al, 2007).

2.2.2 ^{125}I-HPLC method for determination of Pls

We have originally developed a unique method to determine total amounts of PlsCho and PlsEtn separately, using radioactive iodine (^{125}I) and high-performance liquid chromatography (^{125}I-HPLC method) (Maeba & Ueta, 2004; Maeba et al, 2012). The method is based on the binding specificity of iodine to Pls. Triiodide (1-) ion (I_3^-) specifically reacts with a vinyl-ether double bond of Pls at molar ratio 1:1 in methanol solution. Pls assay based on this principle had already been established (Williams et al., 1962). However, the "cold" iodine method has some problems of low detectable Pls and interfering substances in the extracted lipids from biomaterials like serum. ^{125}I-HPLC method permitted the highly sensitive and accurate determination of serum (or plasma) Pls by using a radioactive iodine (^{125}I), and

removing interfering substances with HPLC. The primary advantage of this method enabled to measure PlsCho and PlsEtn separately. ^{125}I-HPLC method has been markedly improved by introducing a quantitative standard (Q.S.) and online detection with flow γ-counter. 1-Alkenyl 2,3-cyclic glycerophosphate was decided as Q.S. in view of both the same quantitative property of Pls and the proper retention time in the HPLC elution profile (**Fig.5, 6**).



R: Hydrocarbon chain

Fig. 5. Chemical structure of 1-Alkenyl 2,3-cyclic glycerophosphate (Q.S.)

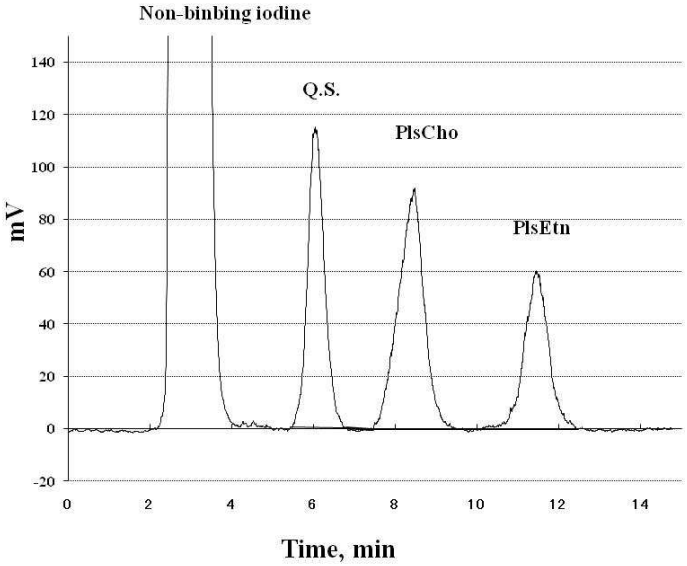


Fig. 6. A typical HPLC elusion profile of Pls and Q.S. detected with flow γ-counter

Online detection with flow γ-counter permitted more precise and safe measurement of Pls-related radio activity. The improved method has been fully validated in terms of selectivity, sensitivity, linearity, precision, accuracy, and has applied to the determination of human serum Pls. To minimize diffusion of radioactive contamination, the treatment column for HPLC waste fluid was prepared. The radioactive iodine adsorbents column reduced the radioactivity in waste fluid less than 500cpm/ml, even when 100 times sample injections. ^{125}I-HPLC method is useful as a continious auto-analytical system for a routine diagnostic test of human serum (or plasma) Pls.

2.3 Pls as a serum (plasma) biomarker

We have focused on the antioxidant property of Pls, and the potential utility of serum Pls as a biomarker for oxidative stress and aging (Brosche, 1997). It is well known that the number and the capability of peroxisome reduce with aging (Poynter & Davnes, 1998), which may induce the reduction of Pls biosynthesis. Decreased antioxidant Pls level may lead to predominant oxidative status in redox balance, which may cause the diseases associated with aging or oxidative stress such as atherosclerosis, metabolic syndrome (MetS) and Alzheimer's disease (**Fig.7**).

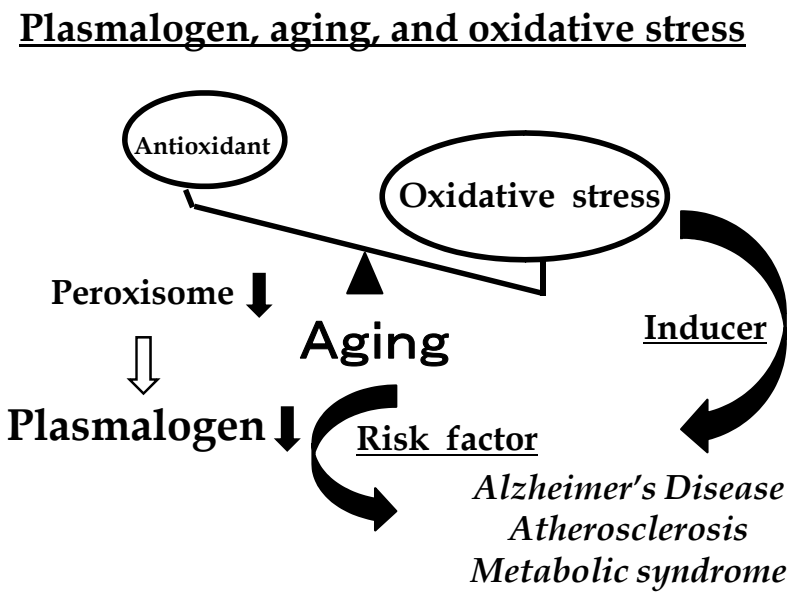


Fig. 7. Hypothesis for Pls deficiency causing life-style related disease

2.3.1 Serum (plasma) Pls

Serum (plasma) Pls are mainly synthesized in and secreted by liver as a structural component of lipoproteins (Vance, 1990), and potentially prevent lipoprotein oxidation relevant to atherosclerosis probably due to the radical scavenging ability of vinyl-ether double bond (Engelmann et al., 1994; Zoeller et al, 1999). The profile of human serum (plasma) Pls is as follows. Pls concentration is 100-300 μM with the content ratio of PlsCho/PlsEtn ranging from 0.5 to 1.5. PlsCho represent approximately 5% of choline glycerophospholipid, while PlsEtn represent 50~60% of ethanolamine glycerophospholipid. Total phospholipids concentration is 2-4 mM in serum (plasma), including 60~75% choline glycerophospholipid and 2~5% ethanolamine glycerophospholipid. The distribution of Pls on lipoprotein classes shows higher proportion of HDL, LDL, and VLDL in the order (Wiesner et al., 2009).

2.3.2 Pls in serum (plasma) or red blood cells of normal subjects

Pls levels in serum (plasma) or red blood cells of normal subjects have been determined with ^{125}I-HPLC method in each generation (**Table 1**).

	Blood serum or plasma						Red blood cells	
	Young		Middle-age		Elderly		Young	
	M	F	M	F	M	F	M	F
n	74	27	383	69	8	65	74	27
Age	23.9	22.7	40.4	34.8***	77.6	72.3*	23.9	22.7
	±3.7	±3.9	±10.7	±10.3	±5.0	±5.1	±3.7	±3.9
PlsCho	98.5	115.2**	62.5	69.8***	54.9	54.7		
μM	±23.0	±24.5	±12.2	±13.0	±10.2	±11.2		
PlsEtn	133.8	142.9	73.3	71.0	68.8	68.5		
μM	±33.3	±38.7	±19.4	±20.5	±13.0	±16.6		
PlsCho+PlsEtn	232.4	258.1	135.8	140.7	123.6	123.2		
μM	±53.3	±61.2	±29.0	±29.8	±21.7	±25.5		
PlsCho/PlsEtn	0.75	0.83*	0.88	1.04***	0.80	0.82	0.29	0.29
ratio	±0.12	±0.14	±0.17	±0.28	±0.11	±0.15	±0.04	±0.03
PL	2.17	2.14	2.99	2.94	3.28	3.44		
mM	±0.34	±0.41	±0.49	±0.33	±0.40	±0.38		
PlsCho/PL	4.6	5.6*	2.2	2.4***	1.7	1.6	5.7	6.5*
mol %	±1.1	±1.6	±0.4	±0.5	±0.3	±0.3	±1.6	±1.6
PlsEtn/PL	6.3	6.9	2.5	2.5	2.1	2.0	19.2	22.3*
mol %	±1.8	±2.2	±0.6	±0.7	±0.5	±0.5	±4.6	±4.8
(PlsCho+PlsEtn)/PL	10.9	12.5	4.6	4.8	3.8	3.6	24.9	28.8*
mol %	±2.7	±3.7	±0.9	±1.1	±0.8	±0.8	±6.0	±6.3

Mean ±S.D., *t*-test between Male and Female in each generation, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 1. Pls levels in serum (plasma) or red blood cells of normal subjects

There were significant differences in almost all Pls parameters as well as age and total phospholipids (PL) among generations, except for PlsEtn level between middle-age and elderly. Pls in serum (plasma) is decreased with aging, especially substantial reduction was observed between young and middle-age. Interestingly, there were significant differences in PlsCho, PlsCho/PlsEtn ratio, and PlsCho/PL mol% between male (M) and female (F) of young and middle-aged normal subjects. However, the sex differences in PlsCho-related parameters disappeared in elderly normal subjects, suggesting that PlsCho biosynthesis may be partly affected by sex hormone like estrogen. Analysis of serum Pls of middle-aged normal subjects (*n*=452) showed a strong correlation between PlsCho and PlsEtn. Correlative analysis of PlsCho/PlsEtn ratio with PlsCho or PlsEtn suggested that the ratio is highly influenced by the variation of PlsEtn (**Fig.8**). PlsCho/PlsEtn ratio in red blood cells considerably differs from that in serum (plasma), indicating predominance of PlsEtn similarly to other many organs, tissues and cells. Both PlsCho/PL and PlsEtn/PL mol% in red blood cells of young female were significantly higher than those of young male, whereas only PlsCho/PL mol% in plasma of young female was significantly higher than that of young male. Analysis of Pls in plasma and red blood cells of young normal subjects (*n*=101) showed a strong correlation between PlsCho and PlsEtn in both specimens. Correlative analysis of PlsCho/PlsEtn ratio with PlsCho or

PlsEtn suggested that the ratio is highly influenced by the variation of PlsCho in the case of red blood cells (n.d.). There were no strong correlations (coefficient >0.3) in all Pls-related parameters between plasma and red blood cells (n.d.).

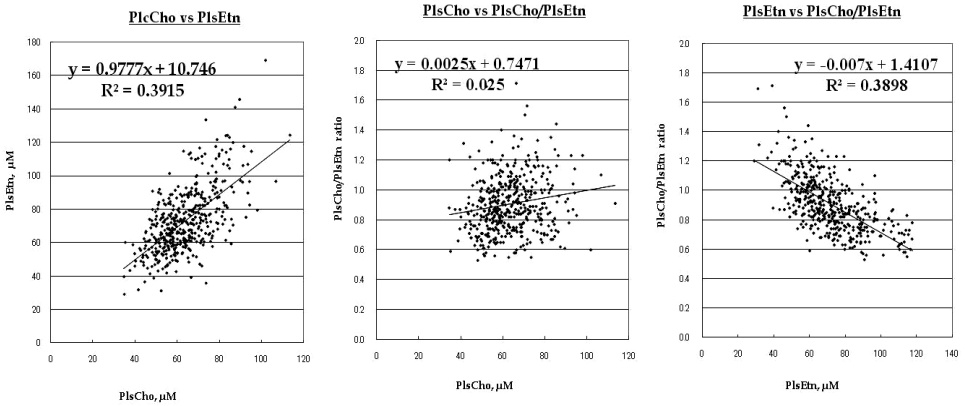


Fig. 8. Relationship between PlsCho and PlsEtn

Next, to investigate the clinical significance of serum Pls, correlative analyses were made of over 40 years old in middle-aged normal subjects (*n*=216). Their clinical and serum biochemical data are shown in **Table 2**. Almost all data showed in the normal range. Total Pls level was 137.0±29.6, and PlsCho/PlsEtn ratio was 0.87±0.19.

n	216	γ-GTP	58±58	Adiponectin	6.9±4.8
(M/F)	(195/21)	(U/l)		(μg/mg)	
Age	48.9±7.1	HDL-C	65±18	PlsCho	62.7±12.9
		(mg/dl)		(μM)	
Body weight	68.5±11.3	LDL-C	124±27	PlsEtn	74.2±19.6
(kg)		(mg/dl)		(μM)	
BMI	24.6±6.0	TG	121±88	PlsCho+PlsEtn	137.0±29.6
(kg/m²)		(mg/dl)		(μM)	
Waist circumstance	83.0±8.9	sdLDL	33.0±12.9	PlsCho/PlsEtn	0.87±0.19
(cm)		(mg/dl)		ratio	
Systolic pressure	126±14	Glu	96±13	PL	3.13±0.47
(mmHg)		(mg/dl)		(mM)	
Diastolic pressure	81±11	UA	6.2±1.5	PlsCho/PL	2.0±0.4
(mmHg)		(mg/dl)		(mol %)	
GOT	25±10	Hcy	11.0±5.0	PlsEtn/PL	2.4±0.6
(U/l)		(μmol/l)		(mol %)	
GPT	28±18	hsCRP	78±67	(PlsCho+PlsEtn)/PL	4.4±1.0
(U/l)		(μg/dl)		(mol %)	

Mean±S.D.

Table 2. Clinical and biochemical data of middle-aged (≧40 years old) normal subjects

Correlative analyses revealed significant positive correlations between PlsCho and HDL-C (r = 0.714) or adiponectin (r = 0.314), and negative correlations between PlsCho and body weight (r = -0.334), waist circumference (r = -0.375), TG (r = -0.327), hsCRP (r = -0.250), respectively. Furthermore, PlsCho/PL mol% correlated with age (r = -0.266), systolic (r = -0.292) and diastolic pressure (r = -0.283), sdLDL-C (r = -0.458), UA (r = -0.255), as well as atherogenic index of plasma, calculated as log (TG/HDL-C) (r = -0.674) (**Table 3**).

	n=216	correlation coefficient >0.25, p< 0.001						
Correlation coefficient	age	Body weight	Waist size	Systolic pressure	Diastolic pressure	HDL-C	LDL-C	sdLDL-C
PlsCho	-0.194	**-0.334**	**-0.375**	-0.134	-0.092	**0.714**	0.036	-0.224
PlsEtn	-0.023	-0.098	-0.118	0.020	0.125	**0.408**	0.120	0.016
PlsCho+PlsEtn	-0.100	-0.210	-0.240	-0.045	0.043	**0.580**	0.096	-0.086
PlsCho/PlsEtn	-0.140	-0.185	-0.196	-0.152	-0.241	0.167	-0.127	-0.242
PL	0.052	0.066	0.068	**0.252**	**0.293**	**0.285**	**0.301**	**0.367**
PlsCho/PL	**-0.266**	**-0.374**	**-0.408**	**-0.292**	**-0.283**	**0.506**	-0.162	**-0.458**
PlsEtn/PL	-0.109	-0.157	-0.162	-0.128	-0.050	**0.307**	-0.033	-0.200
(PlsCho+PlsEtn)/PL	-0.191	**-0.271**	**-0.290**	-0.215	-0.162	**0.427**	-0.095	**-0.337**

Correlation coefficient	TG	AIP	Glu	UA	hsCRP	Adipo-nectin	Hcy
PlsCho	**-0.327**	**-0.576**	-0.168	-0.151	**-0.250**	**0.314**	-0.233
PlsEtn	-0.038	-0.248	0.107	-0.012	-0.105	0.103	-0.194
PlsCho+PlsEtn	-0.166	**-0.414**	-0.001	-0.073	-0.178	0.205	-0.230
PlsCho/PlsEtn	**-0.265**	-0.241	**-0.265**	-0.119	-0.032	0.172	0.031
PL	**0.382**	0.188	0.101	**0.253**	-0.068	-0.024	-0.063
PlsCho/PL	**-0.542**	**-0.674**	-0.236	**-0.318**	-0.226	**0.319**	-0.179
PlsEtn/PL	-0.271	**-0.396**	0.042	-0.172	-0.054	0.125	-0.158
(PlsCho+PlsEtn)/PL	**-0.421**	**-0.561**	-0.081	**-0.255**	-0.138	0.226	-0.183

AIP; atherogenic index of plasma, calculated as log (TG/HDL-C)

Table 3. Correlations between Pls and clinical or serum parameters of middle-aged (≧40) normal subjects

Surprisingly, the correlation between PlsCho and HDL-C was stronger rather than that between PlsCho and PlsEtn (**Fig.8, 9**). Indeed, PlsCho preferentially distribute in HDL class compared to other lipoprotein fractions, but this fact cannot explain the strong correlation of PlsCho with HDL-C, because PlsEtn as well as PlsCho also show preferential distribution in HDL compartment.

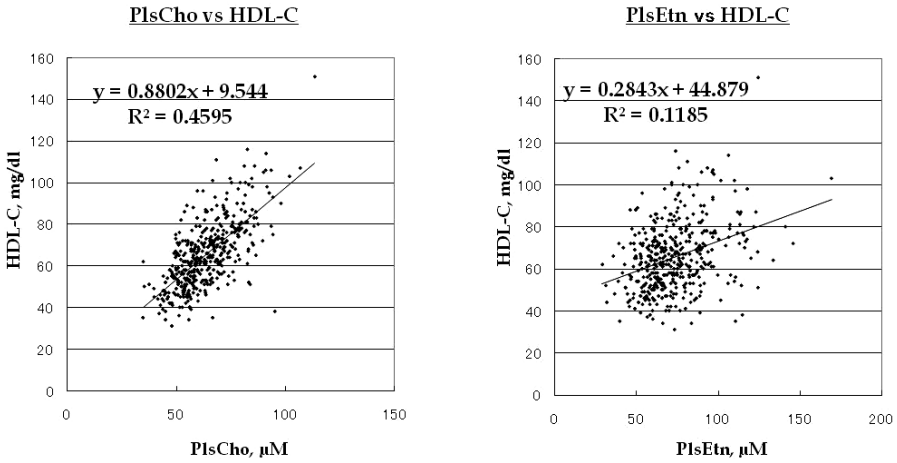


Fig. 9. Relationship between Pls and HDL

2.3.3 Pls in serum of CAD patients

Pls levels in serum and lipoproteins were determined for the CAD patients referred for coronary angiography (*n*=50), and compared with those of patients with (≧75%) and without (<50%) significant stenosis (**Table 4-1**). Serum PlsCho was significantly decreased in CAD patients with ≧75% stenosis compared to patients without significant stenosis (<50%). Total phospholipids (PL), cholesterol (CH), triglyceride (TG), and protein content as well as Pls were determined in HDL and LDL fractions from patients' serum. The ratios of Pls to each lipid (mol %) or protein (μmol/g) in lipoprotein particles were compared between patients with and without significant stenosis. The CAD patients with ≧75% stenosis showed significant reduction in PlsCho/protein ratio in both HDL and LDL, indicating that PlsCho content of each lipoprotein particle is entirely decreased. Concomitantly, tocopherols (V.E.), lipid-soluble antioxidant vitamin, levels in serum were determined, and compared with those of patients with (≧75%) and without (<50%) significant stenosis (**Table 4-2**). There were no significant differences in all tocopherol homolog between them. These results demonstrate the specific reduction in serum PlsCho in CAD patients, and show clinical utility of PlsCho as a serum biomarker for atherogenic status.

Next, to investigate the relationship between Pls and other serum lipids or lipoproteins, correlative analyses were made of another CAD patients referred for coronary angiography (*n*=148). Their serum lipids and lipoproteins data including Pls are shown in **Table 5**.

Interestingly, in addition to the strong correlation of PlsCho with HDL (Maeba et al., 2007), LDL particle size significantly correlated with PlsCho/PlsEtn ratio as well as TG and HDL-C (**Table 6**). Small dense LDL (sdLDL) is well-known risk factor for atherosclerosis, and frequently observed in dyslipidemia of type-2 diabetes or MetS, which is characterized by low HDL and high TG (Nozue et al, 2007). As PlsEtn is presumably a precursor of PlsCho in biosynthetic pathway, PlsCho/PlsEtn ratio can be regarded as an indicator of transfer rate

from PlsEtn to PlsCho. Therefore, the cause or effect of declining transfer rate is assumed to be potentially related to the appearance of atherogenic sdLDL, but it is obscure how Pls are involved in the alteration of lipoprotein metabolism.

Serum

stenosis	*n* (M/F)	Age	PlsCho μM	PlsEtn μM	PlsCho+PlsEtn μM
≧75%	30 (20/10)	67.8±10.3	39.2±10.5**	63.0±14.5	102.2±21.6
<50%	20 (11/9)	61.0±17.7	49.0±12.4	66.9±18.8	115.9±25.7
stenosis	PlsCho/PlsEtn ratio	PL mM	PlsCho/PL mol %	PlsEtn/PL mol %	(PlsCho+PlsEtn)/PL mol %
≧75%	0.64±0.16	2.34±0.39	1.7±0.5	2.8±0.7	4.5±1.1
<50%	0.78±0.28	2.59±0.54	1.9±0.4	2.6±0.7	4.5±0.9

HDL

stenosis	PlsCho/PlsEtn ratio	PlsCho/PL mol %	PlsEtn/PL mol %	PlsCho/CH mol %	PlsEtn/CH mol %
≧75%	0.72±0.20	1.7±0.4	2.5±0.8	1.5±0.4	2.1±0.7
<50%	0.86±0.34	1.7±0.4	2.2±0.6	1.5±0.3	1.9±0.6
stenosis	PlsCho/TG mol %	PlsEtn/TG mol %	PlsCho/protein μmol/g	PlsEtn/protein μmol/g	
≧75%	14.6±8.2	21.4±13.8	11.3±3.5*	16.3±6.1	
<50%	12.7±4.8	16.1±7.1	14.2±4.9	17.7±6.4	

LDL

stenosis	PlsCho/PlsEtn ratio	PlsCho/PL mol %	PlsEtn/PL mol %	PlsCho/CH mol %	PlsEtn/CH mol %
≧75%	0.80±0.19	1.4±0.3	1.8±0.3	0.6±0.1	0.7±0.2
<50%	0.94±0.30	1.6±0.4	1.8±0.4	0.7±0.2	0.7±0.2
stenosis	PlsCho/TG mol %	PlsEtn/TG mol %	PlsCho/protein μmol/g	PlsEtn/protein μmol/g	
≧75%	3.3±1.4	4.2±1.8	24.9±6.0*	31.6±6.5	
<50%	4.3±1.9	4.8±2.2	29.8±7.3	32.8±7.4	

Mean±S.D. t-test between patients with and without significant stenosis, * $p < 0.05$ ** $p < 0.01$

Table 4-1. Comparison of Pls levels in serum and lipoproteins between CAD patients with and without significant stenosis

stenosis	α-Toc. μM	β-Toc. μM	γ-Toc. μM	δ-Toc. μM
≧75%	29.6±5.8	0.6±0.2	1.9±0.7	0.1±0.1
<50%	30.4±8.5	0.6±0.2	2.1±0.8	0.1±0.1

Table 4-2. Comparison of V.E levels in serum between patients with and without significant stenosis

n (M/F)	148 (110/38)	LDL size, nm	25.5 ± 0.5
Age	65.2±12.2	MDA-LDL, U/l	105 ± 40
CAD ≥75% stenosis	63.5%	LP (a), mg/dl	26 ± 21
PlsCho, μM	65 ± 18	TG, mg/dl	123 ± 80
PlsEtn, μM	83 ± 33	LPL mass, ng/ml	42.7 ±13.3
PlsCho +PlsEtn, μM	148 ± 50	apo A-I, mg/dl	119 ± 22
PlsCho/PlsEtn ratio	0.83 ± 0.17	apo A-II, mg/dl	24 ± 6
PL, mM	2.2 ± 0.4	apo B, mg/dl	102 ± 26
TC, mg/dl	189 ± 40	apo B-48, mg/dl	6.9 ± 3.2
LDL-C, mg/dl	119 ± 45	apo C-II, mg/dl	4 ± 2
HDL-C, mg/dl	47 ± 11	apo C-III, mg/dl	9 ± 3
RLP-C, mg/dl	5 ± 2	apo E, mg/dl	4.3 ± 1.3

Mean±S.D.

Table 5. Serum lipids and lipoproteins data of CAD patients

Correlation coefficient	RLP-C	TG	apo C-II	HDL-C	PlsCho/PlsEtn ratio
LDL particle size	-0.452	-0.442	-0.431	0.415	0.402

RLP-C; remnant-like particle-cholesterol

Table 6. Parameters indicating significant strong correlation (coefficient > 0.4, $p<0.001$) with LDL particle size

Most of the PlsCho in the plasma membrane are considered to be probably made by *N*-methylation of PlsEtn in three steps by reaction with *S*-adenosylmethionine (AdoMet), rather than base exchange via exchange of choline (Cho) for ethanolamine (Etn) or serine (Ser), or the Kennedy pathway by reaction of cytidine monophosphate with PlsEtn (Horrocks et al, 1986). Serum PlsCho is also possibly derived from *N*-methylation of PlsEtn, catalyzed by a hepatocyte-specific enzyme, phosphatidylethanolamine *N*-methyltransferase (PEMT) (Fig.10). Interestingly, PEMT knockout mice reveal that PEMT is required for hepatic secretion of triacylglycerol (TG) in very low density lipoproteins (VLDL) (Noga et al, 2002). This finding suggests that PlsCho may have an essential role in lipoprotein metabolism, because PEMT is responsible for ~30% of phosphatidylcholine (PC), major Cho-containing glycerophospholipid, formed in liver, while PlsCho is primarily made from PlsEtn probably via PEMT pathway. Furthermore, a polymorphism of the human *PEMT* gene is associated with diminished activity and may confer susceptibility to nonalcoholic fatty liver disease (NAFLD) (Song et al, 2005). This observation also suggests the importance of PlsCho in the transport of TG from liver, and the participation of the lack of PlsCho in the onset of metabolic syndrome (MetS), because NAFLD shares many features of the MetS, such as abdominal obesity, type 2 diabetes, dyslipidemia, and insulin resistance (Pacifico et al, 2011). Human *PEMT* gene is regulated by estrogen (Resseguie et al, 2007), which may explain the sex difference in serum levels

of PlsCho, despite no significant sex difference in serum levels of PC, and provide an evidence supporting the involvement of PEMT pathway in the synthesis of PlsCho. Moreover, a nutritional insufficiency in the methyl donor such as methionine or choline also causes lack of PlsCho and elevated homocysteine (Hcy), a risk factor for cardiovascular disease. These reports may provide the physiopathological basis for the validity of PlsCho as a biomarker for atherogenic status.

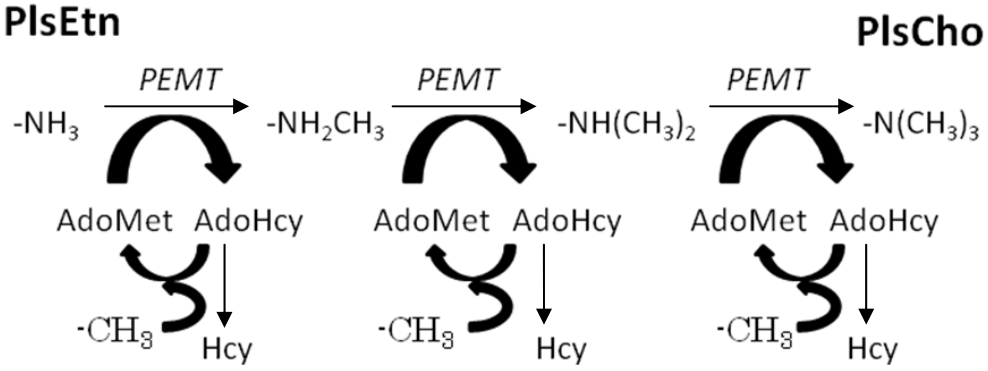


Fig. 10. PEMT pathway for conversion of PlsEtn to PlsCho

2.3.4 Intervention study of increasing serum Pls

Myo-inositol (MI) is one of 9 isomers of inositol, and is the most abundant in nature including human body. Besides dietary intake, MI is synthesized from glucose in vivo. MI contributes to numerous functions as a precursor molecule, such as signal transduction through biosynthesis of inositol phospholipids, or ca^{2+} homeostasis through biosynthesis of inositol phosphates (Vanhaesebroeck et al., 2001). Moreover, MI itself plays an important role in normal functions of CNS as an organic osmolyte (Gullans & Verbalis, 1993), and also possibly involves in myelin formation (Berry et al., 2003). Pls are major component of myelin membranes, and essential for myelin formation and functions. In animal studies, oral administration of MI showed to increase Pls level in rat brain (Hoffman-Kuczynski & Reo, 2004). Therefore, we have undertaken MI intervention study to investigate whether MI administration improves the dyslipidemia of MetS through facilitating Pls biosynthesis, and to explore the relationship between serum Pls and sdLDL. Clinical background of study subjects is shown in **Table 7**. Almost all subjects (n=17) were hyperlipidemia, and half of the subjects were diagnosed as MetS. Treatment of MI 5g daily for 1 week, followed by MI 10g daily for 1 week markedly reduced sdLDL, and significantly increased PlsCho as well as PlsCho/PL and blood glucose particularly for MetS subjects, without alteration in the levels of TG and HDL-C (**Table 8**). This result suggests that facilitating PlsCho biosynthesis potentially prevents from atherogenic status trough reducing sdLDL. The increase in Pls levels after MI treatment tended to be inversely proportional to the serum Pls levels before treatment (Fig.11). Serum Pls reached a plateau level of around 150 μM with MI treatment, which corresponds to the average level observed in normal subjects (Maeba et al., 2008). Pls biosynthesis is strictly regulated and the Pls levels in cells or tissues are physiologically kept constant (Liu et al., 2005). Our result appears to support these facts.

n (M/F)	17 (15/2)
Age	43.6 ± 6.6
Rate of obesity (BMI≧25)	65%
Rate of hyperlipidemia	94%
Rate of diabetes mellitus	18%
Rate of hypertension	29%
Rate of metabolic syndrome (MetS) (according to Japanese guideline*)	47%
Rate of current cigarette smoking	71%

* Japanese criteria for MetS
MetS is diagnosed when 1) plus more than two of the risk determinants among 2) – 4).
1) Waist circumstance: M≧85 cm, F≧90 cm
2) TG≧150 mg/dl and/or HDL-C<40 mg/dl
3) Systolic pressure≧130mmHg and/or Diastolic pressure≧85 mmHg
4) Fasting glucose≧110 mg/dl

Table 7. Clinical background of study subjects

	MetS (*n* = 8)		non- MetS (*n* = 9)	
	before	after	before	after
BMI, kg/m^2	29.3±3.0	29.7±3.7 (101.4)	25.3±2.3	25.2±2.6 (99.6)
Waist circumstance, cm	99.6±6.3	99.9±9.1 (100.3)	85.0±4.6	82.9±4.0 (97.5)
Systolic pressure, mmHg	136±13	140±26 (102.9)	132±11	125±9 (94.7)
Diastolic pressure, mmHg	85±12	93±16 (109.4)	78±7	81±5 (103.8)
Blood glucose, mg/dl	104.1±16.6	87.5±11.6 (84.1)*	96.2±18.6	97.7±17.4 (101.6)
hsCRP, mg/dl	0.316±0.315	0.171±0.137 (54.1)	0.056±0.029	0.453±0.995 (808.9)
apo A-I, mg/dl	121.3±21.6	122.5±15.2 (101.0)	147.6±14.7	146.0±16.4 (98.9)
apo B, mg/dl	142.6±43.4	130.6±40.4 (91.6)	123.9±41.2	119.8±48.9 (96.7)
apo E, mg/dl	6.0±1.2	5.8±1.3 (96.7)	4.3±1.5	4.2±2.0 (97.7)
TG, mg/dl	230.1±89.9	239.5±142.4 (104.1)	151.8±87.4	135.3±100.8 (89.1)
TC, mg/dl	261.9±69.7	246.1±62.6 (94.0)	256.3±66.1	249.3±85.2 (97.3)
HDL-C, mg/dl	44.9±11.1	45.5±11.3 (100.9)	58.2±7.1	58.6±6.7 (100.7)
LDL-C, mg/dl	185.5±71.4	166.1±68.0 (89.5)	174.4±58.0	164.4±70.8 (94.3)
sdLDL-C, mg/dl	56.4±25.0	39.2±19.7 (69.5)	37.5±21.4	32.5±23.3 (86.7)
PL, mM	2.8±0.5	2.8±0.5 (100.0)	2.9±0.5	2.8±0.8 (96.6)
PlsCho, μM	40.9±9.0	51.5±3.6 (125.9)*	50.7±12.5	57.3±18.5 (113.0)
PlsEtn, μM	44.9±13.1	53.9±6.7 (120.0)	56.6±20.6	60.0±20.3 (106.0)
PlsCho+ PlsEtn, μM	85.8±20.3	105.4±8.6 (122.8)*	107.2±30.3	117.3±37.4 (109.4)
PlsCho/PL, mol%	1.5±0.2	1.9±0.2 (130.3)***	1.7±0.4.	2.1±0.5.(115.8)
PlsEtn/PL, mol%	1.6±0.4	2.0±0.4 (124.4)	2.0±0.9	2.2±0.8 (106.4)
(PlsCho+PlsEtn)/PL, mol%	3.1±0.5	3.9±0.6 (127.2)**	3.8±1.3	4.2±1.2 (111.0)
PlsCho/PlsEtn ratio	0.94±0.23	0.97±0.12 (103.2)	0.94±0.21	0.98±0.17 (104.3)

Values are mean±S.D., and values in parentheses are relative percentage to each value before treatment.
t-test between before and after treatment, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 8. Effect of *myo*-inositol treatment on clinical and serum biochemical parameters of the hyperlipidemic subjects with (MetS) and without metabolic syndrome (non-MetS)

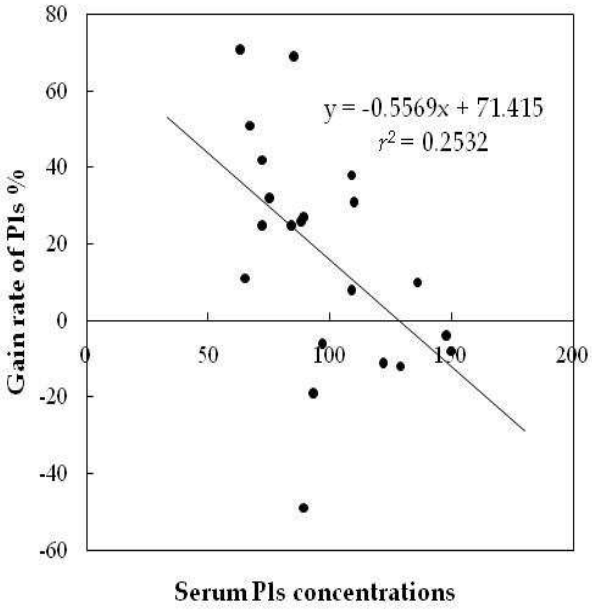


Fig. 11. Relationship between the gain rate of Pls after MI treatment and serum Pls levels before treatment

3. Conclusion

We have established auto-analytical ^{125}I-HPLC system for a routine diagnostic test of human serum (or plasma) Pls. We have applied the system to the determination of serum (or plasma) Pls from normal subjects and CAD patients, and found that serum (or plasma) PlsCho is a novel and reliable biomarker for MetS and atherosclerosis. Intervention study of increasing PlsCho by MI treatment demonstrated clinical utility of PlsCho as a serum (or plasma) biomarker for atherogenic status. Improvement of PlsCho deficiency is important for preventing from life-style related disease associated with aging and oxidative stress. MI intake is one of the effective ways to enhance Pls biosynthesis.

4. Acknowledgement

We especially thank the subjects who participated in these studies. We thank Mr. Yuya Yamazaki of the Life Science Department, Advanced Materials R&D Laboratory, ADEKA Co., for developing ^{125}I-HPLC method. These works were supported by "Knowledge Cluster Initiative" (2nd stage, "Sapporo Bio-cluster Bio-S") of the Ministry of Education, Science, Sports and Culture of Japan. Part of these works was enabled by a Grant-in-Aid for the Technology Development Project for the Creation of Collective Private Agribusiness (H15-7) from the Ministry of Agriculture, Forestry and Fisheries, Japan.

5. References

Berry, G.; Wu, S.; Buccafusca, R.; Ren, J.; Gonzales, L.; Ballard, P.; Golden, J.; Stevens, M. & Greer, J. (2003). Loss of murine Na^+/myo-inositol cotransporter leads to brain myo-inositol depletion and central apnea, *J Biol Chem,* Vol.278, pp. 18297-18302

Brites, P; Waterham, H. & Wanders, R. (2004). Functions and biosynthesis of plasmalogens in heath and disease, *Biochim Biophys Acta,* Vol.1636, pp. 219-231

Brosche, T. (1997). Plasmalogen phospholipids – facts and theses to their antioxidative qualities, *Arch Gerontol Geriatr,* Vol.25, pp. 73-81

Engelmann, B.; Brautigam, C. & Thiery, J. (1994). Plasmalogen phospholipids as potential protectors against lipid peroxidation of low density lipoproteins, *Biochem Biophys Res Commun,* Vol.204, pp.1235-1242

Glaser, P. & Gross, R. (1994). Plasmenylethanolamine facilitates rapid membrane fusion: a stopped-flow kinetic investigation correlating the propensity of a major plasma membrane constituent to adapt an H_{II} phase with its ability to promote membrane fusion, *Biochemistry,* Vol.33**,** pp. 5805-5812

Goodenow, D.; Cook, L.; Liu, J.; Lu, Y.; Jayasinghe, D.; Ahiahonu, P.; Heath, D.; Yamazaki, Y.; Flax, J.; Krenitsky, K.; Sparks D.; Lerner, A.; Friedland, R.; Kudo, T.; Kamino, K.; Morihara, T.; Takeda, M. & Wood, P. (2007). Peripheral ethanolamine plasmalogen deficiency: A logical causative factor in alzheimer's disease and dementia, J Lipid Res, Vol.48, pp. 2485-2498

Gorgas, K.; Teigler, A.; Komljenovic, D. & Just, W. (2006). The ether lipid-deficient mouse: Tracking down plasmalogen functions, *Biochim Biophys Acta,* Vol.1763, pp. 1511-1526

Gullans, S. & Verbalis, J. (1993). Control of brain volume during hyperosmolar and hypoosmolar conditions, *Annu Rev Med,* Vol.44, pp. 289-301

Hoffman-Kuczynski, B. & Reo, N. (2004). Studies of myo-inositol and plasmalogen metabolism in rat brain, *Neurochem Re,s* Vol.29, pp. 843-855

Horrocks, L. (1968). The alk-1-enyl group content of mammalian myelin phosphoglycerides by quantitative two-dimensional thin-layer chromatography, *J Lipid Res,* Vol.9, pp. 469-472

Horrocks, L.; Yeo, Y.; Harder, H.; Mozzi, R. & Goracci, G. (1986). Choline plasmalogen, glycerophospholipid methylation, and receptor-mediated activation of adenylate cyclase, In: *Advances in cyclic nucleotide and protein phsophorylation research*, Greengard, P. & Robison, G., pp.263-291, Raven Press, New York

Ingrand, S.; Wahl, A.; Favreliere, S.; Barbot, F. & Tallineau, C. (2000). Quantification of long-chain aldehydes by gas chromatography coupled to mass spectrometry as a tool for simultaneous measurement of plasmalogens and their aldehydic breakdown products, *Anal Biochem,*Vol.280, pp. 65-72

Lee, T. (1998). Biosynthesis and possible biological functions of plasmalogens, *Biochim Biophys Acta,* Vol.1394, pp. 129-145

Lessig, J. & Fuchs, B. (2009). Plasmalogens in biological systems: Their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis, *Curr Med Chem,* Vol.16, pp. 2021-2041

Liu, D.; Nagan, N.; Just, W.; Rodemer, C.; Thai, T. & Zoeller, R. (2005). Roles of dihydroxyacetonephosphate acyltransferase in the biosynthesis of plasmalogens and nonether glycerolipids, *J Lipid Res,* Vol.46, pp.727-735

Maeba, R. & Ueta, N. (2003a). Ethanolamine plasmalogen prevent the oxidation of cholesterol by reducing the oxidizability of cholesterol in phospholipids bilayers, *J Lipid Res,* Vol.44, pp. 164-171

Maeba, R. & Ueta, N. (2003b). Ethanolamine plasmalogen and cholesterol reduce the total membrane oxidizability measured by the oxygen uptake method, *Biochem Biophys Res Commun,* Vol.302, pp. 265-270

Maeba, R. & Ueta, N. (2004). Determination of choline and ethanolamine plasmalogens in human plasma by HPLC using radioactive triiodide (1-) ion ($^{125}I_3^-$), *Anal Biochem,* Vol.331, pp. 169-176

Maeba, R.; Maeda, T.; Kinoshita, M.; Takao, K.; Takenaka, H.; Kusana, J.; Yoshimura, N.; Takeoka, Y.; Yasuda, D.; Okazaki, T. & Teramoto, T. (2007). Plasmalogens in human serum positively correlate with high-density lipoprotein and decrease with aging, *J Atheroscler Thromb,* Vol.14, pp. 12-18

Maeba, R.; Hara, H.; Ishikawa, H.; Hayashi, S.; Yoshimura, N.; Kusano, J.; Takeoka, Y.; Yasuda, D.; Okazaki, T.; Kinoshita, M. & Teramoto, T. (2008). *Myo*-inositol treatment increases serum plasmalogens and decreases small dense LDL, particularly in the hyperlipidemic subjects with metabolic syndrome, *J Nutr Sci Vitaminol,* Vol.54, pp. 196-202

Maeba, R.; Yamazaki, Y.; Nezu, T. & Okazaki, T. (2012). Improvement and validation of ^{125}I-high-performance liquid chromatography method for determination of total human serum choline and ethanolamine plasmalogens, *Ann Clin Biochem,* Vol.49, pp. 86-93

Nagan, N. & Zoeller, R. (2001). Plasmalogens: biosynthesis and functions, *Prog Lipid Res,* Vol.40, pp. 199-229

Noga, A.; Zhao, Y. & Vance, D. (2002). An unexpected requirement for phosphatidylethanolamine *N*-methyltransferase in the secretion of very low density lipoproteins, *J Biol Chem,* Vol.277, pp. 42358-42365

Nozue, T.; Michishita, I.; Ishibashi, Y.; Ito, S.; Iwaki, T.; Mizuguchi, I.; Miura, M.; Ito, Y. & Hirano, T. (2007). Small dense low-density lipoprotein cholesterol is a useful marker of metabolic syndrome in patients with coronary artery disease, *J Atheroscler Thromb,* Vol.14, pp. 202-207

Pacifico, L.; Nobili, V.; Anania, C.; Verdecchia, P. & Chiesa, C. (2011). Pediatric nonalcoholic fatty liver disease, metabolic syndrome and cardiovascular risk, *World J Gastroenterol*, Vol.17, pp. 3082-3091

Poynter, M. & Davnes, R. (1998). Peroxisome proliferators-activated receptor α activation modulates cellular redox status, represses nuclear factor-κB signaling, and reduces inflammatory cytokine production in aging, *J Biol Chem,* Vol.273, pp. 32833-32841

Resseguie, M.; Song, J.; Niculescu, M.; Costa, K.; Randall, T. & Zeisel S. (2007). Phosphatidylethanolamine *N*-methyltransferase (*PEMT*) gene expression is induced by estrogen in human and mouse primary hepatocytes, *FASEB J*, Vol.21, pp. 2622-2632

Song, J.; Costa, K.; Fischer, L.; Kohlmeier, M.; Kwock, L.; Wang, S. & Zeisel S. (2005). Polymorphism of the *PEMT* gene and susceptibility to nonalcoholic fatty liver disease (NAFLD), *FASEB J*, Vol.19, pp. 1266-1271

Vance, J. (1990). Lipoproteins secreted by cultured rat hepatocytes contain the antioxidant 1-alk-1-enyl-2-acylglycerophosphoethanolamine, *Biochim Biophys Acta,* Vol.1045, pp. 128-134

Vanhaesebroeck, B.; Leevers, S.; Ahmadi, K.; Timms, J.; Katso, R.; Driscoll, P.; Woscholski, R.; Parker, P. & Waterfield, M. (2001). Synthesis and function of 3-phosphorylated inositol lipids, *Annu Rev Biochem*, Vol.70, pp. 535-602

Wanders, R. & Waterham H. (2006). Peroxisomal disorders: The single peroxisomal enzyme deficiencies, *Biochim Biophys Acta,* Vol.1763, pp. 1707-172 169-176

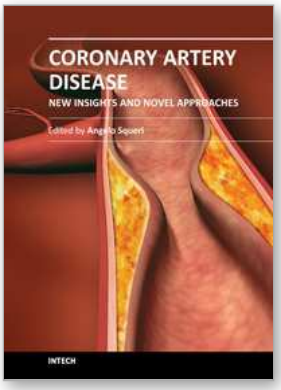
Wiesner, P.; Leidl, K.; Boettcher, A.; Schmitz, G. & Liebisch, G. (2009). Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry, *J Lipid Res,* Vol.50, pp. 574-585

Williams, J.; Anderson, C. & Jasik, A. (1962). A sensitive and specific method for plasmalogens and other enol ethers, *J Lipid Res,* Vol.3, pp. 378-381

Zemski, B. & Murphy, R. (2004). Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids, *J Am Soc Mass Spectrom,* Vol.15, pp. 1499-1508

Zoeller, R.; Morand, O. & Raetz, C. (1988). A possible role for plasmalogens in protecting animal cells against photosensitized killing, *J Biol Chem*, Vol.263, pp. 11590-11596

Zoeller, R.; Lake, A.; Nagan. N.; Gaposchkin, D.; Legner, M. & Lieberthal, W. (1999). Plasmalogens as endogenous antioxidants: somatic cell mutants reveal the importance of the vinyl ether, *Biochem J,* Vol.338, pp.769-776



Coronary Artery Disease - New Insights and Novel Approaches
Edited by Dr. Angelo Squeri

ISBN 978-953-51-0344-8
Hard cover, 260 pages
Publisher InTech
Published online 16, March, 2012
Published in print edition March, 2012

Coronary Artery disease is one of the leading causes of death in industrialized countries and is responsible for one out of every six deaths in the United States. Remarkably, coronary artery disease is also largely preventable. The biggest challenge in the next years is to reduce the incidence of coronary artery disease worldwide. A complete knowledge of the mechanisms responsible for the development of ischaemic heart disease is an essential prerequisite to a better management of this pathology improving prevention and therapy. This book has been written with the intention of providing new concepts about coronary artery disease pathogenesis that may link various aspects of the disease, going beyond the traditional risk factors.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821