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An Application of Apo(a) Isoforms for the Clinical Assessment of Lp(a)

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To examine whether or not Lp(a) is applicable as a diagnostic marker for atherosclerosis, we studied the correlation between Lp(a) levels and molecular weights of apo(a) isoforms in sera from both normal healthy adults and diabetic patients. Serum Lp(a) level was measured by turbidimetric immunoassay (TIA) and the molecular weight of apo(a) isoform was determined by Western blotting analysis. The serum Lp(a) levels of the diabetic patients (25.0 mg/dl ± 2.2 [mean ± SE], n = 54) were significantly higher than those of the normal subjects (14.4 mg/dl ± 0.57, n = 500). With respect to the correlation between serum Lp(a) levels and the molecular weights of apo(a) isoforms, there was an inverse correlation in sera from normal subjects (n = 298), whereas there was no correlation in sera from the diabetic patients. Statistical significant inverse correlation (r = –0.91, y = 224.25 – 3.07x) was especially observed in 50 representative apo(a) isotypes from the normal subjects. By applying a standardized curve based on the significant inverse correlation to serum Lp(a) levels, 40.7% (22/54) of the diabetic patients were revealed to have an abnormally high value of serum Lp(a). Moreover, it was found that the significantly higher mean value of serum Lp(a) in the diabetic group was caused by the 22 patients with higher value of Lp(a). The present findings suggest that determination of apo(a) isoform size provides estimation of the serum Lp(a) value and that the inverse correlation curve between serum Lp(a) level and the molecular weight of apo(a) isoform may be applicable to the clinical use of Lp(a). J. Clin. Lab. Anal. 14:53–58, 2000.

Key words: Lp(a) level; apo(a) isoform; inheriting plasma protein; inverse correlation

INTRODUCTION

Lipoprotein (a) [Lp(a)] was discovered as an inheriting plasma protein (1). The Lp(a) molecule consists of two major structural subunits of apolipoprotein(a) [apo(a)] domain and "LDL-like" domain with apo B-100. Because the serum Lp(a) level closely correlates to the molecular weight of apo(a) isoprotein designated "apo(a) isoform," which has been supposed to be dependent on the numbers of repeated kringle-IVs located on the apo(a) gene, the serum Lp(a) level and molecular size of apo(a) isoform significantly exhibit the polymorphism (2–8). Besides, Lp(a) has been widely accepted as an independent serum lipoprotein since its serological characteristics are quite different from those of other serum lipoproteins(9,10).

There are several reports on a correlation between the serum Lp(a) level and several heart diseases (11–15), suggesting that the high serum Lp(a) level may be associated with the progression of coronary artery dysfunction. Moreover, the correlation between the high serum Lp(a) levels and diabetic patients, especially those with macro-angiopathic disease, has been reported (16,17). For determination of the serum Lp(a) level, quantitative assays using the specific polyclonal or

Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); TIA, turbidimetric immunoassay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TTBS, Tween Tris-buffered saline; PVDF, polyvinylidenefluoride; ABC method, avidin-biotin complex method.

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monoclonal antibody against apo(a) are, currently, routinely performed (18). However, since serum Lp(a) level is originally correlated to the molecular weight of the apo(a) isoform and is individually different even in healthy subjects (19–21), the quantitative evaluation only by a comparison with a mean value quoted from the general population is not suitable for clinical use of Lp(a).

To study whether or not Lp(a) is a candidate for a clinical marker for atherosclerosis, we investigated the relationship between serum Lp(a) levels and the molecular weight of the apo(a) isoform in normal healthy subjects. By using the inverse correlation between them as a standard curve, the Lp(a) levels in sera from diabetic patients were tentatively estimated on the basis of the apo(a) isoform size. The abnormally high values of Lp(a) observed in the diabetic patients suggest that the standardized curve based on the inverse correlation between the Lp(a) level and the molecular size of apo(a) isoform may be useful to clinically estimate individual Lp(a) levels.

MATERIALS AND METHODS

Sera

Fresh sera were obtained from 500 normal healthy adults who lacked any obvious clinical presentation [male: 228; female: 272; age 33.9 ± 10.8 years (SD)] previously confirmed to be normolipidemic and 54 (Type 1: 4; Type 2: 50) diabetic patients (male: 30; female: 24; age 53.9 ± 14.1 years) were studied. This study was approved by the ethics committees of the relevant institutions and was performed in compliance with the Declaration of Helsinki.

Determination of Serum Lp(a) Concentration

Serum Lp(a) concentration was determined with a commercial Lp(a) assay kit (Cosmo Bio Co., Tokyo, Japan) (18). Lp(a) at 0.5 to 150 mg/dl was detectable by turbidimetric immunoassay (TIA) using the kit.

Western Blotting to Analyze Apo(a) Isoform

Two μl of serum sample dissolved in 48 μl of SDS-PAGE buffer (10 mM Tris-HCl pH 8.0, containing 1 mM EDTA, 2.5% SDS, 10% glycerol, and 5% 2-mercaptoethanol) was incubated for 30 min at 60°C. Then one μl of the sample mixture was applied on 4–15% gradient polyacrylamide gel (Pharmacia, Uppsala, Sweden). The electrophoresis procedure involved the following three steps, which consisted of 15°C/1Vh/10mA, 15°C/1 Vh/1 mA and 15°C/300 Vh/10 mA. Biotinylated SDS-PAGE standards (High Range, Bio-Rad, CA) and purified human apolipoprotein B-100 (Organon Teknika, Tokyo, Japan) were used as a molecular size marker. After electrophoresis, separated proteins were electrophoretically transferred to PVDF-membrane (Immobilon-P, Millipore, Bedford, MA) under a condition of 25 mA /gel for 90 min at 15°C. All procedures for electrophoresis were performed with PhastSystem (Pharmacia, Uppsala, Sweden).

The PVDF-membrane was soaked in TTBS (0.25% Tween-20 in 20 mM Tris-HCl, pH 7.2 containing 0.9% NaCl) containing 5% of nonfat dry milk for 2 hr, then was washed three times by TTBS. The first immunological reaction was performed with TTBS containing 1 μg ml^{-1} of purified goat anti-human Lp(a) antibody for 4 hr. After washing, the membrane was again soaked in the second reaction mixture consisted of 0.5% nonfat dry milk-TTBS with 0.5 μg ml^{-1} of biotinylated rabbit antigoat IgG antibody (Vector, CA) for 1.5 hr. After washingthe apo(a) moiety was visualized by ABC method (22) with a POX-conjugated streptoavidin (Amersham, Tokyo, Japan) and a Konica immunostain HRP kit (Konica, Tokyo, Japan). Finally the apo(a) isoform was classified into seven types according to the molecular weight reported by Uterman et al. (8). To confirm the condition on SDS-PAGE, some electrophoresed gels loaded in parallel were also stained by an optimized silver staining method with PhastSystem.

Statistical Analysis

Data were expressed as the mean ± SE or SD. A P value of less than 0.05 calculated by nonparametric Mann-Whitney U-test and a P value of less than 0.005 calculated by the Bonferroni/Dunn test were considered significant. Regression calculated by Spearman's single rank test using a P value with a 95% of confidential rate was also expressed. StatView software (Abacus Concepts, CA) was used for all analyses.

RESULTS

Determination of Lp(a) Level in Sera From Normal Healthy Adults and Diabetic Patients

To determine the serum Lp(a) level, sera from 500 normal subjects and 54 diabetic patients were obtained. Mean (± SE) levels of Lp(a) were determined by TIA . There was a significant difference ($P < 0.001$) in the Lp(a) level between the normal subjects (14.4 ± 0.57 mg/dl) and the patients (25.0 ± 2.2 mg/dl), whereas there was no gender difference in both subjects (Fig.1). As shown in Figure 1, the levels of Lp(a) in the normal subjects were widely distributed from 1.0–130 (mg/dl), whereas those from the diabetic patients were from 2.0–89.0. The highest frequency of Lp(a) levels in both subjects was found at a range of 5.0–15.0 (mg/dl). However, Lp(a) levels at less than 25.0 mg/dl were found in 85% (475/500) of sera from the normal subjects, as compared to 61.1% (33/54) of sera from the diabetic patients.

Western Blotting to Analyze Apo(a) Isoform(s)

To study the correlation between the serum Lp(a) levels and the molecular weight of apo(a) isoforms, apo(a) molecules in sera from normal subjects and diabetic patients were analyzed by Western blotting. Two hundred and ninety-eight

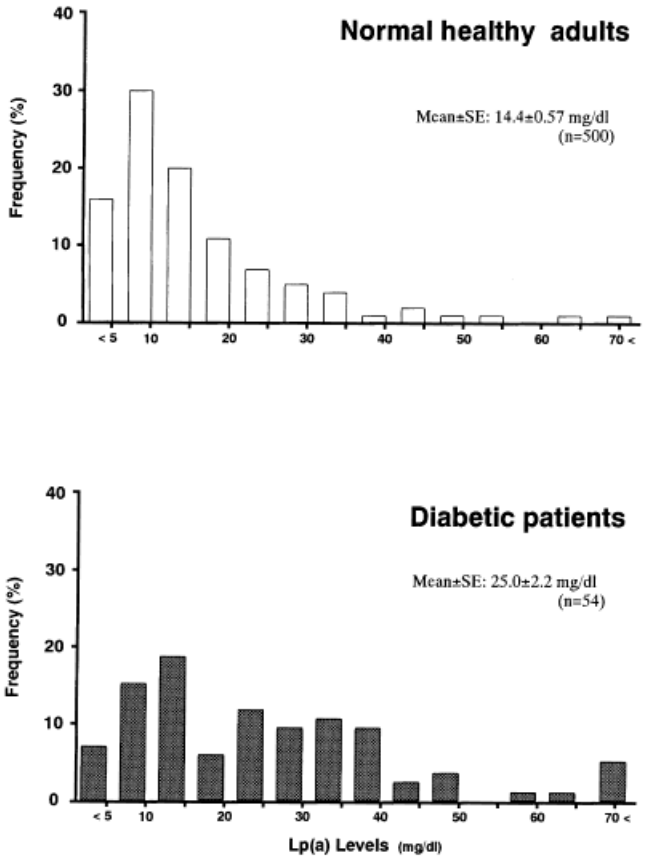

Fig. 1. Histogram of Lp(a) levels in normal healthy adults (n = 500) and diabetic patients (n = 54). The serum Lp(a) levels (mg/dl) were measured by a TIA method. The mean (± SE) in the diabetic patients was significantly higher than that of the normal healthy adults by analysis of the Mann-Whitney U-test ($P < 0.0001$).

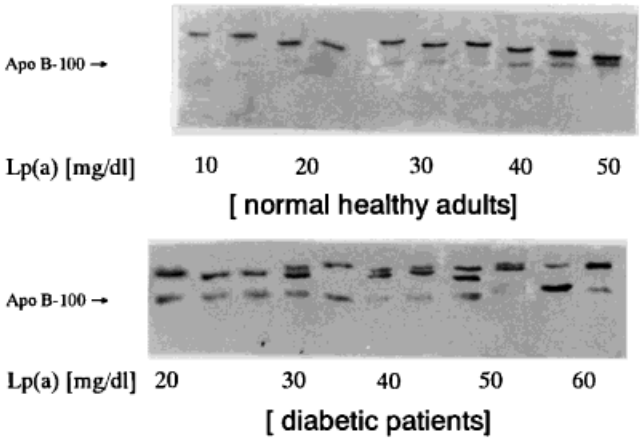

Fig. 2. Polymorphism of apo(a) isoforms on the analysis by Western blotting. Each apo(a) isoform size in the normal subjects showed to become gradually smaller in relation to the elevation of serum Lp(a) levels, respectively. In contrast, apo(a) isoforms in the diabetic patients constantly showed high molecular weights despite having high levels of serum Lp(a). →: Apo B-100 (as a molecular weight marker).

apo(a) isoforms selected randomly from the 500 normal subjects were classified into 7 isotypes according to the previous report (8). Various apo(a) isotypes such as *S4*, *S4/S3*, *S4/S2*, *S3*, *S3/S2*, *S3/S1*, *S2*, *S2/S1*, *S1* and *B* were observed in 116 (39.1%), 45 (15.3%), 25 (8.4%), 32 (10.7%), 29 (9.9%), 2 (0.8%), 23 (7.6%),14 (4.6%), 10 (3.4%) and 2 (0.8%), respectively. The intratypical Lp(a) levels (mean ± SD) of the apo(a) isotypes were 11.2 ± 5.2 for *S4*, 18.2 ± 7.1 for *S4/S3*, 34.4 ± 8.4 for *S4/S2*, 25.2 ± 6.9 for *S3*, 48.9 ± 16.4 for *S3/S2*, 16.1 ± 7.5 for *S3/S1*, 46.9 ± 7.5 for *S2* isotype, 69.3 ± 21.5 for *S2/S1* isotype, 63.2 ± 18.3 mg/dl for *S1*, and 85.7 ± 15.3 mg/dl for *B*, respectively. With respect to the relationship between the serum Lp(a) levels and the molecular weight of the apo(a) isoforms, smaller molecular mass the apo(a) molecule was found in the serum with the higher Lp(a) level as shown in Figure 2. To confirm the relationship between the serum Lp(a) level and the molecular weight of apo(a) isoform, 50 representative isotypes showed a single apo(a) isoform band, such as *S4*, *S3*, *S2*, *S1*, and *B* were again selected from the 298 normal subjects. The mean(± SD) value of them was 2.0 to 19.0 mg/dl (9.8 ± 5.5[mean ± SD], n = 14) for *S4*, 15.0 to 42.3 mg/dl (25.9 ± 8.0, n = 13) for *S3*, 38.0 to 60.0 mg/dl (47.4 ± 7.9, n = 11) for *S2*, 40.2 to 97.0 mg/dl (63.3 ± 18.3, n = 10) for *S1*, and 70.5 and 101.0 mg/dl for B, respectively. By analysis of the Bonferroni/Dunn test, a significant difference of Lp(a) levels was clearly shown between the representative apo(a) isotypes (Fig. 3). Moreover, the serum Lp(a) levels showed inversely proportional to the molecular weights of the apo(a) isotypes and the linear regression analysis of the relationship by Spearman's rank correlation test gave a significant correlation: $y = 224.25 – 3.07x$ with coefficient of $r = –0.91$ ($P < 0.0001$).

Relationship Between Lp(a) Levels and the Molecular Weight of Apo(a) Isoforms in Diabetic Patients

On Western blotting analysis, the density of the apo(a) isoforms in sera from the 54 diabetic patients was found to be similar to that from the normal subjects. However, the apo(a) isoforms such as *S4* or *S3* with high molecular size were observed in 96.3% (52/54) of the diabetic patients although high Lp(a) levels were also observed. The Lp(a) levels were plotted against the molecular weights of apo(a) isoforms in the diabetic patients (Fig. 4). As shown in Figure 4, 40.7% (22/54) of the Lp(a) levels among the diabetic patients were apparently higher than the mean ± 1 SD levels at the corresponding apo(a) isotypes from the normal subjects. The frequency of an abnormally high value of Lp(a) levels observed was up to 33.3% (6/18) of sera with a single *S4* isotype, 36.4% (4/11) of sera a single *S3* isotype, 52.9% (9/17) of sera with combined *S3/S4* isotypes, 25% (1/4) of sera with combined *S2/S4* isotypes, 50% (1/2) of sera *S2/S3* isotypes and

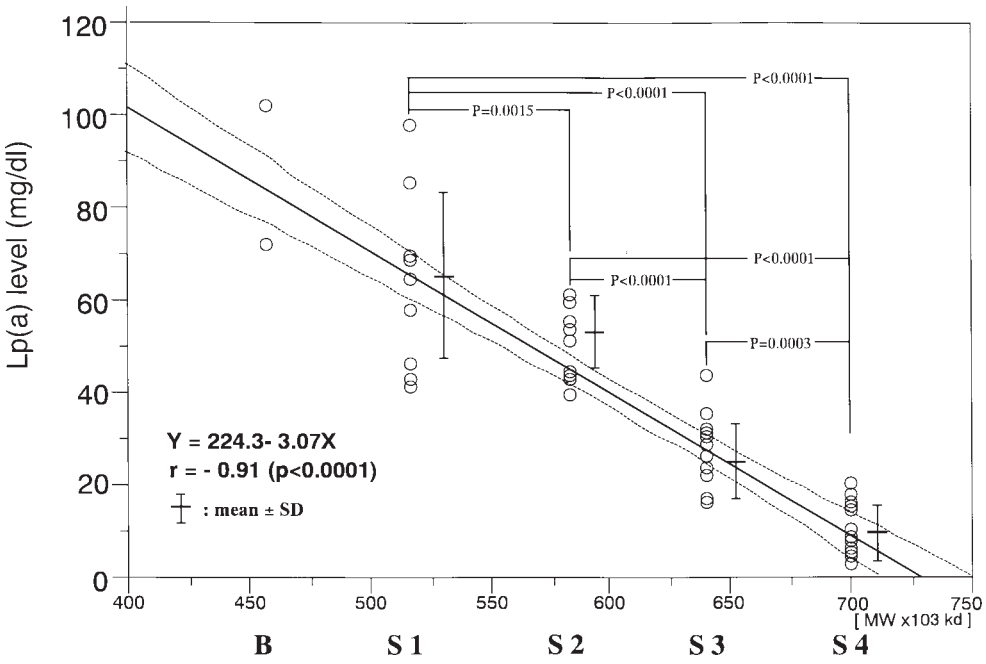

Fig. 3. The correlation between serum Lp(a) levels and the molecular weights of the apo(a) isotypes in 50 representative apo(a) isotypes extracted from the normal subjects. The statistical significance of serum Lp(a) levels in each apo(a) isotype was analysed by the Bonferroni/Dunn test with the standard P value of < 0.005, and the inverse correlation curve was calculated by Spearman's rank correlation; Solid line = regression line, dotted line = 95% confidence intervals curves (tied $P < 0.0001$).

one serum with *B/S4* isotypes, respectively. Many of the diabetic patients with an *S4* and/or *S3* apo(a) isotype showed abnormally high Lp(a) levels.

DISCUSSION

There have been many reports that high serum Lp(a) is closely associated with atherosclerosis (11–15), suggesting that Lp(a) may be a candidate for use as a clinical indicator for the assessment of atherosclerosis. On the other hand, various micro- and/or macro-angiopathic complications in diabetic patients have been reported (22–25) and the high levels of serum Lp(a) in the diabetic patients have been considered to be possibly associated with the progression of their angiopathical atherosclerosistic dysfunction (16,17). However, a quantitative determination of serum Lp(a) levels is not enough to reflect the clinical state of atherosclerosis because serum Lp(a) levels are closely correlated with the molecular size of apo(a) isoforms, which vary among individuals (2–8).

Many investigations concerning the influence of aging or gender difference on serum Lp(a) levels have been reported (26–34). Lp(a) levels in infants (26,27) and elderly subjects (28,29) have been reported to be significantly lower than those of healthy adults. Although several reports have shown higher levels of serum Lp(a) in women (32,33) and postmenopausal women (30,34), the influence of gender difference is still uncertain (30,31). In this study, the mean(± SE) values of Lp(a) levels in 500 healthy normal subjects and 54 diabetic patients were 14.4 ± 0.57 mg/dl and 25.0 ± 2.2 mg/dl, respectively. Although the mean age of the diabetic patients was significantly higher than that of the normal subjects ($P < 0.001$), the Lp(a) levels in the diabetic patients were significantly higher than those of the normal subjects ($P < 0.001$). Since there was no significant gender difference on the Lp(a) levels in the diabetic patients, these findings have supported previous reports of the higher Lp(a) levels in diabetic patients (16,17).

We also analyzed 298 apo(a) isoforms among the normal subjects by Western blotting and investigated the relationship between the molecular weight of the apo(a) isoform and the level of serum Lp(a). In the findings obtained, smaller molecular mass of apo(a) molecule found in sera showed higher serum levels of Lp(a) (8,19–21), indicating that each size of genetically-controlled apo(a) isoforms is closely correlated with the level of serum Lp(a). Moreover, the Lp(a) levels obtained from 50 normal sera with representative apo(a) isotypes were significantly different depending on the apo(a) isotype. The inverse correlation between the molecular weights of apo(a) isoform and the levels of serum Lp(a) was supported by the statistical significance(Fig. 3) (35). The mean (± SD) value of serum Lp(a) in every apo(a) isotype in the representative 50 subjects was not significantly different from those of the 298 subjects. In contrast, many of apo(a) isoforms in 54 diabetic patients were found to consist of *S4* or *S3* with a higher molecular weight although they showed the higher Lp(a) levels (Fig. 2). Therefore, the correlation between the

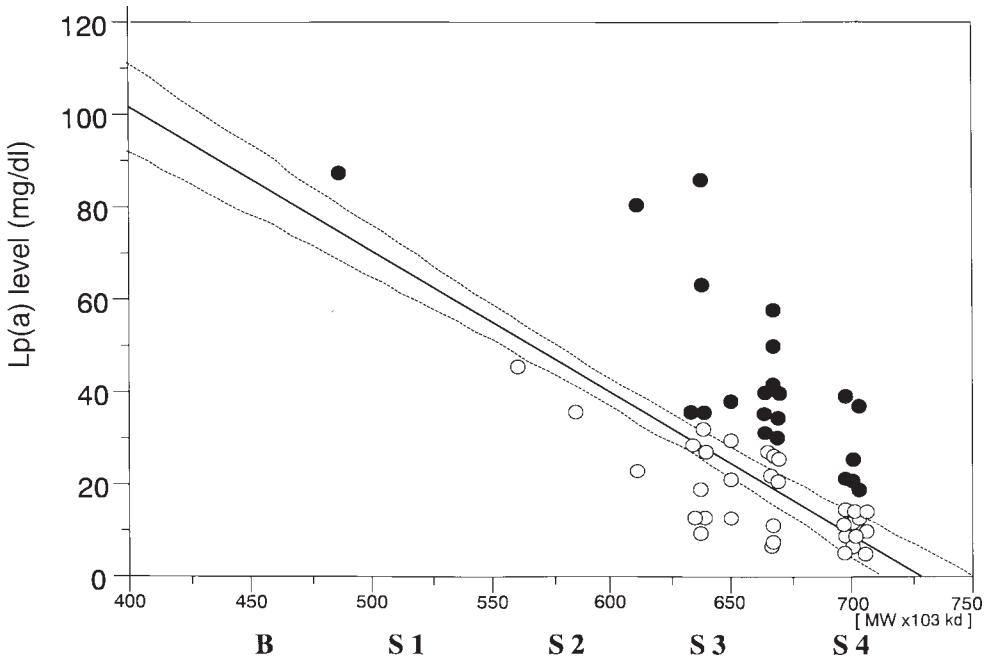

Fig. 4. Evaluation of serum Lp(a) levels in the diabetic patients. The Lp(a) levels which were higher than the range of mean ± 1SD at the corresponding apo(a) isotypes were expressed as a closed circle, ●.

serum Lp(a) levels and the molecular weights of the apo(a) isotypes in the diabetic patients was apparently different from that of the normal subjects (data not shown).

To distinguish between normal and abnormal value of Lp(a) levels in the diabetic patients, the Lp(a) levels were plotted against the molecular weights of the apo(a) isotypes. According to classification of the serum level of each apo(a) isotype, 40.7% (22/54) of sera from the patients was clearly revealed quantitative by using a standardized curve based on the significant inverse correlation from the representative 50 subjects. From the findings, it was determined that the significantly higher mean value (25.0 ± 2.2 mg/dl) of serum Lp(a) obtained from the diabetic group was caused by the 22 patients with higher Lp(a) values. Therefore, on the basis of apo(a) isoform sizes, the high value of serum Lp(a) observed in the 22 diabetic patients was concluded to be abnormal. Although there was no significant correlation between the abnormally high Lp(a) levels and their diabetic complications, such as retinopathy, nephropathy, and neuropathy, these findings in the diabetic patients may be associated with the progression of diabetic angiopathy.

The present findings strongly suggest that determination of the molecular size of apo(a) isoform is important, because the serum Lp(a) level is estimated by determination of the molecular size of the apo(a) isoform. Moreover, the serum Lp(a) level based on the standardized curve may be applicable to the clinical use of Lp(a). To clarify the clinical significance of Lp(a), it may be necessary to confirm whether or not the high serum Lp(a) level in a patient is in an inheriting value or in an abnormally high value. In terms of clinical assessment of Lp(a), therefore, it may be also important to establish the inheriting correlation between serum Lp(a) levels and the apo(a) isoform sizes.

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