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# Serum Choline Plasmalogen is a Reliable Biomarker for Atherogenic Status

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## 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 (<sup>125</sup>I-HPLC method) (Maeba & Ueta, 2004; Maeba et al, 2012). The method has improved as auto-analytical system by introducing online detection with flow  $\gamma$ -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).

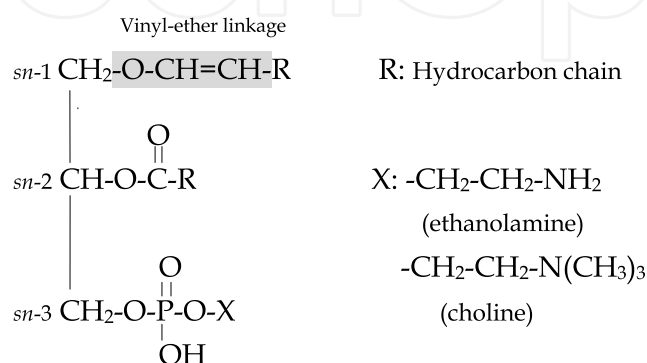


Fig. 1. Chemical structure of PLs

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  $\Delta 1$  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.

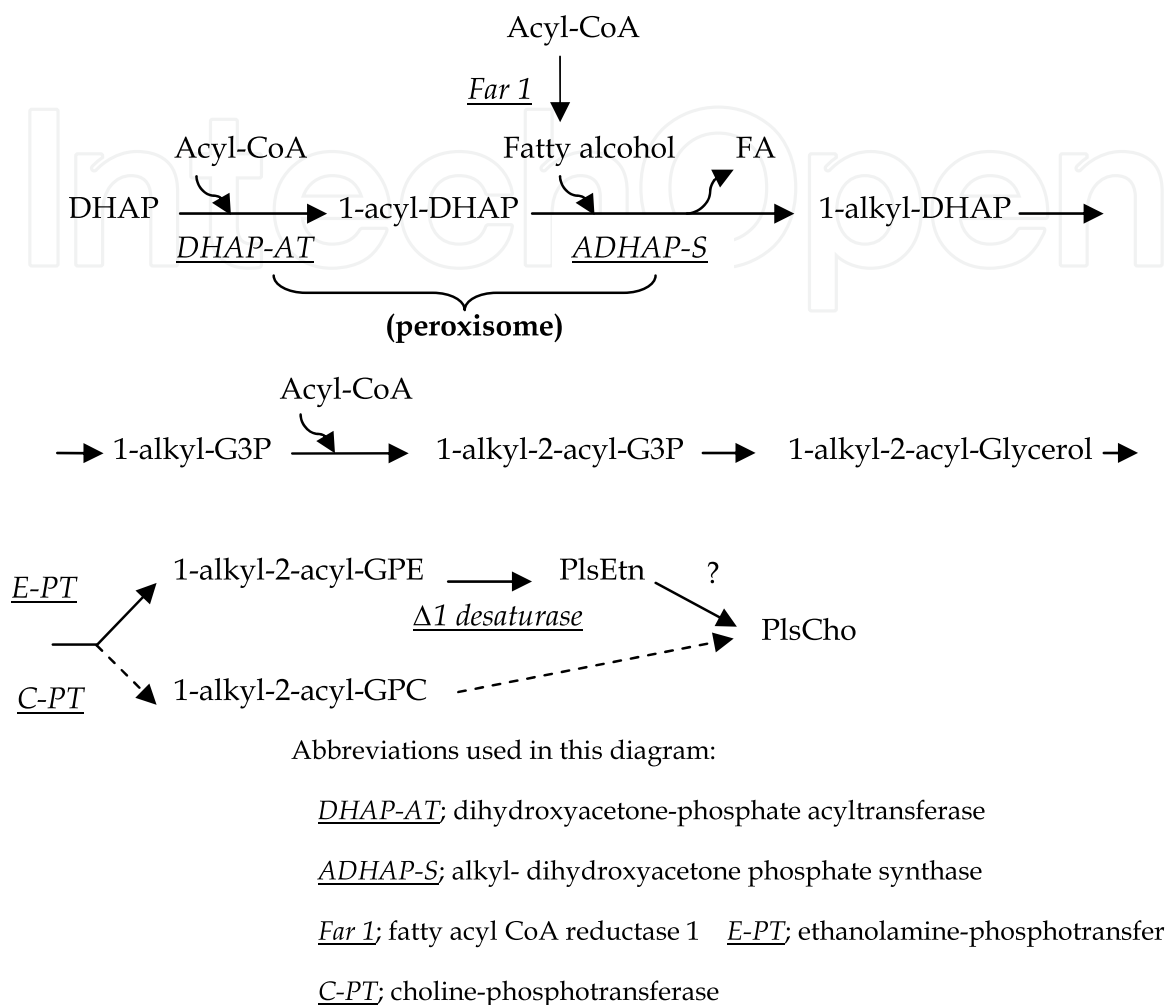


Fig. 3. Biosynthetic pathway of Pls

## 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).

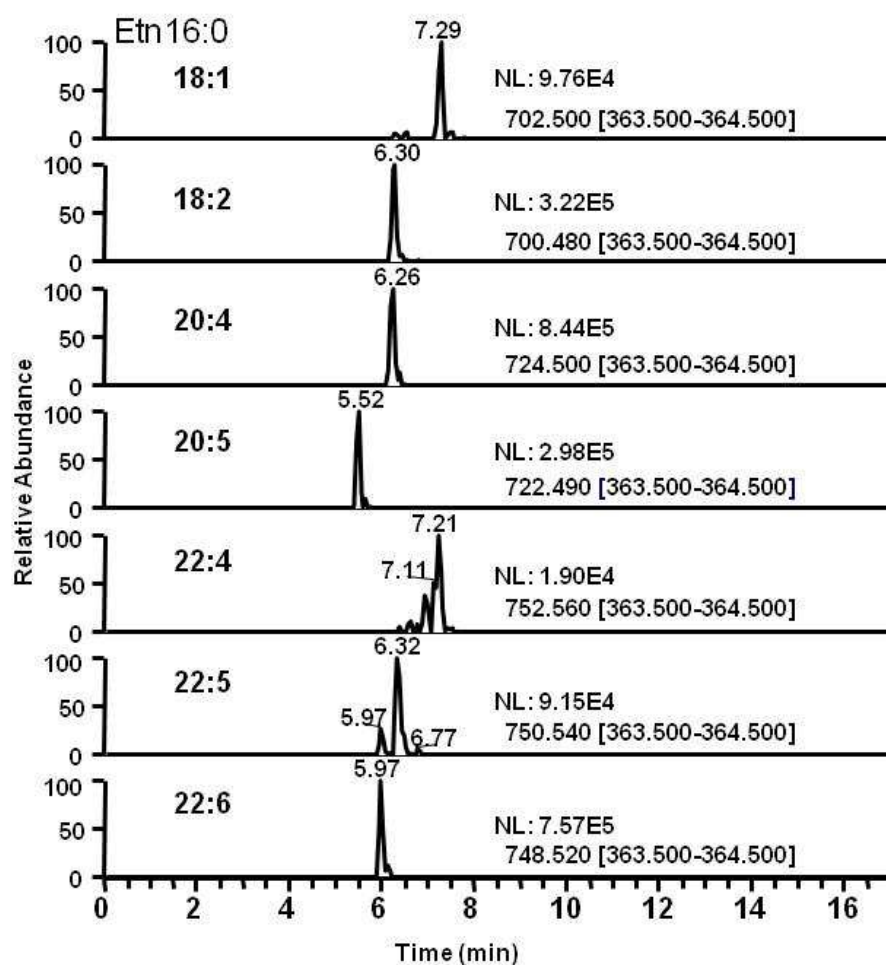


Fig. 4. UPLC-MS/MS analysis of PlsEtn 16:0 molecular species

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 <sup>125</sup>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 (<sup>125</sup>I) and high-performance liquid chromatography (<sup>125</sup>I-HPLC method) (Maeba & Ueta, 2004; Maeba et al, 2012). The method is based on the binding specificity of iodine to Pls. Triiodide (I<sub>3</sub><sup>-</sup>) ion 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. <sup>125</sup>I-HPLC method permitted the highly sensitive and accurate determination of serum (or plasma) Pls by using a radioactive iodine (<sup>125</sup>I), and

removing interfering substances with HPLC. The primary advantage of this method enabled to measure PlsCho and PlsEtn separately.  $^{125}\text{I}$ -HPLC method has been markedly improved by introducing a quantitative standard (Q.S.) and online detection with flow  $\gamma$ -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).

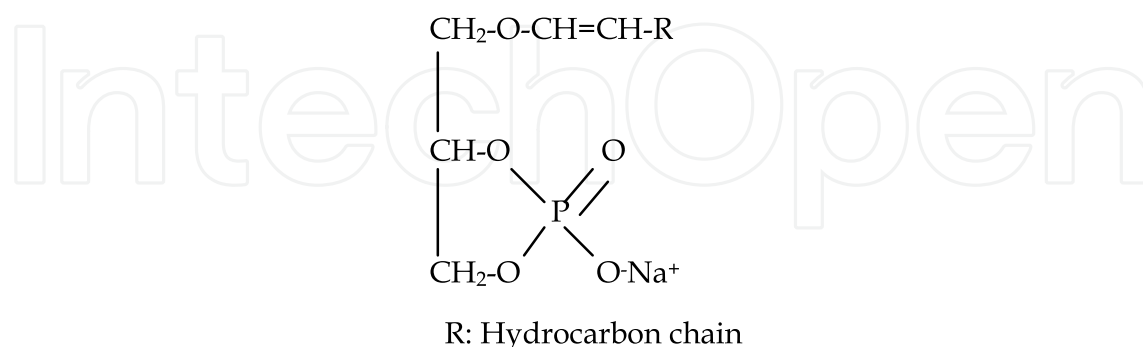


Fig. 5. Chemical structure of 1-Alkenyl 2,3-cyclic glycerophosphate (Q.S.)

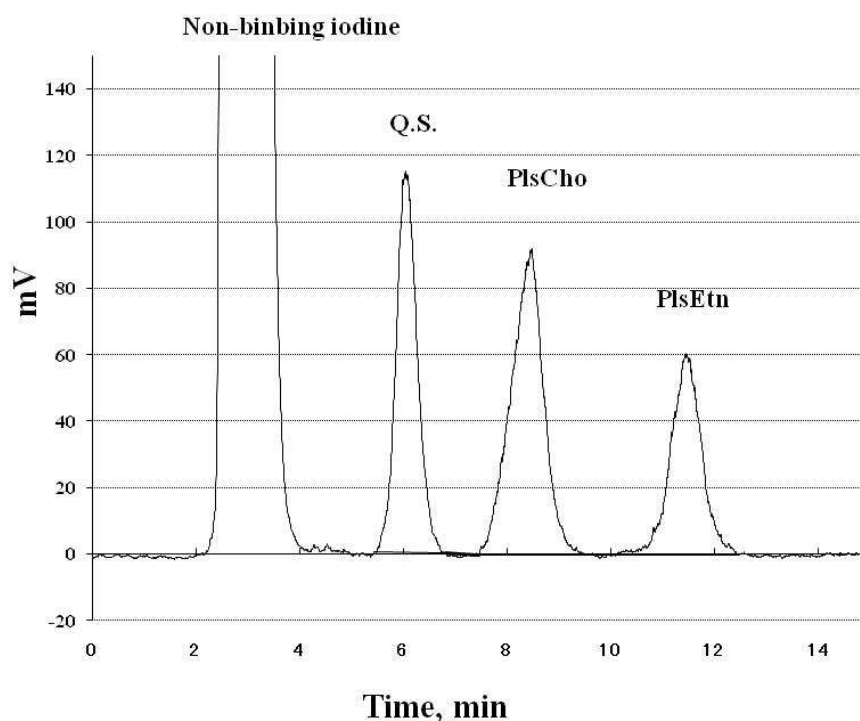


Fig. 6. A typical HPLC elution profile of Pls and Q.S. detected with flow  $\gamma$ -counter

Online detection with flow  $\gamma$ -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.  $^{125}\text{I}$ -HPLC method is useful as a continuous 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).

### Plasmalogen, aging, and oxidative stress

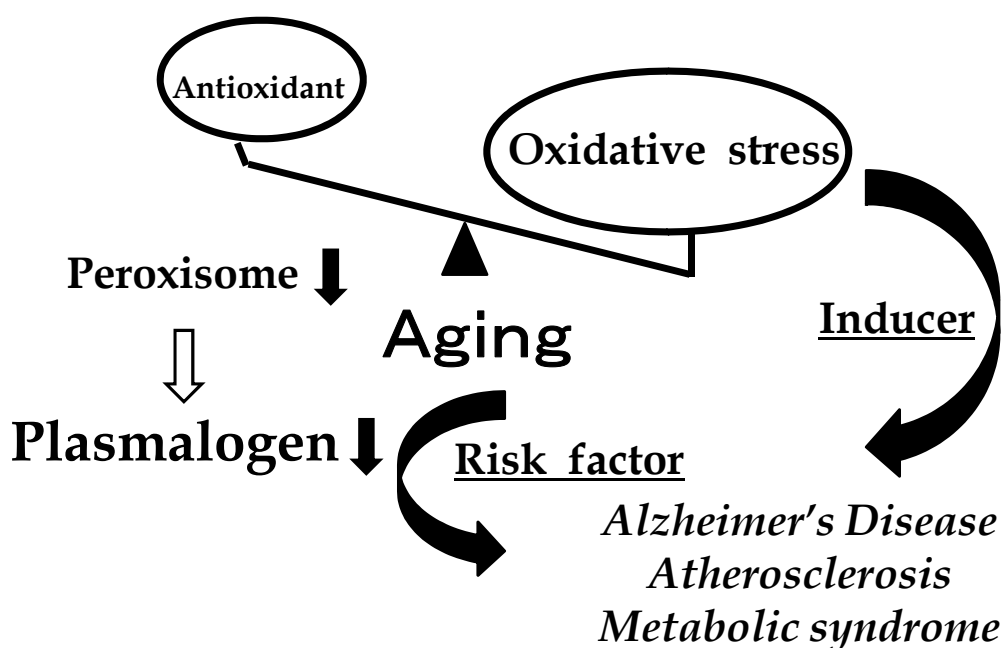


Fig. 7. Hypothesis for Pls deficiency causing life-style related disease

#### 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  $\mu\text{M}$  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  $^{125}\text{I}$ -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
<i>n</i>	74	27	383	69	8	65	74	27
Age	23.9 ±3.7	22.7 ±3.9	40.4 ±10.7	34.8*** ±10.3	77.6 ±5.0	72.3* ±5.1	23.9 ±3.7	22.7 ±3.9
PlsCho μM	98.5 ±23.0	115.2** ±24.5	62.5 ±12.2	69.8*** ±13.0	54.9 ±10.2	54.7 ±11.2		
PlsEtn μM	133.8 ±33.3	142.9 ±38.7	73.3 ±19.4	71.0 ±20.5	68.8 ±13.0	68.5 ±16.6		
PlsCho+PlsEtn μM	232.4 ±53.3	258.1 ±61.2	135.8 ±29.0	140.7 ±29.8	123.6 ±21.7	123.2 ±25.5		
PlsCho/PlsEtn ratio	0.75 ±0.12	0.83* ±0.14	0.88 ±0.17	1.04*** ±0.28	0.80 ±0.11	0.82 ±0.15	0.29 ±0.04	0.29 ±0.03
PL mM	2.17 ±0.34	2.14 ±0.41	2.99 ±0.49	2.94 ±0.33	3.28 ±0.40	3.44 ±0.38		
PlsCho/PL mol %	4.6 ±1.1	5.6* ±1.6	2.2 ±0.4	2.4*** ±0.5	1.7 ±0.3	1.6 ±0.3	5.7 ±1.6	6.5* ±1.6
PlsEtn/PL mol %	6.3 ±1.8	6.9 ±2.2	2.5 ±0.6	2.5 ±0.7	2.1 ±0.5	2.0 ±0.5	19.2 ±4.6	22.3* ±4.8
(PlsCho+PlsEtn)/PL mol %	10.9 ±2.7	12.5 ±3.7	4.6 ±0.9	4.8 ±1.1	3.8 ±0.8	3.6 ±0.8	24.9 ±6.0	28.8* ±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.).

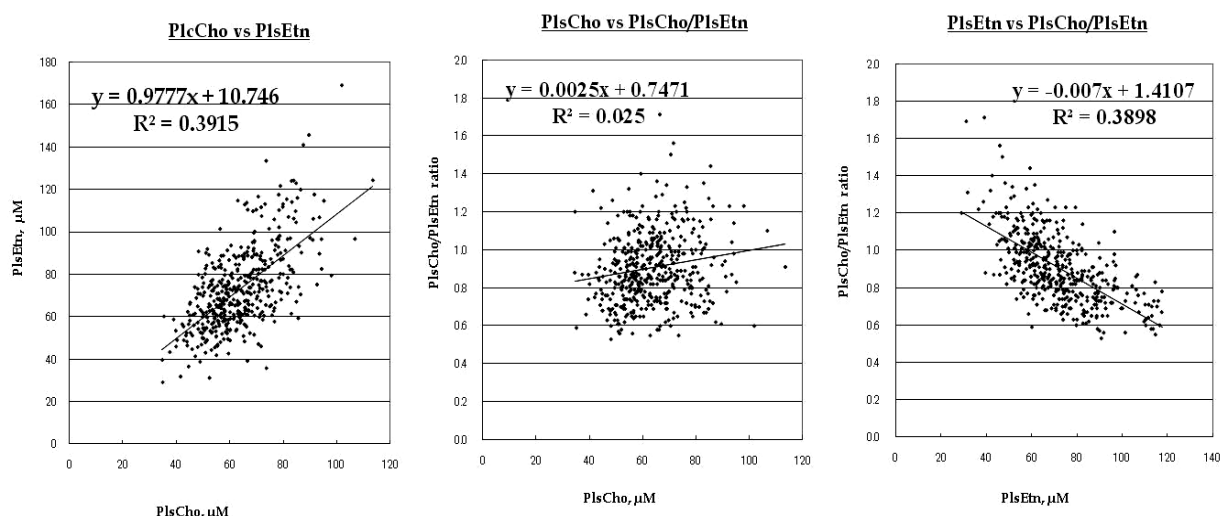


Fig. 8. Relationship between PlsCho and PlsEtn

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  $137.0 \pm 29.6$ , and PlsCho/PlsEtn ratio was  $0.87 \pm 0.19$ .

n	216	$\gamma$ -GTP	$58 \pm 58$	Adiponectin	$6.9 \pm 4.8$
(M/F)	(195/21)	(U/l)		( $\mu\text{g}/\text{mg}$ )	
Age	$48.9 \pm 7.1$	HDL-C	$65 \pm 18$	PlsCho	$62.7 \pm 12.9$
		(mg/dl)		( $\mu\text{M}$ )	
Body weight	$68.5 \pm 11.3$	LDL-C	$124 \pm 27$	PlsEtn	$74.2 \pm 19.6$
(kg)		(mg/dl)		( $\mu\text{M}$ )	
BMI	$24.6 \pm 6.0$	TG	$121 \pm 88$	PlsCho+PlsEtn	$137.0 \pm 29.6$
( $\text{kg}/\text{m}^2$ )		(mg/dl)		( $\mu\text{M}$ )	
Waist circumference	$83.0 \pm 8.9$	sdLDL	$33.0 \pm 12.9$	PlsCho/PlsEtn	$0.87 \pm 0.19$
(cm)		(mg/dl)		ratio	
Systolic pressure	$126 \pm 14$	Glu	$96 \pm 13$	PL	$3.13 \pm 0.47$
(mmHg)		(mg/dl)		(mM)	
Diastolic pressure	$81 \pm 11$	UA	$6.2 \pm 1.5$	PlsCho/PL	$2.0 \pm 0.4$
(mmHg)		(mg/dl)		(mol %)	
GOT	$25 \pm 10$	Hcy	$11.0 \pm 5.0$	PlsEtn/PL	$2.4 \pm 0.6$
(U/l)		( $\mu\text{mol}/\text{l}$ )		(mol %)	
GPT	$28 \pm 18$	hsCRP	$78 \pm 67$	(PlsCho+PlsEtn)/PL	$4.4 \pm 1.0$
(U/l)		( $\mu\text{g}/\text{dl}$ )		(mol %)	

Mean $\pm$ S.D.

Table 2. Clinical and biochemical data of middle-aged ( $\geq 40$  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).

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

Correlation coefficient	TG	AIP	Glu	UA	hsCRP	Adiponectin	Hcy
PlsCho	<b>-0.327</b>	<b>-0.576</b>	-0.168	-0.151	<b>-0.250</b>	<b>0.314</b>	-0.233
PlsEtn	-0.038	-0.248	0.107	-0.012	-0.105	0.103	-0.194
PlsCho+PlsEtn	-0.166	<b>-0.414</b>	-0.001	-0.073	-0.178	0.205	-0.230
PlsCho/PlsEtn	<b>-0.265</b>	-0.241	<b>-0.265</b>	-0.119	-0.032	0.172	0.031
PL	<b>0.382</b>	0.188	0.101	<b>0.253</b>	-0.068	-0.024	-0.063
PlsCho/PL	<b>-0.542</b>	<b>-0.674</b>	-0.236	<b>-0.318</b>	-0.226	<b>0.319</b>	-0.179
PlsEtn/PL	-0.271	<b>-0.396</b>	0.042	-0.172	-0.054	0.125	-0.158
(PlsCho+PlsEtn)/PL	<b>-0.421</b>	<b>-0.561</b>	-0.081	<b>-0.255</b>	-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 ( $\geq 40$ ) 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.

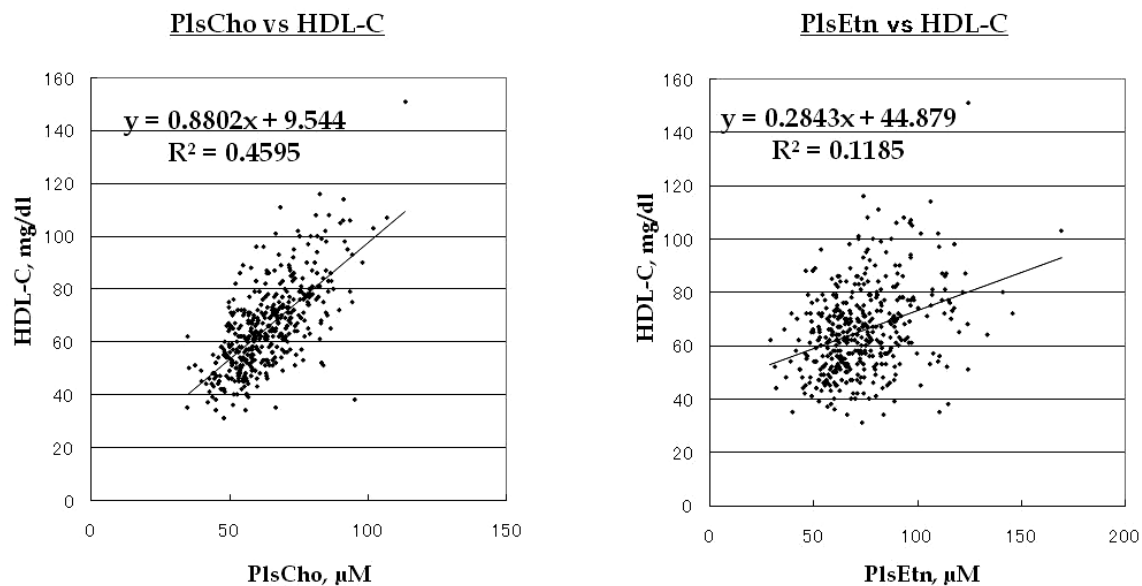


Fig. 9. Relationship between Pls and HDL

### 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 ( $\geq 75\%$ ) and without ( $< 50\%$ ) significant stenosis (**Table 4-1**). Serum PlsCho was significantly decreased in CAD patients with  $\geq 75\%$  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 ( $\mu\text{mol/g}$ ) in lipoprotein particles were compared between patients with and without significant stenosis. The CAD patients with  $\geq 75\%$  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 ( $\geq 75\%$ ) 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.

<b>Serum</b>					
	<i>n</i> (M/F)	Age	PlsCho μM	PlsEtn μM	PlsCho+PlsEtn μM
stenosis					
≥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
	PlsCho/PlsEtn ratio	PL mM	PlsCho/PL mol %	PlsEtn/PL mol %	(PlsCho+PlsEtn)/PL mol %
stenosis					
≥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
<b>HDL</b>					
	PlsCho/PlsEtn ratio	PlsCho/PL mol %	PlsEtn/PL mol %	PlsCho/CH mol %	PlsEtn/CH mol %
stenosis					
≥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
	PlsCho/TG mol %	PlsEtn/TG mol %	PlsCho/protein μmol/g	PlsEtn/protein μmol/g	
stenosis					
≥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	
<b>LDL</b>					
	PlsCho/PlsEtn ratio	PlsCho/PL mol %	PlsEtn/PL mol %	PlsCho/CH mol %	PlsEtn/CH mol %
stenosis					
≥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
	PlsCho/TG mol %	PlsEtn/TG mol %	PlsCho/protein μmol/g	PlsEtn/protein μmol/g	
stenosis					
≥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

	α-Toc. μM	β-Toc. μM	γ-Toc. μM	δ-Toc. μM
stenosis				
≥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

<i>n</i> (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.

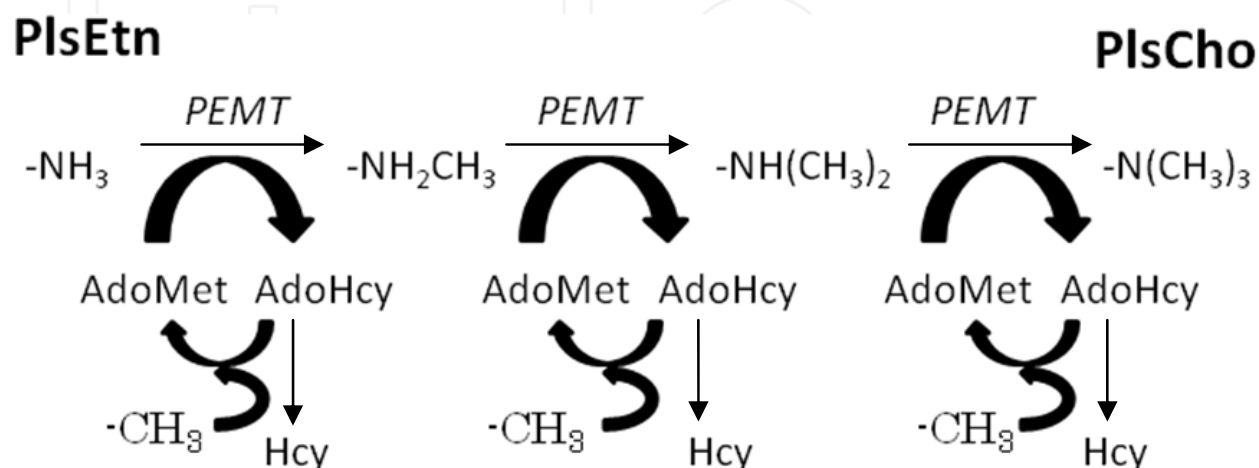


Fig. 10. PEMT pathway for conversion of PlsEtn to PlsCho

### 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  $\text{Ca}^{2+}$  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 through 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  $\mu\text{M}$  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.

<i>n</i> (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 circumference: M ≥ 85 cm, F ≥ 90 cm
- 2) TG ≥ 150 mg/dl and/or HDL-C < 40 mg/dl
- 3) Systolic pressure ≥ 130 mmHg and/or Diastolic pressure ≥ 85 mmHg
- 4) Fasting glucose ≥ 110 mg/dl

Table 7. Clinical background of study subjects

	MetS ( <i>n</i> = 8)		non- MetS ( <i>n</i> = 9)	
	before	after	before	after
BMI, kg/m <sup>2</sup>	29.3±3.0	29.7±3.7 (101.4)	25.3±2.3	25.2±2.6 (99.6)
Waist circumference, 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)

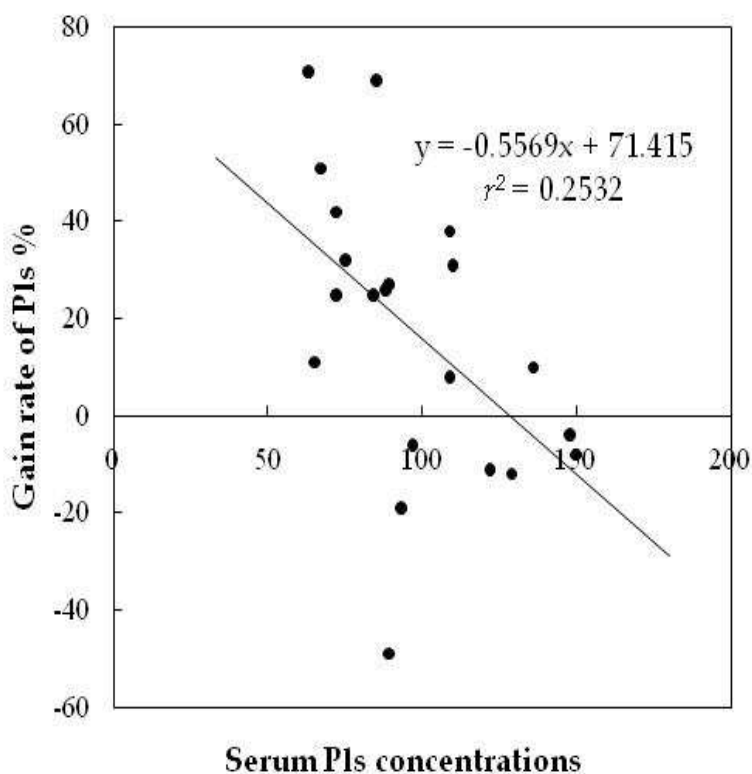


Fig. 11. Relationship between the gain rate of Pls after MI treatment and serum Pls levels before treatment

### 3. Conclusion

We have established auto-analytical  $^{125}\text{I}$ -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  $^{125}\text{I}$ -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.



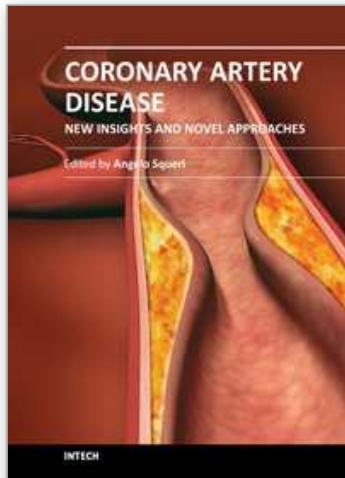
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ISBN 978-953-51-0344-8

Hard cover, 260 pages

**Publisher** InTech

**Published online** 16, March, 2012

**Published in print edition** March, 2012

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