Lipoprotein-Associated Phospholipase A$_2$ Activity Is a Marker of Small, Dense LDL Particles in Human Plasma

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Background: Recent clinical studies showed that lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$) is a predictor for incident atherosclerotic disease. We have previously shown that among the LDL subfractions, Lp-PLA$_2$ activity is preferentially associated with the atherogenic small, dense (sdLDL) particles in vitro. We investigated whether Lp-PLA$_2$ could be a marker of sdLDL in human plasma.

Methods: One hundred and seventy-six individuals participated in the study. LDL subclass analysis was performed by polyacrylamide gel electrophoresis. Lp-PLA$_2$ activity and mass were determined in total plasma and in apolipoprotein B-depleted plasma (HDL-Lp-PLA$_2$). Non–HDL-Lp-PLA$_2$ activity and mass were calculated by subtracting the HDL-Lp-PLA$_2$ from total plasma Lp-PLA$_2$.

Results: On the basis of the LDL subclass analysis, participants were categorized into phenotype A and non-A (total cholesterol mass of the sdLDL subfractions ≤0.155 and >0.155 mmol/L, respectively). Unlike total plasma Lp-PLA$_2$ mass, total plasma Lp-PLA$_2$ activity and non–HDL-Lp-PLA$_2$ activity and mass were significantly higher in persons with phenotype non-A compared with persons with phenotype A, whereas HDL-Lp-PLA$_2$ activity and mass were lower in persons with phenotype non-A compared with phenotype A. Total plasma activity and non–HDL-Lp-PLA$_2$ activity and mass, but not Lp-PLA$_2$ mass, were correlated with sdLDL-cholesterol mass, proportion, and mean LDL particle size. In multiple regression analysis, total plasma and non–HDL-Lp-PLA$_2$ activities were the second best predictors of the presence of sdLDL particles in human plasma after serum triglyceride concentrations. At serum triglyceride concentrations >1.356 mmol/L, total plasma and non–HDL-Lp-PLA$_2$ activity added significantly to the prediction of the presence of sdLDL in plasma.

Conclusions: Lp-PLA$_2$ activity, but not the enzyme mass, is a marker of sdLDL in human plasma.
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LDL-cholesterol is a major target in the guidelines for the prevention of coronary artery disease (CAD)[3] *(1)*. However, plasma LDL-cholesterol concentrations are insufficient to identify individuals with incident CAD events because ~50% of all CAD events occur in persons with normal or even low LDL-cholesterol concentrations *(2)*. This has led to the hypothesis that other factors may be involved in the pathogenesis of atherosclerosis and CAD.

LDL is a heterogeneous population of particles with respect to size, density, and chemical composition. These differences have led to the recognition of 2 distinct phenotypes: phenotype A, which is associated with large, buoyant LDL particles; and phenotype B, in which small, dense LDL (sdLDL) particles predominate *(3)*. Several studies have shown that sdLDL particles are more atherogenic than large, buoyant LDL *(4–13)*.

Platelet-activating factor (PAF) acetylhydrolase (EC 3.1.1.47) has Ca^{2+}-independent phospholipase A$_2$ activity and degrades PAF and oxidized phospholipids by catalyzing the hydrolysis of the ester bond at the *sn-2* position

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[3] Nonstandard abbreviations: CAD, coronary artery disease; sdLDL, small, dense LDL; PAF, platelet-activating factor; Lp-PLA$_2$, lipoprotein-associated phospholipase A$_2$; apo, apolipoprotein; IDL, intermediate-density lipoprotein; and Lp(a), lipoprotein(a).

(14, 15). PAF-acetylhydrolase in plasma is complexed to lipoproteins; thus it is also referred to as lipoprotein-associated phospholipase A_2 (Lp-PLA_2) *(16, 17)*. Lp-PLA_2 is associated mainly with apolipoprotein B (apoB)-containing lipoproteins and primarily with LDL, whereas a small proportion of circulating enzyme activity is also associated with HDL *(14)*. Recent data from large Caucasian population studies consistently indicated a positive association between plasma Lp-PLA_2 mass or activity and the risk for incident atherosclerotic events *(18–24)*.

Using a gradient ultracentrifugation technique, we have previously shown that in plasma of either normolipidemic volunteers *(16, 25)* or patients with various types of dyslipidemia *(26, 27)*, the majority of the LDL-associated Lp-PLA_2 activity was bound to sdLDL particles. However, it remains to be established whether the plasma enzyme activity or mass could be a marker of these atherogenic sdLDL particles in plasma. We therefore investigated possible correlations between plasma Lp-PLA_2 activity or mass with sdLDL particles with the aim to determine whether the Lp-PLA_2 activity or mass could be a marker of the sdLDL particles in plasma.

Materials and Methods

STUDY POPULATION

A total of 176 individuals (108 women and 68 men) attending the Outpatient Lipid Clinic of the University Hospital of Ioannina participated in the present study. Exclusion criteria were previous atherosclerotic disease (myocardial infarction, unstable angina, ischemic stroke, peripheral arterial disease, percutaneous transluminal coronary angioplasty, and coronary artery bypass graft), diagnosed diabetes mellitus [fasting glucose >6.875 mmol/L (>125 mg/dL)], liver disease [serum aminotransferase activity >3-fold above the upper limit of the reference interval, e.g., >120 U/L (reference interval, 5–40 U/L)], renal disease [serum creatinine >15 mg/L (reference interval, 6–12 mg/L)], and hypothyroidism [thyroid-stimulating hormone >5 mIU/L (reference interval, 0.5–4.8 mIU/L)]. Moreover, patients receiving drugs that could affect lipid metabolism as well as renal or hepatic function were also excluded from the present study. The Ethics Committee of the University Hospital of Ioannina gave approval for the study, and all participants gave written consent.

LDL SUBCLASS ANALYSIS

LDL subclass analysis was performed electrophoretically by use of high-resolution 3% polyacrylamide gel tubes and the Lipoprint LDL System (Quantimetrix), according to the manufacturer's instructions *(28)*. In this method, VLDL remains in the origin [retention factor (R_f) = 0.0], whereas HDL migrates at the front (R_f = 1.0). In between, several bands can be detected: MID bands C, B, and A, which correspond mainly to intermediate-density lipoprotein (IDL), as well as up to 7 LDL bands. The LDL1 and LDL2 bands correspond to large, buoyant LDL particles, whereas bands LDL3 to -7 correspond to sdLDL particles. We determined the cholesterol mass (in mmol/L) of each lipoprotein subfraction, the mean LDL particle size (in Å), and the proportion (%) of the cholesterol mass of sdLDL subfractions (LDL3 to -7) over the total LDL-cholesterol mass. According to the LDL electrophoretic profile, 2 phenotypes were defined: phenotype A (total cholesterol mass of the sdLDL subfractions ≤0.155 mmol/L) and phenotype non-A (total cholesterol mass of the sdLDL subfractions >0.155 mmol/L).

SUBFRACTIONATION OF PLASMA LIPOPROTEINS BY ULTRACENTRIFUGATION

Lipoproteins were fractionated by isopycnic density gradient ultracentrifugation as described previously *(16)*. Total plasma containing lipoprotein(a) [Lp(a)] concentrations <8 mg/L and the HDL-containing supernatant, after treatment of plasma with magnesium chloride–dextran sulfate (to precipitate all apoB-containing lipoproteins), were subjected separately to ultracentrifugation. After ultracentrifugation, 30 fractions of 0.4 mL each were collected and analyzed for their protein content. When plasma was used, equal volumes of gradient fractions 1 to 12 were pooled to form the following apoB-containing subfractions: VLDL + IDL (d <1.019 kg/L), LDL-1 (d = 1.019–1.023 kg/L), LDL-2 (d = 1.023–1.029 kg/L), LDL-3 (d = 1.029–1.039 kg/L), LDL-4 (d = 1.039–1.050 kg/L), and LDL-5 (d = 1.050–1.063 kg/L) *(26)*. When the HDL-containing supernatant was used, equal volumes of gradient fractions 13 to 23 were pooled to form the following apoAI-containing subfractions: HDL-2b (d = 1.063–1.091 kg/L), HDL-2a (d = 1.091–1.100 kg/L), HDL-3a (d = 1.100–1.133 kg/L), HDL-3b (d = 1.133–1.156 kg/L), and HDL-3c (d = 1.156–1.179 kg/L) *(29)*. All subfractions were dialyzed extensively at 4 °C in 10 mmol/L phosphate-buffered saline (per liter, 16.4 g of NaCl, 2.8 g of NaH_2PO_4, 3.6 g of $Na_2HPO_4 \cdot 2 H_2O$, pH 7.4) containing 2 mmol/L EDTA, filter-sterilized, and maintained at 4 °C under nitrogen until analysis. Under these storage conditions, no oxidation has been reported to occur *(30)*.

MEASUREMENT OF PLASMA Lp-PLA_2 ACTIVITY

We determined the Lp-PLA_2 activity in total plasma, in apoB-depleted plasma after sedimentation of all apoB-containing lipoproteins with dextran sulfate–magnesium chloride (HDL-Lp-PLA_2 activity), and in lipoprotein subfractions by trichloroacetic acid precipitation with [³H]-PAF (100 μmol/L final concentration) as the substrate *(16)*. The reaction was performed for 10 min at 37 °C, and Lp-PLA_2 activity is reported as nmol PAF degraded per minute per milliliter of plasma or milligram of protein in the LDL subfraction. The non–HDL-Lp-PLA_2 activity was calculated by subtracting the HDL-Lp-PLA_2 activity from the total plasma enzyme activity. The Lp-PLA_2 specific activity is reported as the ratio of the enzyme activity to the enzyme mass ($nmol \cdot ng^{-1} \cdot min^{-1}$).

MEASUREMENT OF PLASMA Lp-PLA$_2$ MASS

We determined the Lp-PLA$_2$ mass in total plasma, in apoB-depleted plasma (HDL-Lp-PLA$_2$ mass), and in the lipoprotein subfractions by use of a dual monoclonal antibody immunoassay standardized to recombinant Lp-PLA$_2$ (PLAC test; diaDexus, Inc.), according to the manufacturer's instructions (31). We used 10 μL of undiluted plasma or apoB-depleted plasma or 10 μL from each lipoprotein subfraction as the source of the enzyme, and calculated the non–HDL-Lp-PLA$_2$ mass by subtracting the HDL-Lp-PLA$_2$ mass from the total plasma enzyme mass.

BIOCHEMICAL MEASUREMENTS

All samples for lipid and lipoprotein measurements were collected after participants had fasted overnight. Serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, Lp(a), and triglycerides were determined as described previously (26). The total cholesterol, triglyceride, and phospholipid content in each lipoprotein subfraction was measured by enzymatic methods using the Bio-Merieux reagents. The protein content of each lipoprotein subfraction was measured by the bicinchoninic acid method (Pierce), and the lipoprotein mass of each subfraction was calculated as the sum of the mass of the individual lipid and protein components (26).

STATISTICAL ANALYSIS

Data are presented as the mean (SD) except for non–gaussian-distributed variables, which are presented as the median (range). Preliminary analyses were performed to ensure no violation of the assumptions of normality and linearity. The Kolmogorov–Smirnoff test was used to evaluate whether each variable followed a gaussian distribution. The relationships among study variables were investigated by use of the Pearson product–moment correlation coefficient, whereas correlations including at least 1 non–gaussian-distributed variable were performed with the Spearman correlation coefficient. The independent samples t-test (or Mann–Whitney U-test when required) was used to assess differences in the variables evaluated in the present study between groups (phenotype A and phenotype non-A groups). Linear multiple regression analyses were performed to explore the relationships between a dependent variable and a set of independent variables (or predictors), after checking for normality and linearity of the variables included. The Kruskal–Wallis test was performed to discriminate differences in non–gaussian-distributed variables between more than 2 groups. A P value <0.05 was considered to be significant. All analyses were carried out with the Statistica 6 softpack.

Results

CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF THE STUDY POPULATION

One hundred and seventy-six individuals participated in the study. The clinical and biochemical characteristics of the study population are shown in Table 1. On the basis of the results of the electrophoretic LDL subclass analysis,

Table 1. Clinical and biochemical characteristics of the study population.[a]

Variable	Total	Phenotype A	Phenotype non-A
No.	176	69	107
Sex, M/F	68/108	25/44	43/64
Age, years	49.2 (12.5)	44 (11.7)	52.4 (12.1)
SBP,[b] mmHg	135.8 (18.7)	132.5 (19.9)	138.2 (17.2)
DBP, mmHg	87.1 (10.5)	85.7 (11.5)	88.1 (10.0)
Waist circumference, cm	106.9 (16.1)	102.9 (19.9)	109.4 (13.6)[c]
Total cholesterol, mmol/L	5.93 (1.15)	5.40 (0.93)	6.26 (1.17)[d]
Triglycerides, mmol/L	2.00 (1.16)	1.34 (0.71)	2.43 (1.21)[d]
LDL-cholesterol, mmol/L	3.72 (0.89)	3.42 (0.76)	3.91 (0.95)[d]
HDL-cholesterol, mmol/L	1.32 (0.30)	1.36 (0.29)	1.29 (0.31)
Non–HDL-cholesterol, mmol/L	4.64 (1.08)	4.07 (0.87)	4.97 (1.07)[d]
Lp(a), mg/L	105 (24–500)	108 (24–500)	103 (24–423)
Glucose, mmol/L	5.47 (0.84)	5.42 (0.68)	5.50 (0.92)
Insulin, mIU/L	13.3 (7.6)	12.2 (7.4)	14.3 (7.7)
HOMA index	3.3 (2.2)	3.0 (2.1)	3.6 (2.2)
VLDL-cholesterol, mmol/L	1.24 (0.26–3.52)	0.95 (0.26–2.18)	1.45 (0.44–3.52)[d]
IDL-cholesterol, mmol/L	1.19 (0.36–3.13)	1.44 (0.52–3.13)	1.09 (0.36–2.1)[d]
Buoyant LDL-cholesterol, mmol/L	1.76 (0.28–3.29)	1.79 (0.28–2.9)	1.74 (0.49–3.29)[d]
sdLDL-cholesterol, mmol/L	0.31 (0–2.05)	0.1 (0–0.156)	0.57 (0.16–2.05)[d]
sdLDL proportion, %	13.58 (0–71.8)	5.23 (0–12.77)	25.4 (7.25–71.8)[d]
Mean LDL size, Å	265 (242–274)	270 (263–274)	261 (242–271)[d]

[a] Values are the mean (SD) or the median (range).
[b] SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA, homeostasis model assessment.
[c,d] Compared with phenotype A: [c] $P <0.04$; [d] $P <0.001$.

we categorized participants into phenotype A (total cholesterol mass of the sdLDL subfractions ≤0.155 mmol/L) and phenotype non-A (total cholesterol mass of the sdLDL subfractions >0.155 mmol/L). Typical electrophoretic lipoprotein profiles of phenotype A and phenotype non-A are illustrated in Fig. 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue12/. Of the 176 participants, 107 (60.8%) presented with phenotype non-A. The lipid and lipoprotein subfraction profiles that were significantly different between phenotype A and non-A individuals are indicated in Table 1.

PLASMA $Lp\text{-}PLA_2$ ACTIVITY AND MASS
Total plasma $Lp\text{-}PLA_2$ activity, but not the enzyme mass, was significantly higher in persons with phenotype non-A than in those with phenotype A (Table 2). As expected, plasma enzyme activity was significantly correlated with total cholesterol ($r = 0.34$), LDL-cholesterol ($r = 0.33$), non–HDL-cholesterol ($r = 0.41$), and triglyceride concentrations ($r = 0.28$), whereas the enzyme mass exhibited weaker but significant correlations with the above lipid fractions [total cholesterol ($r = 0.22$), LDL-cholesterol ($r = 0.21$), non–HDL-cholesterol ($r = 0.2$), triglycerides ($r = 0.20$)]. The $Lp\text{-}PLA_2$ mass was significantly correlated with $Lp\text{-}PLA_2$ activity ($r = 0.49$). The specific activity of total plasma $Lp\text{-}PLA_2$ was significantly higher in persons with phenotype non-A than in those with phenotype A (Table 2).

The lack of difference in the plasma $Lp\text{-}PLA_2$ mass between the 2 groups, despite the significant difference in the enzyme activity, led us to determine the enzyme mass and activity of HDL and then to calculate the non-HDL mass and activity. As shown in Table 2, the $HDL\text{-}Lp\text{-}PLA_2$ activity and mass were significantly lower in persons with phenotype non-A than in persons with phenotype A. It is important to note that in the total population, the $HDL\text{-}Lp\text{-}PLA_2$ activity represented 4.9 (0.8)% of the total plasma enzyme activity, whereas the $HDL\text{-}Lp\text{-}PLA_2$ mass represented 28.5 (2.0)% of total plasma enzyme mass. Thus, the $HDL\text{-}Lp\text{-}PLA_2$ activity does not significantly contribute to the total plasma enzyme activity, whereas the $HDL\text{-}Lp\text{-}PLA_2$ mass significantly influences the total

plasma enzyme mass. Furthermore, the $HDL\text{-}Lp\text{-}PLA_2$ specific activity in the total population as well as in each group was significantly lower than total plasma specific activity. Finally, we observed no difference in the $HDL\text{-}Lp\text{-}PLA_2$ specific activity between persons with phenotype A and non-A (Table 2).

Non–$HDL\text{-}Lp\text{-}PLA_2$ activity and mass were significantly higher in persons with phenotype non-A than in persons with phenotype A (Table 2). Non–$HDL\text{-}Lp\text{-}PLA_2$ activity was correlated with total cholesterol ($r = 0.33$), LDL-cholesterol ($r = 0.32$), non–HDL-cholesterol ($r = 0.41$), triglycerides ($r = 0.30$), and HDL-cholesterol ($r = -0.23$). Non–$HDL\text{-}Lp\text{-}PLA_2$ mass was correlated with waist circumference ($r = 0.48$), systolic and diastolic blood pressure ($r = 0.37$ and 0.36, respectively), total cholesterol ($r = 0.42$), non–HDL-cholesterol ($r = 0.50$), and triglycerides ($r = 0.47$). Non–$HDL\text{-}Lp\text{-}PLA_2$ specific activity in the total population as well as in each group was significantly higher than that of total plasma $Lp\text{-}PLA_2$ or $HDL\text{-}Lp\text{-}PLA_2$ (Table 2). It should be noted that the dextran sulfate–magnesium chloride reagent used for the precipitation of apoB-containing lipoproteins and preparation of the apoB-depleted plasma does not significantly affect the assays for either $Lp\text{-}PLA_2$ activity or $Lp\text{-}PLA_2$ mass (data not shown).

$Lp\text{-}PLA_2$ ACTIVITY AND MASS IN LIPOPROTEIN SUBFRACTIONS
In an effort to further investigate the above differences in the $Lp\text{-}PLA_2$ activity and mass observed between plasma and HDL, we performed lipoprotein subfractionation, using an isopycnic density gradient ultracentrifugation method. To study the distributions of $Lp\text{-}PLA_2$ activity and mass among the apoB-containing lipoprotein subspecies, we subjected total plasma to ultracentrifugation. The $Lp\text{-}PLA_2$ activity and mass were preferentially associated with the dense LDL-5 subfraction in persons with either phenotype A or non-A. From these experiments we were able to calculate the molar ratio between LDL particles and $Lp\text{-}PLA_2$. This calculation revealed that 1 molecule of $Lp\text{-}PLA_2$ corresponds to ~100 particles of LDL-5 and to 4000 particles of either LDL-2 or LDL-3. Importantly, our results showed that the specific activity of $Lp\text{-}PLA_2$ asso-

Table 2. Mean (SD) Lp-PLA₂ activity and mass in the study population.

	Total	Phenotype A	Phenotype non-A
Plasma $Lp\text{-}PLA_2$ activity, $nmol \cdot mL^{-1} \cdot min^{-1}$	53.8 (15.9)	48.5 (12.3)	56.9 (17.3)[a]
Plasma $Lp\text{-}PLA_2$ mass, $\mu g/L$	284.4 (83.2)	279.2 (87.4)	285.4 (82.3)
Plasma $Lp\text{-}PLA_2$ specific activity, $nmol \cdot ng^{-1} \cdot min^{-1}$	0.197 (0.058)	0.182 (0.050)	0.206 (0.060)[b]
$HDL\text{-}Lp\text{-}PLA_2$ activity, $nmol \cdot mL^{-1} \cdot min^{-1}$	2.5 (0.9)	2.8 (1.1)	2.4 (1.0)[c]
$HDL\text{-}Lp\text{-}PLA_2$ mass, $\mu g/L$	80.2 (26.4)	85.7 (29.6)	71.4 (13.8)[b]
$HDL\text{-}Lp\text{-}PLA_2$ specific activity, $nmol \cdot ng^{-1} \cdot min^{-1}$	0.037 (0.011)	0.040 (0.012)	0.031 (0.010)
Non–$HDL\text{-}Lp\text{-}PLA_2$ activity, $nmol \cdot mL^{-1} \cdot min^{-1}$	51.4 (16.1)	45.7 (12.4)	54.6 (17.4)[a]
Non–$HDL\text{-}Lp\text{-}PLA_2$ mass, $\mu g/L$	218.8 (90.2)	179.7 (75)	250.0 (90.8)[b]
Non–$HDL\text{-}Lp\text{-}PLA_2$ specific activity, $nmol \cdot ng^{-1} \cdot min^{-1}$	0.269 (0.165)	0.295 (0.222)	0.249 (0.089)

[a-c] Compared with phenotype A: [a] $P < 0.001$; [b] $P < 0.02$; [c] $P < 0.01$.

ciated with LDL-5 particles was 3-fold lower than that of large, buoyant LDL particles. Shown in Fig. 1 are bar graphs representing the total protein profile of apoB-containing lipoprotein subspecies as well as the profiles of the Lp-PLA$_2$ activity, mass, and specific activity in plasma from a phenotype non-A person.

We next studied the distribution of Lp-PLA$_2$ among the apoAI-containing lipoproteins, using apoB-depleted plasma to avoid any contamination by HDL-Lp-PLA$_2$ of the LDL-associated enzyme during ultracentrifugation (29, 32). The Lp-PLA$_2$ activity and mass were preferentially associated with the HDL-3c subfraction in persons of either phenotype (A or non-A). According to our calculations, 1 molecule of Lp-PLA$_2$ corresponds to ~10 particles of HDL-3c or to 1500 particles of the HDL-3a subfraction. Importantly, the specific activity of Lp-PLA$_2$ associated with the HDL-3c subfraction was 2- to 6-fold lower than in the other HDL subfractions. It should be noted that we found no difference in the above variables between phenotype A and non-A individuals. Shown in Fig. 2 are representative profiles of the distribution of total protein in the apoAI-containing lipoprotein subspecies

and of the Lp-PLA$_2$ activity, mass, and specific activity in plasma of a phenotype non-A individual.

Lp-PLA$_2$ AS A MARKER OF sdLDL

The preferential association of Lp-PLA$_2$ activity and mass with LDL-5 particles and the differences in the enzyme activity and mass observed between phenotype A and non-A individuals prompted us to investigate whether Lp-PLA$_2$ could be a marker for sdLDL in plasma. Univariate analysis revealed that the sdLDL-cholesterol mass and proportion were positively correlated with total plasma Lp-PLA$_2$ activity as well as with non–HDL-Lp-PLA$_2$ activity or mass, whereas mean LDL particle size was significantly negatively correlated with the above variables (Table 3). It should be noted that we found no correlation between total plasma Lp-PLA$_2$ mass and sdLDL-cholesterol mass, LDL particle size, or sdLDL proportion (Table 3). Furthermore, we found no correlation between the cholesterol mass of buoyant LDL particles and Lp-PLA$_2$ activity or mass. Multiple regression analysis showed that plasma Lp-PLA$_2$ activity is the second best predictor of the presence of sdLDL particles
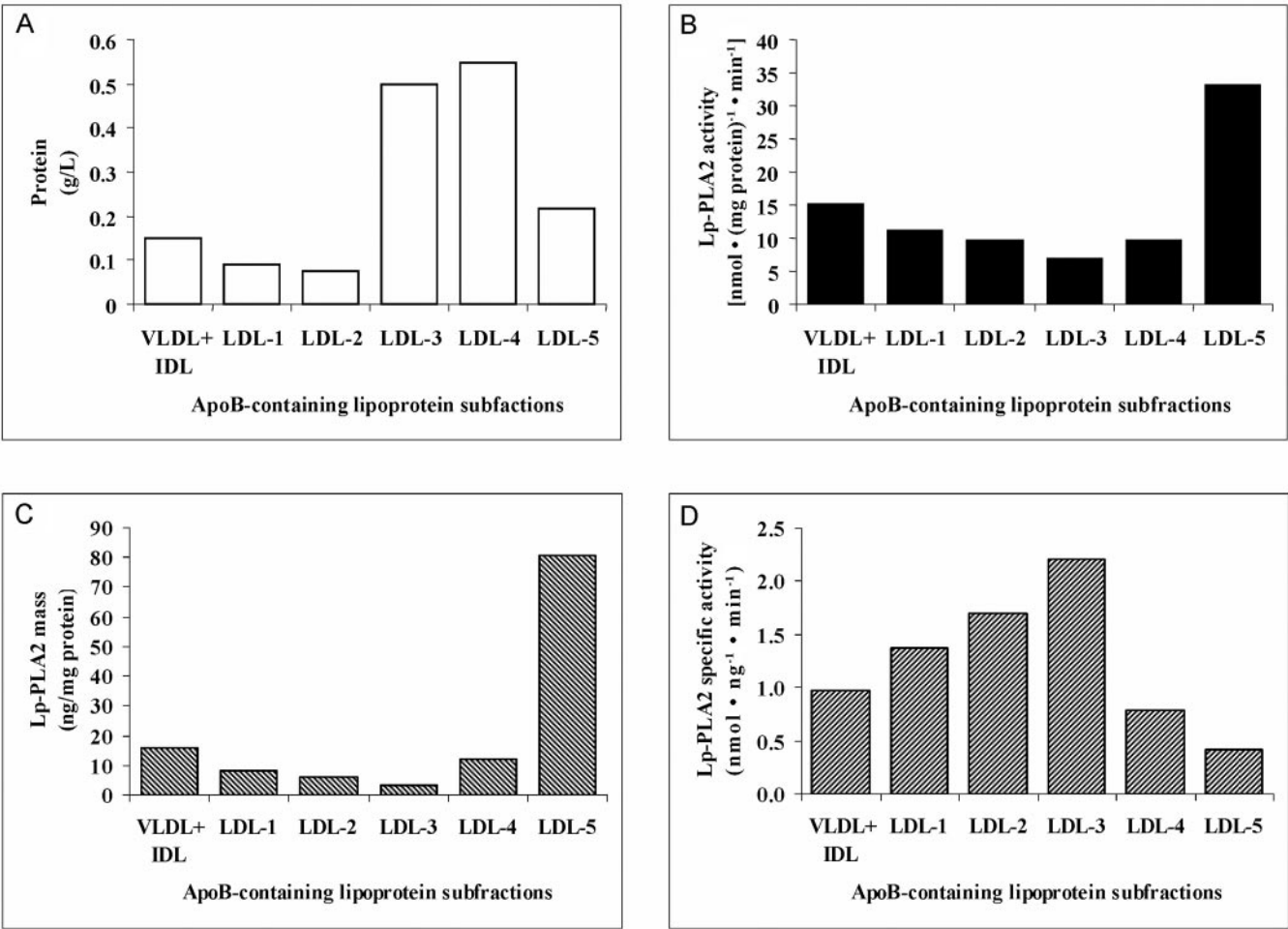

Fig. 1. Bar graphs illustrating the distribution profiles of total protein (A), Lp-PLA$_2$ activity (B), Lp-PLA$_2$ mass (C), and Lp-PLA$_2$ specific activity (D) among apoB-containing lipoprotein subspecies in plasma of a phenotype non-A individual.
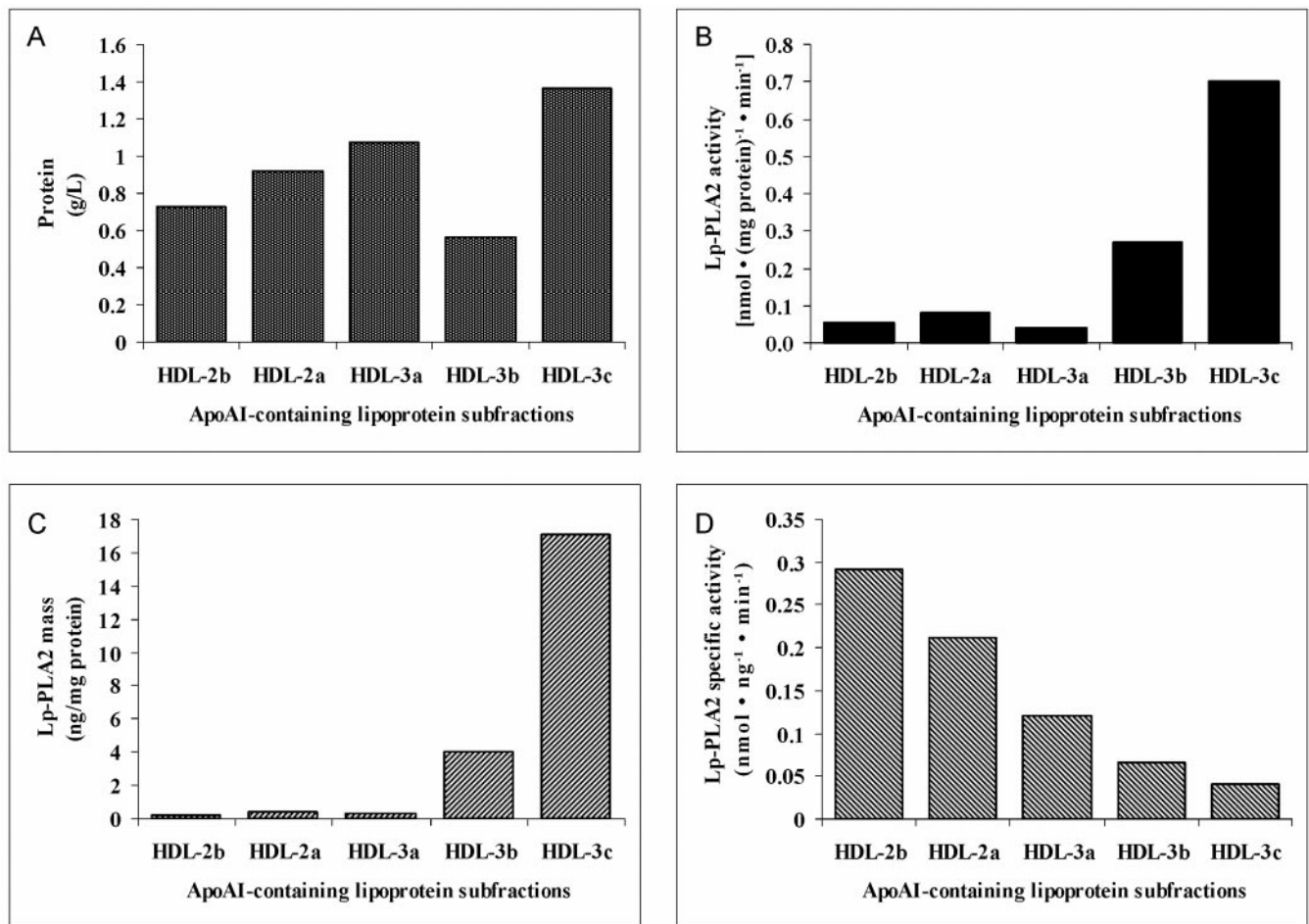
Fig. 2. Bar graphs illustrating the distribution profiles of total protein (*A*), Lp-PLA$_2$ activity (*B*), Lp-PLA$_2$ mass (*C*), and Lp-PLA$_2$ specific activity (*D*) among apoAI-containing lipoprotein subspecies in plasma of a phenotype non-A individual.

in human plasma after serum triglyceride concentrations (Table 4, model 1). We obtained similar results for the non–HDL-Lp-PLA$_2$ activity (Table 4, model 2). When we included non–HDL-Lp-PLA$_2$ mass in the model (model 3), the only predictor of the presence of sdLDL particles was serum triglycerides (Table 4). It should be noted that when LDL-cholesterol was included in the model instead of non–HDL-cholesterol, the results of the multiple re-

Table 3. Spearman correlation coefficient for sdLDL-cholesterol mass, sdLDL proportion, and LDL particle size.

Variable	sdLDL-cholesterol		LDL particle size		sdLDL proportion	
	ρ	*P*	ρ	*P*	ρ	*P*
Age	0.309	0.001	−0.302	0.001	0.295	0.001
Total cholesterol	0.524	0.001	−0.372	0.001	0.376	0.001
Triglycerides	0.592	0.001	−0.628	0.001	0.612	0.001
HDL-cholesterol	−0.06	NS[a]	0.181	0.02	−0.153	0.048
Non–HDL-cholesterol	0.582	0.001	−0.43	0.001	0.436	0.001
LDL-cholesterol	0.37	0.001	−0.21	0.007	0.209	0.007
Plasma Lp-PLA$_2$ activity	0.36	0.001	−0.324	0.001	0.325	0.001
Non–HDL-Lp-PLA$_2$ activity	0.374	0.001	−0.343	0.001	0.341	0.001
Plasma Lp-PLA$_2$ mass	0.192	NS	−0.1446	NS	0.1665	0.04
Non–HDL-Lp-PLA$_2$ mass	0.448	0.007	−0.532	0.001	0.492	0.002
HDL-Lp-PLA$_2$ activity	−0.1	NS	0.16	NS	−0.2	0.011
HDL-Lp-PLA$_2$ mass	−0.18	NS	0.19	NS	−0.265	NS

[a] NS, not significant.

Table 4. Multiple linear regression analysis for the prediction of sdLDL proportion and LDL particle size.

	sdLDL proportion		LDL particle size	
	β	P	β	P
Model 1[a]				
Triglycerides	0.549	0.001	−0.509	0.001
Plasma Lp-PLA₂ activity	0.174	0.017	−0.148	0.04
Model 2[b]				
Triglycerides	0.546	0.001	−0.507	0.001
Non–HDL-Lp-PLA₂ activity	0.175	0.017	−0.148	0.04
Model 3[c]				
Triglycerides	0.532	0.009	−0.522	0.008

[a] Variables included in the model: triglycerides, HDL-cholesterol, non–HDL-cholesterol, age, and plasma Lp-PLA₂ activity.

[b] Variables included in the model: triglycerides, HDL-cholesterol, non–HDL-cholesterol, age, and non–HDL-Lp-PLA₂ activity.

[c] Variables included in the model: triglycerides, HDL-cholesterol, non–HDL-cholesterol, age, and non–HDL-Lp-PLA₂ mass.

gression analysis did not alter, indicating that LDL-cholesterol concentrations do not contribute to the prediction of the presence of sdLDL particles in plasma (data not shown).

To investigate the impact of the total plasma or non–HDL-Lp-PLA₂ activity as well as the impact of the non–HDL-Lp-PLA₂ mass in combination with triglyceride concentrations on the prediction of the presence of sdLDL in plasma, we divided the participants into 4 groups according to the Lp-PLA₂ and triglyceride concentrations (Fig. 3). We used 1.356 mmol/L as the cutoff for the serum triglycerides because it has been reported that the existence of sdLDL particles in plasma is associated with serum triglyceride concentrations >1.356 mmol/L (3). We also used the mean total plasma and nonHDL-Lp-PLA₂ activities as cutoffs, as well as the non–HDL-Lp-PLA₂ mass. As shown in Fig. 3, at serum triglyceride concentrations >1.356 mmol/L, total plasma and non–HDL-Lp-PLA₂ activity significantly added to the prediction of the presence of sdLDL in plasma. By contrast, the non–HDL-Lp-PLA₂ mass did not contribute to the predictive efficiency of serum triglyceride concentrations (Fig. 2 in the online Data Supplement). Finally, at serum triglyceride concentrations <1.356 mmol/L, neither Lp-PLA₂ activity nor the enzyme mass significantly contributed to the predictive efficiency of serum triglyceride concentrations for the presence of sdLDL in plasma (Fig. 3; also see Fig. 2 in the online Data Supplement).

Discussion

Our results show for the first time that plasma Lp-PLA₂ activity is a marker of sdLDL in human plasma. This is consistent with the preferential distribution of Lp-PLA₂ in the sdLDL subfraction observed in our gradient ultracentrifugation experiments (16, 25, 26, 29). However, serum triglyceride concentrations are a better predictor of sdLDL; in fact, an increased plasma triglyceride concen-

tration is biochemically a prerequisite for the formation of sdLDL particles. Thus, other than triglyceride concentrations, the total plasma Lp-PLA₂ activity appears to be the best marker of sdLDL in plasma. However, a limitation of this study might be the fact that most of the participants (78% of total) exhibited various types of dyslipidemia, whereas only 38 participants were normolipidemic. Thus, additional studies are necessary to show whether the present results could be extended to normolipidemic individuals.

The present study also shows that the enzyme mass is preferentially distributed on sdLDL particles. However, the Lp-PLA₂ activity in these particles is much less than that expected from the enzyme mass carried by sdLDL particles. Indeed, the specific activity of the Lp-PLA₂ associated with LDL-5 is 2- to 4-fold lower than that associated with the other apoB-containing lipoprotein subfractions. This phenomenon could be attributable to a possible sequestration of enzyme molecules on LDL-5, leading to an inability of these particles to reach their maximum activity. However, this is unlikely because, according to our results, not all LDL-5 particles contain Lp-PLA₂. Indeed, we show for the first time that among 100 sdLDL particles, only 1 contains an Lp-PLA₂ molecule. The possibility that the above phenomenon may be attributable to any influence of plasma factors on the Lp-PLA₂ mass assay can be excluded because we obtained similar results when we measured the enzyme mass either in total plasma or in lipoprotein subfractions completely separated from the other plasma components. Another possibility could be the well-known increased susceptibility of sdLDL to oxidation (33) because, according to data published previously by our group and others, during lipoprotein oxidation the enzyme activity is significantly reduced (34, 35). We found no evidence of oxidation in any lipoprotein subfraction, however. Consequently, the above hypothesis is unlikely. It has been reported previously that the lipid composition of lipoproteins could influence the Lp-PLA₂ activity (36). Thus, the reduced Lp-PLA₂ specific activity in the sdLDL fraction could be attributed to the well-known differences in lipid composition of sdLDL particles compared with buoyant LDL particles (16). An alterative suggestion could be the existence of an inactive form of Lp-PLA₂ in plasma that could be preferentially associated with lipoprotein subfractions migrating at the dense portion of the gradient, i.e., LDL-5 and HDL. Indeed, according to our results, HDL-Lp-PLA₂ activity does not significantly contribute to the total plasma enzyme activity, whereas the HDL-Lp-PLA₂ mass significantly influences the total plasma enzyme mass. Consistent with this suggestion is the lack of any correlation between total plasma enzyme mass and sdLDL, whereas we observed a significant correlation between the non–HDL-Lp-PLA₂ mass and sdLDL. Furthermore, the above findings could at least partially explain the relatively poor correlation between total plasma Lp-PLA₂ activity and mass. In support of the
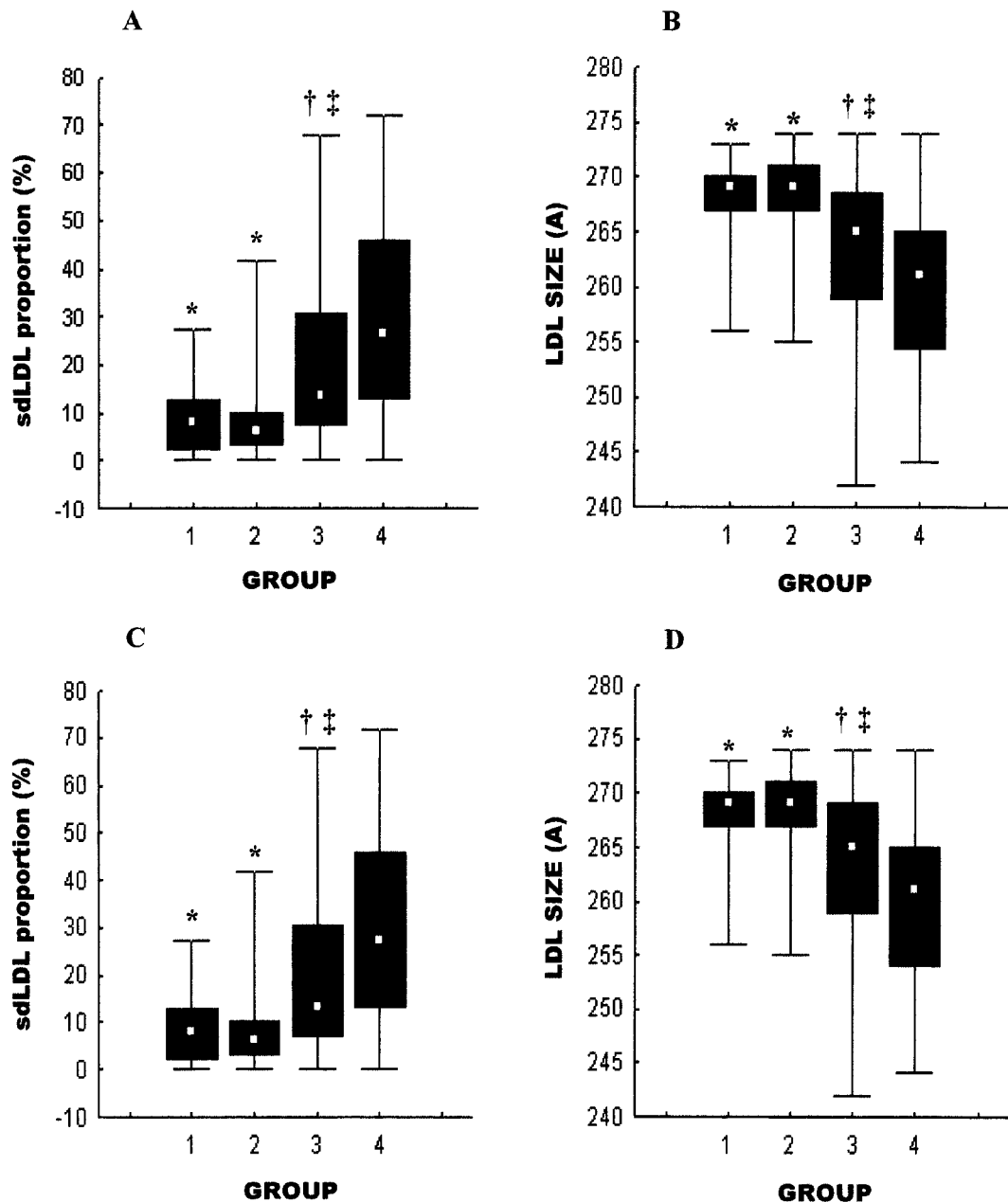
A

B

C

D

Fig. 3. Kruskal–Wallis analysis of the sdLDL proportion (*A* and *C*) and LDL particle size (*B* and *D*) among participants divided into groups according to serum triglyceride concentrations and total plasma Lp-PLA$_2$ activity (*A* and *B*) or non–HDL-Lp-PLA$_2$ activity (*C* and *D*).

□, median; ■, 25th–75th percentiles; *error bars*, minimum–maximum. (*A* and *B*), *group 1*, Lp-PLA2 activity <53.8 nmol · mL^{-1} · min^{-1} and triglycerides ≤1.356 mmol/L; *group 2*, Lp-PLA2 activity >53.8 nmol · mL^{-1} · min^{-1} and triglycerides ≤1.356 mmol/L; *group 3*, Lp-PLA2 activity <53.8 nmol · mL^{-1} · min^{-1} and triglycerides >1.356 mmol/L; *group 4*, Lp-PLA2 activity >53.8 nmol · mL^{-1} · min^{-1} and triglycerides >1.356 mmol/L. ∗, *P* <0.01 compared with groups 3 and 4; †, *P* <0.01 compared with groups 3 and 4; ‡, *P* <0.01 compared with group 4. (*C* and *D*), *group 1*, non–HDL-Lp-PLA2 activity <51.4 nmol · mL^{-1} · min^{-1} and triglycerides ≤1.356 mmol/L; *group 2*, non–HDL-Lp-PLA2 activity >51.4 nmol · mL^{-1} · min^{-1} and triglycerides ≤1.356 mmol/L; *group 3*, non–HDL-Lp-PLA2 activity <51.4 nmol · mL^{-1} · min^{-1} and triglycerides >1.356 mmol/L; *group 4*, non–HDL-Lp-PLA2 activity >51.4 nmol · mL^{-1} · min^{-1} and triglycerides >1.356 mmol/L. ∗, *P* <0.01 compared with groups 3 and 4; †, *P* <0.01 compared with groups 3 and 4; ‡, *P* = 0.001 compared with group 4.

above assumption is the finding that the specific activity of HDL-Lp-PLA$_2$ is ~8-fold less than that of non–HDL-Lp-PLA$_2$. Furthermore, among all HDL subfractions, the specific activity of the dense HDL-3c was lower than that of the larger HDL particles despite the fact that it has the highest molar ratio (1 molecule of Lp-PLA$_2$ corresponds to ~10 particles of HDL-3c). Overall, it is apparent that

the Lp-PLA$_2$ specific activity continuously decreased as the lipoprotein particle size decreased, i.e., from the LDL-3 to the HDL-3c subfraction (Figs. 1 and 2). This phenomenon could be attributed to well-described structural alterations in the lipoprotein particles as their size decreases, which may affect the catalytic efficiency of Lp-PLA$_2$.

Unlike Lp-PLA₂ activity, the enzyme mass failed to predict the presence of sdLDL in human plasma; thus, the ability of the enzyme mass to predict the risk for CAD reported in various clinical trials is unlikely to be related to sdLDL (18–22). In the recent Ludwigshafen risk and cardiovascular health study, Lp-PLA₂ activity was associated with the severity of CAD as well as the number of coronary vessels with significant stenoses (23). In this study, the serum triglyceride concentrations were higher in all patient groups than in the controls; thus, the possibility that the association of Lp-PLA₂ activity with angiographic CAD is at least partially attributable to the ability of Lp-PLA₂ to predict the existence of sdLDL can not be excluded.

On the basis of the results of the above studies as well as the findings of the present study, we suggest that the predictive efficiency of plasma Lp-PLA₂ activity or mass concerning the risk for atherosclerotic disease could be different and highly dependent on other, currently unknown, variables. Thus, Lp-PLA₂ activity could be a marker for at least one well-established atherogenic factor, the presence of sdLDL particles, and it could be a useful substitute for the evaluation of the sdLDL concentrations in plasma, in addition to the serum triglyceride concentrations. However, it remains to be established whether Lp-PLA₂ activity plays a causal role in the atherogenicity of sdLDL or whether it is simply a marker of these particles. Additionally, it remains to be established whether the biochemical and biological properties, and hence the atherogenic characteristics, of the sdLDL particles that carry Lp-PLA₂ are different from the characteristics of those particles not carrying the enzyme. In contrast to Lp-PLA₂ activity, the pathophysiologic basis of the well-established efficiency of Lp-PLA₂ mass to predict atherosclerotic events needs further investigation, particularly considering the possibility that an inactive form of enzyme exists in plasma, as the results of the present study suggest.

In conclusion, the present study shows for the first time that Lp-PLA₂ activity, but not enzyme mass, is a marker for sdLDL in human plasma. Further studies involving larger number of participants are necessary to further establish the predictive efficiency of Lp-PLA₂ activity as a marker for sdLDL in human plasma.

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