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Instability of Lipoprotein(a) in Plasma Stored at −70 °C: Effects of Concentration, Apolipoprotein(a) Genotype, and Donor Cardiovascular Disease

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Background: **There is considerable evidence to suggest that plasma lipoprotein(a) [Lp(a)] concentration is a cardiovascular risk factor. Confusing results in epidemiologic studies, however, suggest that the effects of storage should be further investigated. The influence of the assay method, the initial plasma Lp(a) concentration, and the apolipoprotein(a) [apo(a)] genotype are all factors that should be considered.**

Methods: **Blood was obtained from 65 survivors of premature myocardial infarction and 95 age-matched controls. The plasma samples were stored in sterile conditions at −70 °C for 5 years in the presence of antioxidant and antiproteolytic substances. Plasma Lp(a) was measured by immunoturbidimetry, and apo(a) alleles were determined by pulsed-field gel electrophoresis and Southern blotting.**

Results: **Plasma Lp(a) was significantly higher in patients. The mean kringle number for the smallest isoform was also lower in patients than in controls, but no differences were found in the distribution of the largest isoform. All patients and controls were heterozygotes. During storage, mean Lp(a) decreased significantly in samples from patients (−23%; $P < 0.001$) but not in samples from controls (−9%; *P*, not significant). This was not related to the kringle number and was limited to samples with initial plasma Lp(a) concentrations between 41 and 345 mg/L.**

Conclusions: **Plasma Lp(a) from patients is less stable than Lp(a) from controls, and the difference is not related to distribution of apo(a) genotypes but may be concentration-dependent. Differential sample stability may complicate the interpretation of several studies.**

The lipid composition of lipoprotein(a) [Lp(a)][1] is similar to that of LDL, but the protein moiety consists of apolipoprotein B covalently linked to the highly polymorphic glycoprotein apolipoprotein(a) [apo(a)] *(1)*. The plasma Lp(a) concentration may vary as much as 1000-fold among study participants. In addition, apo(a) has a striking homology to plasminogen and is known to have important physiologic interactions with the coagulation and fibrinolytic systems *(2, 3)*. Consequently, it has been postulated that this particle is a potential bridge between the fields of atherosclerosis and thrombosis *(4)*.

The role of apo(a) as potential bridge between atherosclerosis and thrombosis has been partially supported by several prospective studies *(5–9)*, and increased plasma Lp(a) has been shown to be associated with an increased prevalence and severity of atherosclerotic vascular disease [for a review, see Ref. *(10)*]. Discrepant views, however, have appeared in the literature *(11, 12)*. These views have caused considerable concern and suggest that further studies are required to clarify this question and to explore the reasons for the discrepancies. The effect of long-term storage on the measurement of plasma Lp(a) concentration is of major concern because most studies are performed with samples that have been stored at different temperatures for different periods of time. However, data in the literature are scarce and somewhat confusing *(12–17)*. Only one of these studies *(17)* deals with possible apo(a) phenotype-specific changes in Lp(a)

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[1] Nonstandard abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); and KIV, kringle IV.

concentration, and none of them investigates the stability of Lp(a) measurements in relation to the apo(a) genotype.

Our aim was to investigate the decrease in Lp(a) values in samples from controls and patients with established cardiovascular disease that had been frozen for 5 years and to analyze the relationship between such decrease and the number of kringle IV (KIV) repeats in the smallest and largest isoforms.

Materials and Methods

STUDY POPULATION AND QUANTIFICATION OF PLASMA Lp(a)

The procedures used in this study complied with the ethics standards of the Hospital Universitari de Sant Joan. As in a previous study *(18)*, we reviewed the medical records of 1254 patients who had experienced a myocardial infarction between 1991 and 1995; we subsequently contacted, and invited to participate, all those male patients under the age of 50 (age at the onset of myocardial infarction, not current age) whose history matched the WHO definition *(19)* and who had no history of familial hypercholesterolemia, renal failure, or liver disease.

In a previous experiment, we found that the expected loss of Lp(a) immunoreactivity in fresh samples (n = 20) that were assayed and then frozen for 1 month at −70 °C and reassayed was not significant and that both measurements correlated well (r = 0.99; 187 ± 162 vs 186 ± 171 mg/L). Therefore, to avoid interassay variation, we decided not to use fresh samples and to store samples for a maximum of 1 month to assay them in the same batch. During this period, 65 patients and 95 controls with no clinical evidence of coronary disease and who were of similar ages (± 4 years) and body mass index (± 1.3) were recruited. EDTA blood was collected and centrifuged for 10 min at 4 °C and 2000*g*. The plasma was separated, and two aliquots were stored at −70 °C. The plasma Lp(a) concentration was then measured twice: (*a*) after 1–25 days of storage and (*b*) after 1990–2014 days, ~5 years. Each aliquot was stored in the presence of a mixture containing an antibiotic, antioxidants, and antiproteolytics (final concentrations: gentamicin, 90 g/L; NaN_3, 0.2 g/L, butylated hydroxytoluene, 45 μmol/L; aprotinin, 2 mg/L; and phenylmethylsulfonyl fluoride, 1 mmol/L). Plasma Lp(a) was measured by immunoturbidimetry in a Cobas instrument (Roche) using antibodies and calibrators supplied by Incstar Corporation *(20)* in the same batch; intraassay CVs were 4.6%, 2.4%, and 8.2% for pooled plasma with mean Lp(a) concentrations of 85, 207, and 825 mg/L, respectively. None of the stored specimens had been thawed before analysis. Plasma was thawed at room temperature and assayed in a time frame of 3 h. The specificity of the apo(a) antibodies and their ability to recognize equally the different apo(a) size isoforms have been described *(20)*. The assay is not affected by the presence of apoprotein B, plasminogen, or hyperlipidemia and shows a good correlation with a double monoclonal ELISA assay used at the Northwest Lipid Research Laboratories *(21)*. As certified by the manufacturer, the source, affinity, and other relevant characteristics of the antibody did not change during the observation time. In addition, the control and calibrator materials supplied with the reagent set behaved consistently throughout the 5 years with no relevant changes in the absorbance readings.

The plasma cholesterol and triglyceride concentrations and cholesterol in the HDL were measured as described *(22)*.

APO(A) GENOTYPE DETERMINATION

apo(a) alleles were determined by a modification of the procedure described by Lackner et al. *(23)*. Leukocytes were isolated from whole blood, suspended to a final cell concentration of 2×10^{10} cells/L, and embedded in low-melting temperature agarose plugs. The separation was performed in a Gene Navigator™ apparatus (Pharmacia) with alternating 4-s pulses at 190 mA for 30 min, followed by 10-s pulses at 170 mA for 18 h and 6-s pulses at 170 mA for 6 h. Premade plugs containing lambda phage concatamers (Pharmacia) were used as size calibrators, and several samples were used as internal standards to assure accurate measurement of migration in different gels. The size-fractionated DNA was blotted to a nylon membrane and hybridized with an [^{32}P]dCTP-radiolabeled human apo(a) KIV-specific single-stranded fragment (MP1) *(23)*, kindly provided by Dr. Helen Hobbs (University of Texas Southwestern Medical Center, Dallas, TX). The apo(a) alleles were designated by the estimated number of KIV-encoding sequences per allele. A single KIV repeat was considered to be 5.5 kb in length.

STATISTICAL ANALYSIS

Values are expressed as the mean ± SD. Statistically significant differences were set at $P < 0.05$. For statistical assessment, the plasma Lp(a) concentrations were log-

Table 1. Characteristics of the study population.[a]

Variable	Patients (n = 65)	Controls (n = 95)	P
Age, years	40.1 ± 9.2	42.4 ± 7.6	NS[b]
Body mass index, kg/m^2	27.1 ± 2.4	26.9 ± 2.9	NS
Kringle number,[c] smallest isoform	18.3 ± 4.5	20.5 ± 4.7	0.008
Kingle number,[c] largest isoform	24.5 ± 4.4	26.2 ± 5.1	NS
Current or former smokers, %	76.9	37.9	0.0001
Hypertension, %	12.3	2.1	0.0001
Type II diabetes mellitus, %	3.1	1.0	NS
Plasma cholesterol, mmol/L	6.02 ± 0.85	5.78 ± 0.82	0.04
Plasma triglycerides, mmol/L	2.64 ± 1.5	1.25 ± 0.92	0.001
HDL-cholesterol, mmol/L	0.95 ± 0.23	1.24 ± 0.12	0.0005

[a] Results are expressed as mean ± SD.

[b] NS, not significant.

[c] All participants had two apo(a) alleles that contained different numbers of KIV repeats; the mean number is indicated.

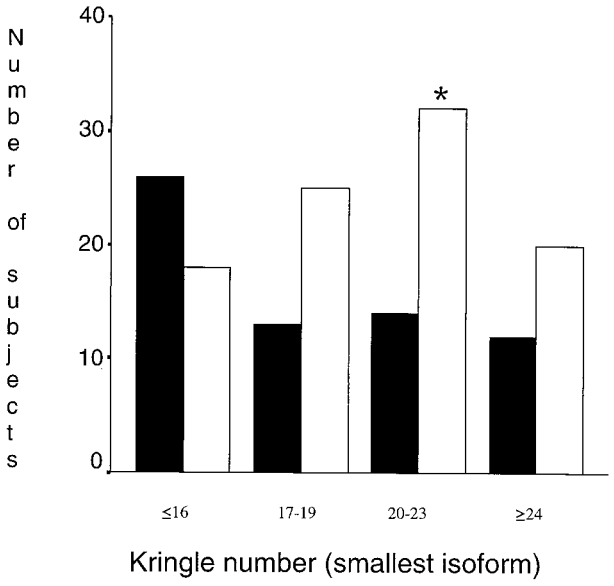

Fig. 1. Relative distribution of the smallest isoforms in the participants (n = 160) divided into quartiles according to the number of KIV repeats.

■, patients; □, controls. ∗, $P < 0.05$.

transformed because they did not follow a gaussian distribution (as assessed by the Kolmogorov–Smirnov test). To evaluate the changes between the first and second measurements, we used the paired Wilcoxon test. The association between variables was measured by linear regression analysis and Spearman rank correlation. The χ^2 test was used to compare frequencies. Participants were divided into quartiles according to the number of KIV repeats in the smallest and largest isoforms, or to the plasma Lp(a) concentration. The Kruskal–Wallis ANOVA by ranks was used to investigate the possible decrease in Lp(a) values among individuals of the different groups considered. The analyses were performed with SPSS-PC (SPSS Inc.).

Results

Patients were young and nonobese, but some conventional risk factors were present. Proportionally more patients than controls smoked or had hypertension. There were also two patients and one control with type II diabetes mellitus (Table 1). Sixty patients were receiving low-dose aspirin, 25 were receiving hypolipidemic agents, and 3 were receiving β-blockers. Most patients stated that they had substantially changed some life-style factors. They had decreased the total caloric intake, and on medical advice, cholesterol-rich foods had been substituted with fish, vegetables, and fruits. Most patients also stated that they had reduced the consumption of coffee and alcohol and increased physical exercise. Patients showed higher plasma triglyceride and cholesterol concentrations and a lower HDL-cholesterol concentration than age-matched controls.

In the whole group, we found a total of 28 apo(a) size alleles with KIV repeats numbers of 12–38. There were no homozygous individuals: they all had two apo(a) alleles that contained different numbers of KIV repeats. The mean kringle number of the largest isoform was the same between groups (Table 1), but for the smallest isoform, it

Table 2. Plasma Lp(a) in survivors of myocardial infarction and controls before and after storage for 5 years at −70 °C.

	Lp(a),[a] mg/L		
	Patients (n = 65)	**Controls (n = 95)**	**All (n = 160)**
First measurement at baseline	302 ± 280[b,c] (275)	177 ± 153 (144)	228 ± 222[d] (163)
Second measurement 5 years later	231 ± 210[b] (178)	162 ± 139 (113)	190 ± 174 (126)

[a] Results are expressed as mean ± SD (median).

[b] Significantly different from values obtained in controls: $P < 0.01$.

[c,d] Significantly different from values obtained in the second measurement: [c] $P < 0.001$; [d] $P < 0.05$.

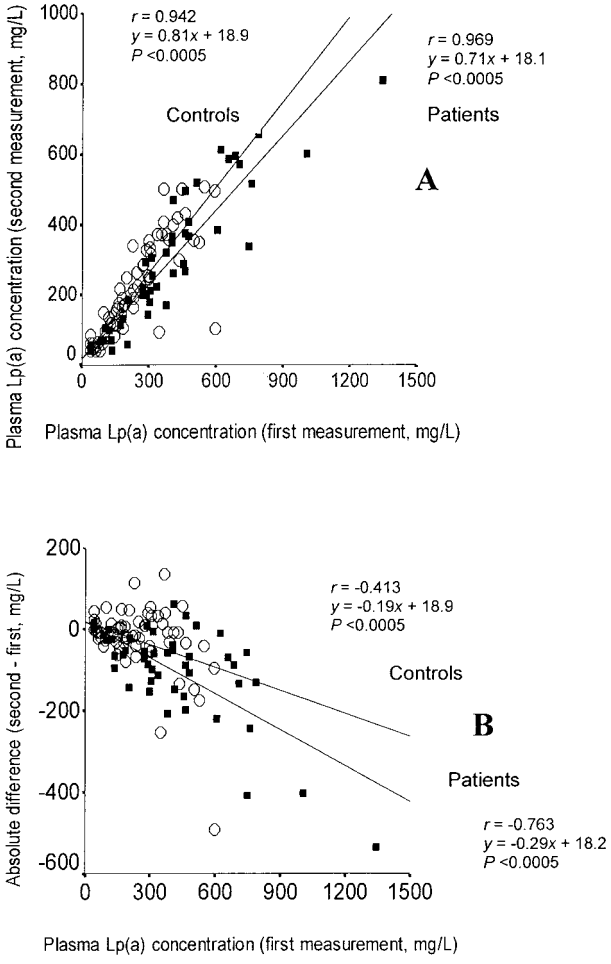

Fig. 2. Regression (*A*) between first and second measurements and bias plot (*B*) comparing the initial measurement and the difference between both measurements in patients (■) and controls (○).

Table 3. Percentage of change in plasma Lp(a) concentration between first and second measurement in each quartile of the kringle number of the smallest and largest isoforms.

	Kringle number (smallest isoform)				*P*
	≤16	**17–19**	**20–23**	**≥24**	**(ANOVA)**
Patients					
n	26	13	14	12	
Mean ± SD, %	−12.6 ± 16.7	−26.8 ± 17.3	−14.7 ± 20.2	−14.5 ± 31.3	0.240
95% confidence interval, %	(−19.3 to −5.8)	(−37.3 to −16.4)	(−26.4 to −3.1)	(−34.4 to 5.3)	
Controls					
n	18	25	32	20	
Mean ± SD, %	−1.5 ± 19.9	−11.7 ± 25.9	2.1 ± 27.6	−1.1 ± 17.7	0.187
95% confidence interval, %	(−11.4 to 8.4)	(−22.4 to −1.0)	(−7.9 to 11.9)	(−9.4 to 7.1)	
	Kringle number (largest isoform)				
	≤22	**23–25**	**26–29**	**≥30**	
Patients					
n	18	22	18	7	
Mean ± SD, %	−16.9 ± 18.3	−22.4 ± 21.9	−6.4 ± 18.9	−20.6 ± 25.6	0.106
95% confidence interval, %	(−26.0 to −7.8)	(−32.1 to −12.6)	(−15.8 to 3.1)	(−44.3 to 3.1)	
Controls					
n	22	26	20	27	
Mean ± SD, %	−2.7 ± 19.5	1.2 ± 31.2	−2.9 ± 14.9	−7.1 ± 26.1	0.675
95% confidence interval, %	(−11.3 to 5.9)	(−11.4 to 13.8)	(−9.9 to 4.1)	(−17.4 to 3.2)	

was lower in patients. The largest isoform (of each participant) was equally distributed in the two groups (data not shown), but there were some differences in the distribution of the smallest isoform (Fig. 1). Patients were relatively more frequent in the group with a kringle number ≤16 and less frequent in the group of those with ≥20. Plasma Lp(a) was also significantly higher in patients (Table 2). There was an inverse relationship between the size of the apo(a) alleles and the plasma concentration of Lp(a) that was significant in patients ($r = -0.421$; $P < 0.005$) but not in controls ($r = -0.112$; $P > 0.05$). The plasma Lp(a) concentration in samples (n = 160) stored for 5 years at −70 °C decreased by 17%, which was significant ($P < 0.05$). In patients, these values decreased more evidently (−23%; $P < 0.001$) than in controls (−9%), in whom the difference did not reach statistical significance (Table 2). There was a strong positive correlation between the first and the second measurements (Fig. 2A). Linear regression analysis for the combined control and patient values, excluding the two aberrant Lp(a) values for control samples and including the measurements of <600 mg/L, gave a nearly perfect correlation ($r = 0.989$; $P < 0.0005$). Patients clearly showed a larger decrease in plasma Lp(a) values between first and second measurements compared with controls. This was even more evident when we plotted the initial measurement vs the absolute difference between both measurements (Fig. 2B). This excess loss of reactivity or decreased Lp(a) was not related to the kringle number of either the smallest or largest isoforms. Samples from patients showed an uniform loss of reactivity of Lp(a) during storage regardless of genotype, and this was considerably higher than in controls (Table 3). Correspondingly, the changes in Lp(a) over time did not correlate with the number of KIV repeats ($r = 0.09$; *P*, not significant).

The difference between patients and controls was also evident when we compared the mean relative percentage of Lp(a) difference between the two measurements in participants divided into quartiles according to the initial plasma Lp(a) concentration (Table 4). There was no appreciable change over time in participants with plasma Lp(a) <41 mg/L (n = 54), who were 34% of the sample. As the initial plasma Lp(a) concentration increased in patients, the relative Lp(a) decrease over time became smaller ($P < 0.05$). This trend, however, was not significant in controls. Table 4 also shows that Lp(a) decreased to a greater extent only in those patients whose initial plasma Lp(a) concentration was between 41 and 345 mg/L. When plasma Lp(a) was either <41 mg/L or >345 mg/L, the decrease of Lp(a) in patients and controls was similar.

Discussion

It is common practice in clinical and epidemiologic studies to store one or more aliquots of plasma from participants. Samples are usually stored for years and at −70 °C or lower. This approach, although used extensively, is not valid when the long-term stability during storage of the component to be measured has not been determined. It is generally assumed, however, that any deterioration affects all of the specimens to the same extent.

Our results indicate that this is not the case for plasma Lp(a) concentrations in patients with established cardiovascular disease. A significant decrease in Lp(a) values

Table 4. Percentage of change in plasma Lp(a) concentration between first and second measurements in each quartile of initial plasma Lp(a) concentration.

	Initial plasma Lp(a) concentration, mg/L			
	≤40	41–163	164–345	≥346
Patients				
n	21	6	14	24
Mean ± SD, %	1.4 ± 6.5	−40.1 ± 38.5	−35.7 ± 40.2	−28.1 ± 30.2
95% confidence interval, %	(−1.3 to 4.0)	(−120.5 to 23.4)	(−89.5 to −18.2)	(−42.3 to −13.6)
Controls				
n	33	20	26	16
Mean ± SD, %	2.7 ± 11.0	−13.5 ± 23.4	−5.1 ± 22.2	−22.9 ± 60.7
95% confidence interval, %	(−1.4 to 6.6)	(−104.2 to 41.2)	(−13.9 to 3.8)	(−66.1 to 12.7)
P	0.485	0.043	0.0038	0.532

was detected in stored samples, but this was more evident in patients than in controls. Not all patient samples and only some control samples showed decreased Lp(a) values after prolonged storage. However, after 5 years of storage, the difference in plasma Lp(a) concentration between patients and controls was still significant. If we assume that Lp(a) values decrease proportionally over time, this difference would disappear in a few more years. The change in measured Lp(a) was not related to the number of KIV repeats in the largest or smallest isoforms. This was unexpected and contradicted previously reported findings, although the participants were quite different in this study and sample storage time was for 5 years compared with 25 months *(17)*. It is well known that there is an inverse correlation between the number of KIV repeats of apo(a) and plasma Lp(a) concentrations *(23, 24)*. Likewise, it is also well known that low-molecular weight apo(a) isoforms with their associated high plasma Lp(a) concentrations are found more frequently among patients with established cardiovascular disease than in healthy controls *(25)*. Our sample further confirmed these two facts (see Fig. 1 and Tables 1 and 2), but their lack of relationship with a decrease in plasma Lp(a) was evident (Table 3).

It is also possible that the higher the Lp(a) concentration, the higher will be the decrease in Lp(a) values or loss of Lp(a) immunoreactivity over time. This was true in terms of the absolute difference between the initial and the final measurement. Again, the samples from patients and controls behaved differently. Both measurements were significantly and negatively correlated in both patients and controls, but the correlation in controls was not significant if the two outlier values observed in Fig. 2B were excluded. When samples were divided into quartiles according to the initial plasma Lp(a) concentration, we found that the relative changes were also different in both groups. If plasma Lp(a) was either very low or very high, patients and controls behaved similarly, but the patients with median values showed a larger decrease in Lp(a) values than controls with similar initial Lp(a) values (Table 4). However, we recognize that there is no logical reason that this effect did not also occur for the highest and lowest quartiles of the Lp(a) concentration range, and it deserves further confirmation.

During the follow-up, the reagents and the control and calibrator materials behaved consistently, which made it unlikely that the discrepancy could be attributable to an artifact of the assay method. Moreover, Lp(a) measured in samples stored for 5 years correlated significantly ($P < 0.0005$) with the initial values (Fig. 2A) in both patients and controls.

Storage conditions were the same for both groups but deserve further discussion. Inappropriate storage of samples may lead to the appearance of fragments that can be detected in immunoblots of reduced 4% polyacrylamide SDS gels. It is also likely to be responsible for high-molecular weight aggregates *(26, 27)*. We did not perform electrophoretic studies to determine whether this is so, but we consider that a significant effect in our case is unlikely because samples were stored in sterile conditions in the presence of antioxidants and proteolytic inhibitors. Although there are no standardized methods for storing samples containing Lp(a), the addition of cryopreservatives should be considered. The immunochemical properties of Lp(a) are preserved at −20 °C for at least 1 year in the presence of 500 mL/L glycerol *(28)*. More recently *(29)*, it has been reported that Lp(a) and apo(a) can be stored for 3 years in trehalose at −80 °C without aggregation or degradation.

We therefore conclude that an unknown factor unrelated to either the number of KIV repeats or to the plasma Lp(a) concentration causes a higher loss of immunoreactivity in samples from patients than in samples from controls. This factor may be related to the difference in the lipoprotein composition of the plasma; patients have higher plasma cholesterol and triglyceride concentrations and consequently a different lipid-to-apoprotein ratio *(22)*. Moreover, other factors associated with plaque formation and the progress of atherosclerosis may possibly change the immunoreactivity of the Lp(a) particle. Our findings may help to explain the failure of some epidemiologic studies that attempted to identify Lp(a) as a risk factor for cardiovascular disease. At the same time, it may strengthen the positive results. Whatever the cause of the

decrease in Lp(a) values, if the differences in plasma Lp(a) concentration are still significant after many years of storage, we may conclude that the initial differences were extremely high.

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