Impact of apolipoprotein(a) isoform size heterogeneity on the lysine binding function of lipoprotein(a) in premature coronary artery disease

Josep M. Simó[a], Jorge Joven*[a], Elisabet Vilella[a], Montserrat Ribas[a], Miguel A. Pujana[b], Indirana M. Sundaram[c], Jeffrey P. Hammel[d], Jane L. Hoover-Plow†[c],

[a] Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, Reus, Spain

[b] Institut de Recerca Oncològica

[c] Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Dept. Molecular Cardiology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio

[d] Dept. of Biostatistics and Epidemiology, Cleveland Clinic Foundation

*Address reprint requests to Dr. Jorge Joven at Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, Calle Sant Joan s/n, 43201, Reus, Spain. Tel +34 977 310300, Fax +34 977 312569, e-mail jjoven@grupsgs.com

†Corresponding Author: Jane Hoover-Plow, Ph.D., Department of Molecular Cardiology/NB50, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio, 44195; Tel (216) 445-8207, Fax (216) 444-9263, e-mail hooverj@.ccf.org

Running Title: Lp(a) LBS function in CAD

Summary

Elevated plasma Lp(a) is an independent risk factor for cardiovascular disease. Unique to Lp(a) is the apoprotein, apo(a) which can vary from 250-800 kDa in molecular weight. Small isoforms are also associated with the risk of cardiovascular disease. The purpose of this study was to examine the association of Lp(a) concentration, apo(a) size, and Lp(a) lysine-binding site(s) (LBS) function in patients with premature heart disease, and age-matched controls. Mean values of Lp(a) were significantly higher in the patients than for the age-matched group. The smallest molecular weight isoform for each subject had significantly fewer kringles for the patients than the age-matched controls. There was a significant correlation between LBS activity and kringle number in the single-banded phenotypes of the patients, but not the controls. LBS activity was significantly higher in patients in the small isoforms (≤18 kringles) compared to controls. The odds ratio for coronary artery disease for high LBS activity and high Lp(a) concentration was 4.4 ($p = 0.002$) and for high LBS activity in small isoforms was 10.1 ($p = 0.002$). In the patients, Lp(a) concentration was higher, apo(a) size was smaller, and LBS activity higher in the small isoforms compared to the controls. This study suggests an association of high LBS activity in small isoforms of Lp(a) with disease in humans.

Key words: Lipoprotein(a), apo(a) size, lysine binding site function, myocardial infarction

Introduction

Numerous studies have demonstrated that plasma lipoprotein (a) [Lp(a)] concentrations are elevated in patients with coronary artery disease [CAD] (for review (1)), but some prospective studies have failed to find an association between initial plasma Lp(a) concentration and the occurrence of myocardial infarction (2). Lp(a) is a class of lipoprotein particles resembling low density lipoproteins (LDL) in which the apoB-100 is covalently linked to a highly polymorphic glycoprotein, apolipoprotein (a) [apo(a)], a multikringle structure with a high degree of homology to plasminogen (3). Apo(a) contains multiple copies of the plasminogen-like kringle 4 (KIV) domain (4). These kringles are similar but not identical to each other and 10 distinct types of kringles have been identified (KIV types 1 through 10). The tandem repeats of KIV type 2 constitute the molecular basis of Lp(a) isoform size heterogeneity and most Caucasian individuals (94%) have two apo(a) alleles that contain different numbers of KIV repeats, ranging from 3 to > 40 (5). The distribution of plasma Lp(a) is highly skewed towards lower levels and in general there exists an inverse association between the number of KIV repeats, the size of apo(a) isoforms, and plasma Lp(a) concentrations (6).

Certain of the kringles of apo(a), like K4 of plasminogen function as lysine binding sites (LBS). The LBS consists of a trough lined by three aromatic residues flanked on one end by two anionic residues and at the other end by two cationic residues (7). The KIV type 10, contributes most of the lysine affinity of apo(a), but KIV types 5-8, also contain LBS (8). The LBS mediate the interaction of Lp(a) with Lys residues in fibrin, cells, and extracellular matrix components (9-12). The LBS activity of Lp(a) has been shown to play a key role in the pathogenic activity of apo(a). Wild-type apo(a) transgenic mice show a marked increase in the development of lipid lesions and deposition of apo(a) in the aorta compared with a strain of mice expressing an apo(a)

in which the two key anionic residues (Asp55, Asp57) were replaced by alanine, resulting in a 70% loss of the LBS activity (13,14). In humans and Rhesus monkeys, a naturally occurring polymorphism has been described for single point substitution (Trp72 to Arg) that nearly eliminates lysine binding of intact Lp(a) (15,16). This finding also suggests that apo(a) isoforms might show variability in LBS function. It has been recently shown that Lp(a) particles containing distinct apo(a) isoforms display functional heterogeneity for fibrin and monocyte binding, with the low molecular mass isoforms having the highest affinity (17). A logical step was to assess LBS function of Lp(a) in patients with CAD, with a recently developed accurate and simple immunoassay that can quantify LBS activity (14,18). Therefore, in this study, we have examined the relationship of apo(a) gene, apo(a) glycoprotein, plasma concentration and LBS activity of Lp(a) in patients who have survived an early onset myocardial infarction.

Materials and Methods

Study population

The procedures used in this study complied with the ethical standards of the Hospital Universitari de Sant Joan. Blood samples were obtained from 95 male patients, who participated in a previous study (19) and had one episode of acute myocardial infarction before the age of 50 years. To ensure genetic predisposition 95 subjects were selected with no history of familial hypercholesterolemia, renal failure, liver disease, hypertension, obesity or diabetes mellitus from a group of 250 patients. The patients were compared with 95 male controls without clinical evidence of coronary disease and having a similar age (± 4 years) and BMI (± 1.1), randomly chosen from the routine health and safety-at-work checks conducted in several industrial companies in our area.

Lipid and Lipoprotein measurement

Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and the lipase/GPO/PAP methods, respectively, as previously described (20). High density lipoprotein (HDL) cholesterol was measured with a homogeneous assay recently described (20).

Quantification of plasma Lp(a)

The concentration of Lp(a) in plasma was determined by immunoturbidimetry using antibodies, calibrators and standards supplied by Incstar Corporation (Stillwater, MN) (21). The specificity of the apo(a) antibodies and ability to recognize equally the different apo(a) size isoforms has already been described (22). 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).

Apo(a) glycoprotein size determination by immunoblotting

Apo(a) phenotyping was performed by sodium dodecyl sulfate (SDS) agarose gel electrophoresis of plasma under reducing conditions followed by immunoblotting as recently described (23). In brief, 125 ng of delipidated Lp(a) was boiled for 10 min in the presence of 10 μM dithiothreitol, 3% SDS, 0.5 mM EDTA and 22.5 mM Tris buffer, pH 8.2, and then bromophenol blue (0.1%), glycerol (1%), and α-iodoacetamide (100 mM) were added at room temperature. Agarose (1.5%) electrophoresis was performed in a GNA chamber (Pharmacia, Uppsala, Sweden) at 120 V, 4^0C and during 6 h. Protein were transferred to nitrocellulose membranes and incubated sequentially with a polyclonal goat anti-human Lp(a) (BiosPacific, Emeryville, CA) and a monoclonal mouse anti-goat IgG coupled with alkaline phosphatase. Bands were made visible colorimetrically and the number of KIV repeats assigned according to standards supplied by Immuno AG (Vienna, Austria).

Apo(a) genotype determination

Apo(a) alleles were determined by a modification of the procedure described by Lackner et al (5). Leukocytes were isolated from whole blood, suspended to a final cell concentration of $2x10^7$ cells/mL and embedded in low-melting-temperature agarose plugs. The separation was performed in a Gene Navigator™ apparatus (Pharmacia) with alternating pulses of 4 s 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 a size standards 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), (5) kindly provided by Dr. Helen Hobbs, Univ. Texas Southwestern Medical Ctr, Dallas. 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.

Measurement of LBS activity

The frozen plasma samples were shipped on dry ice to Cleveland with no evidence of thawing during transit. The samples were stored at -70°C and assayed within 10 wk. of arrival. The samples were thawed only once and assayed the same day. The LBS function of Lp(a) was measured with a quantitative LBS-Lp(a) immunoassay as previously described (14). The maximum absorbance of each sample, taken at the plateau of the concentration curve (4-80 mg/L), was compared to an isolated Lp(a) reference standard. Thus, the measurement of LBS activity in this assay is not concentration dependent and LBS activity can be quantified in plasma samples with as little as 2-5 mg/L of Lp(a) protein (14). The limit of the Lp(a) concentration assay was 49 mg/L, so for samples < 49 mg/L, the plasma was assayed at 0, 1:2, and 1:4 dilutions. In the LBS-Lp(a) immunoassay (14), two-lysine analogues, ε-aminocaproic acid (EACA) and lysine, produced similar dose-dependent inhibition of the LBS antibody binding to Lp(a). The average IC_{50} for EACA was 0.5 and for lysine 3.7 mmol/L. These values are in excellent agreement with values (1.3 and 3.8 mmol/L for EACA and lysine, respectively) obtained in our lysine-bead assay (24) where Lp(a) is in solution. In a preliminary study, we determined that LBS activity did not change significantly with up to 12 weeks of storage at -70°C.

Statistical analysis

Values are expressed as the mean ± SD or SEM as indicated. Statistically significant differences were set at $p < 0.05$. Multiple Kolmogorov-Smirnov one-sample tests were performed to determine the fit of the data to a normal distribution. Accordingly, the differences between groups were assessed using ANOVA or Mann-Whitney's U test following the indications of the F test. The association between variables was measured by linear regression analysis and Spearman rank correlation. The association between the likelihood of CVD and the level of LBS activity was measured by odds ratios with 95% confidence intervals within high and low levels of Lp(a) concentration and the smallest kringle number. Tests for associations

and for differences in the odds ratios were carried out using Wald statistics from logistic regression models. The analyses were performed with SPSS-PC (SPSS Inc., Chicago, IL) and SAS version 6.12 (SAS Institute, Cary, NC).

Results

Description of the subjects

Patients with myocardial infarction were young (45 yr. ± 6, mean ± SD), and the mean age of the control group matched patients, 45 ± 5. There were no differences in body-mass index between the groups considered and the percentage of current or former smokers was significantly ($p < 0.005$) higher (81.2%) in patients with myocardial infarction than in controls (46.2%). Patients also showed a higher ($p<0.05$) enrichment of cholesterol (5.76 ± 0.70 mmol/L) and triglyceride (2.09±1.33 mmol/L) in apo B containing lipoproteins and lower HDL-cholesterol (1.00 ± 0.23 mmol/L) as compared with the control group (5.49 ± 0.91, 1.66 ± 1.17 and 1.11 ± 0.25 mmol/L, respectively).

Differences in plasma Lp(a) concentration and apo(a) size

The mean and median Lp(a) concentration was significantly higher in patients than age-matched controls (Table 1). In the control group, > 77% of the individuals had plasma Lp(a) concentrations less than 300 mg/L whereas only 51% of patients had plasma Lp(a) levels in this range ($p < 0.05$). The distribution of plasma Lp(a) concentration for each group is shown in Figure 1. Plasma Lp(a) concentrations were not normally distributed, and only patients had Lp(a) levels > 800 mg/L. Skewness and kurtosis were higher in patients than in controls. In the whole group (all subjects), we found a total of 31 apo(a) size alleles with KIV repeat numbers ranging from 8 to 39, 96% of the subjects had two apo(a) alleles that contained different numbers of KIV repeats, and the number of homozygous subjects did not differ between groups. The largest isoform (of each subject) was equally distributed in the two groups (Fig. 2), but patients showed a significantly higher prevalence of isoforms ≤ 22 in the smallest isoform (for each subject) than controls ($p = 0.037$). Likewise, the mean kringle number of the largest isoform was not different between groups (Table 1), but the mean kringle number for the smallest isoform was higher ($p < 0.05$) in controls.

There was an inverse relationship between the size of the apo(a) alleles and the plasma concentration of Lp(a), which is consistent with data found in other studies (22,25). The Spearman rank correlation values for the relationship between the sum of the apo(a) allele sizes was significant in patients (-0.377, $p < 0.0005$) but not in controls (-0.116, $p > 0.05$). As a whole group (all subjects), there was no substantial correlation between Lp(a) concentrations and the number of KIV repeats in the largest isoform (per subject), but plasma Lp(a) concentrations (y) correlated negatively with the number of KIV repeats in the smallest isoform (per subject) (x) [y(mg/L) = 581.44 - 16.76x; $r = 0.315$, $p < 0.0005$]**.** Lp(a) concentration at various kringle numbers is depicted in Fig. 3. Lp(a) concentration is significantly higher for the isoforms of 16-18 and 19-22 kringles (Fig. 3) in the patients than the controls. The apo(a) size of $\leq$ 22 KIV kringles was more prevalent in patients (74%) than controls (62%). Lp(a) concentration was significantly higher in the patients (400 ± 344, n = 72) with smaller apo(a) isoforms (≤ 22 kringle number) compared to the age-matched controls (180 ± 166, n = 69), but not with larger isoforms (> 22 kringles).

LBS activity and its relationship to Lp(a) concentration and size

Although there was a trend toward higher values in patients, the mean LBS activity of Lp(a) was not significantly different between groups (Table 1). The frequency of LBS activity was normally distributed in both groups (data not shown), but the peak frequency of LBS activity was shifted toward higher LBS activity values in the patients (70-80%) compared to the control group (50-60%). There was a strong and positive correlation between plasma Lp(a) concentration and the LBS activity ($r = 0.524$, $p < 0.0005$ for the whole group), that was observed in both groups (Figure 4). We have observed a negative association of LBS activity with expressed isoform in single-banded phenotypes (Figure 5) but this association was only significant ($r = -0.632$, $p = 0.0005$) in the patients (Figure 5). Thus, in the patients, both Lp(a)

concentration and LBS activity of the small isoforms were higher than in controls. LBS activity of the single-banded phenotypes was 49% for patients and 47% for controls, but if we consider only those with kringle numbers $\leq$ 22 the difference between patients (65% ± 2, mean ± SEM, n = 48) and controls (54% ± 2, n = 38) is statistically significant. As found in other studies, Lp(a) concentration and apo(a) size are independent risk factors (model 1) for CVD in this study (Table 2). LBS activity is not an independent risk factor, but high LBS activity becomes a significant risk factor when considered with high Lp(a) concentration ($p = 0.003$) or low kringle number ($p = 0.004$) (Table 2). The odds ratio for high LBS activity and high Lp(a) concentration is 4.4 and for high LBS activity and low kringle number the odds ratio is 10.1.

Discussion

In this study of patients with an early onset myocardial infarction, we document elevated plasma Lp(a), a preponderance of small apo(a) isoforms, and an association of elevated Lp(a) and high LBS activity with the small isoforms compared to controls. This study demonstrates an association of the LBS function of Lp(a) with cardiovascular disease in humans. Numerous and extensive clinical studies have indicated that elevated plasma Lp(a), particularly values above 300 mg/L, is a major independent risk factor for CAD. While this generalization is not supported in all studies or all populations, many investigators have reached similar conclusions (reviewed in (1)). Several studies also demonstrate a high prevalence of low molecular weight apo(a) isoforms in patients with CAD (26,27). In a recent study, Gazzaruso et al (28) found that Lp(a) with small apo(a) isoforms (280-640 kDa) was associated with two or more stenosed coronary vessels (75% of patients), compared to only 24% of the patients with high molecular weight isoforms (655-790 kDa). African-Americans (29) and African populations (30) have high concentrations of Lp(a), but the elevated Lp(a) is of the larger isoform size and is not as great a coronary disease risk as elevated small size isoforms found in Caucasians (31). In our study, patients with apo(a) kringle numbers between 16 and 22 of the smallest isoform had significantly elevated Lp(a) concentration compared to controls. Our results indicate that it is not only the elevated concentration of Lp(a), but elevated Lp(a) in subjects with small apo(a) isoforms contribute to the CAD risk.

The importance of the LBS function of Lp(a) in the development of atherosclerosis has been demonstrated in transgenic mice. Atherosclerosis is reduced in transgenic mice carrying apo(a) with a mutation in the KIV-10 kringle in which ASP 55 and ASP 57 are replaced by

ALA, and LBS activity is reduced (13,14). In another study (32), a recombinant adenovirus with an apo(a) construct was injected into the apoB transgenic mice. Three apo(a) constructs were studied, wild-type, a mutation of TRP 72 substituted for ARG in the KIV-10 kringle, and the Rhesus monkey construct with the TRP to ARG mutation in KIV-10 and the absence of a KV kringle. Both of these mutations have markedly reduced LBS activity (14). Lesion formation was reduced in both mutant constructs compared to the wild-type apo(a). In a clinical study, Karmansky et al. (33) examined lysine-binding species of Lp(a) in two groups of subjects with CAD, one with severe (2 or 3-vessel) and the other with moderate (one vessel) disease. The Lp(a) concentration of both the Lys(+) and Lys(-) species were higher in patients with severe disease than patients with moderate disease. These authors did not examine apo(a) size in this study. In a cell culture system, we have demonstrated (34) that modifications of the LBS function, alter retention in the extracellular matrix. Modifications of Lp(a) which selectively increase LBS function also selectively increase binding to the matrix.

In addition to the observation in several studies (26,27,35) that the low-molecular isoforms are associated with CAD, studies have demonstrated that smaller molecular forms of Lp(a) that have a higher affinity for fibrin (36) and a greater antifibrinolytic effect (37) than large isoforms, and 04small molecular weight forms have a lower binding affinity for macrophages than the higher molecular weight forms (17). Our results suggest that properties which increase retention in the vessel wall (LBS function) and binding to fibrin and cells (small isoforms) are associated with Lp(a) of patients with CAD.

Although the distribution of the alleles in our study was in Hardy Weinberg equilibrium, the apo(a) isoforms detectable by immunoblotting are not. This suggests that the methods currently used to analyze the apo(a) protein do not detect all of the apo(a) gene products. Since a

significant percentage of isoforms may not be detectable the analysis was made with subjects showing the single banded phenotype, and the conclusions are similar to those obtained with the genotype. This is not surprising if we accept that the detection of an isoform is related to its size, and undetected isoforms may be from larger alleles than those coding for detectable proteins. Other authors (5,38) consider the apo(a) gene itself as an important determinate of the plasma levels of Lp(a) and consequently of LBS activity. In our study the percent of individuals with single-banded phenotypes, similar to that observed in other studies (39-41) could represent a lack of sensitivity to detect all of the apo(a) genetic products with the electrophoresis and immunodetection methods (42). Other factors, such as, rapid clearance, inefficient synthesis or secretion could also affect plasma concentrations.

The increased LBS activity found in the small isoforms of the patients could be due to either sequence polymorphisms or post-translational modifications. Sequence polymorphisms of Lp(a) which have been identified to date do not exhibit enhanced LBS function (15,16,43). The Lp(a) LBS immunoassay measures the LBS activity of Lp(a). Certain kringles of apo(a) (KIV types 5-8, LBS II) have LBS activity and are not available at the surface of most intact Lp(a) particles or are occupied by lysines from apoB. The LBS II is unavailable in the intact Lp(a), but can be measured in the isolated apo(a), and could be the site of enhanced LBS activity in the small isoforms. It is unclear whether the LBS II is available in vivo under certain circumstances, such as after enzymatic or chemical modifications. Substitutions of amino acids in the LBS pocket of KIV 10 which could enhance LBS activity, e.g. K1 of plasminogen has different amino acids in the LBS pocket which results in enhanced LBS affinity compared to K4 of plasminogen and KIV type 10 of apo(a). According to the study of Rejante and Llinas (44), NMR experiments indicate the ligand-binding site of plasminogen kringle 1 is a shallow cavity

composed of Pro33, Phe36, Trp62, Tyr64, Tyr72 and Tyr74 with the double charged anionic and cationic center configured by the side chain of Asp55 and Asp57, and Arg34 and Arg71. In Plg K4 and Lp(a) KIV 10, there are two amino acid substitutions (7,45,46) which may account for the reduced of affinity of Plg K4 compared to K1, and these are His33 instead of Pro33 and a Phe64 instead of the Tyr in K4 compared to K1 of plasminogen. Substitutions of these two amino acids in Lp(a) KIV 10 from His33 to Pro33, and Phe64 to Tyr64 could increase the affinity of the LBS. In plasminogen K1 these substitutions direct the ligand amino group toward Arg 34 and Arg71 (3). KV of Lp(a) could also be a potential site of mutations which could enhance LBS activity in Lp(a) (47). We previously demonstrated that certain enzymatic and chemical modification of Lp(a) may alter LBS activity, such as phospholipase A_2 or oxidation (18), and small isoforms may be more susceptible to these types of modifications resulting in altered LBS activity. Determination of the sequence polymorphisms and susceptibility to modification of low molecular weight isoforms of Lp(a) in patients with CAD and their effect on LBS function will be important to our understanding of the pathogenicity of Lp(a).

Acknowledgments

This work was supported in part by the Fondo de Investigación Sanitaria, the National Institutes of Health Grant HL 18577, and the American Heart Association, Ohio Valley Affiliate.

Reference List

1. Kronenberg F, Steinmetz A, Kostner GM, Dieplinger H: Lipoprotein(a) in health and disease. Crit Rev Clin Lab Sci 1996; 33: 495-543.
2. Ridker PM, Hennekens CH, Stampfer MJ: A prospective study of lipoprotein(a) and the risk of myocardial infarction. JAMA 1993; 270: 2195-2199.
3. Scanu AM, Edelstein C: Kringle-dependent structural and functional polymorphism of apolipoprotein(a). Biochim Biophys Acta 1995; 1256: 1-12.
4. McLean JW, Tomlinson JE, Kuang W-J, et al: cDNA sequence of human apolipoprotein(a) is homologous to plasminogen. Nature 1987; 330: 132-137.
5. Lackner C, Boerwinkle E, Leffert CC, Rahmig T, Hobbs HH: Molecular basis of apolipoprotein (a) isoform size heterogeneity as revealed by pulsed-field gel electrophoresis. J Clin Invest 1991; 87: 2153-2161.
6. Utermann G, Menzel HJ, Kraft HG, Duba HC, Kemmler HG, Seitz C: Lp(a) glycoprotein phenotypes: Inheritance and relation to Lp(a)-lipoprotein concentrations in plasma. J Clin Invest 1987; 80: 458-465.
7. Wu T-P, Padmanabhan K, Tulinsky A, Mulichak AM: The refined structure of the ε-aminocaproic acid complex of human plasminogen kringle 4. Biochemistry 1991; 30: 10589-10594.
8. Ernst A, Helmhold M, Brunner C, Pethö-Schramm A, Armstrong VW, Müller H-J: Identification of two functionally distinct lysine-binding sites in kringle 37 and in kringles 32-36 of human apolipoprotein(a). J Biol Chem 1995; 270: 6227-6234.
9. Rouy D, Koschinsky ML, Fleury V, Chapman J, Anglés-Cano E: Apolipoprotein(a) and plasminogen interactions with fibrin: A study with recombinant apolipoprotein(a) and

isolated plasminogen fragments. Biochemistry 1992; 31: 6333-6339.

10. Klezovitch O, Edelstein C, Scanu AM: Evidence that the fibrinogen binding domain of Apo(a) is outside the lysine bidning site of kringle IV-10: A study involving naturally occurring lysine binding defective lipoprotein(a) phenotypes. J Clin Invest 1996; 98: 185-191.
11. Miles LA, Fless GM, Scanu AM, et al: Interaction of Lp(a) with plasminogen binding sites on cells. Thromb Haemost 1995; 73: 458-465.
12. Miles LA, Sebald MT, Fless GM, et al: Interaction of lipoprotein(a) with the extracellular matrix. Fibrinolysis Proteolysis 1998; 12: 79-87.
13. Boonmark NW, Lou XJ, Yang ZJ, et al: Modification of apolipoprotein(a) lysine binding site reduces atherosclerosis in transgenic mice. J Clin Invest 1997; 100: 558-564.
14. Hoover-Plow JL, Boonmark N, Skocir P, Lawn R, Plow EF: A quantitative immunoassay for the lysine-binding function of lipoprotein(a): Application to recombinant apo(a) and lipoprotein(a) in plasma, and modified lipoprotein(a). Arterioscler Thromb Vasc Biol 1996; 16: 656-664.
15. Scanu AM, Pfaffinger D, Lee JC, Hinman J: A single point mutation (Trp72-->Arg) in human apo(a) kringle 4-37 associated with a lysine binding defect in Lp(a). Biochim Biophys Acta 1994; 1227: 41-45.
16. Scanu AM, Miles LA, Pfaffinger D, et al: Rhesus monkey Lp(a) binds to lysine-Sepharose and U937 monocytoid cells less efficiently than human Lp(a). Evidence for a dominant role of kringle 4_{37}. J Clin Invest 1993; 91: 283-291.
17. Kang C, Durlach V, Soulat T, Fournier C, Anglés-Cano E: Lipoprotein(a) isoforms display differences in affinity for plasminogen-like binding to human mononuclear cells.

Arterioscler Thromb Vasc Biol 1997; 17: 2036-2043.

18. Hoover-Plow J, Skocir P: Enzymatic and chemical modifications of lipoprotein(a) selectively alter its lysine-binding functions. Biochim Biophys Acta Lipids Lipid Metab 1998; 1392: 73-84.
19. Joven J, Simó JM, Vilella E, et al: Lipoprotein(a) and the significance of the association between platelet glycoprotein IIIa polymorphisms and the risk of premature myocardial infarction. Atherosclerosis 1998; 140: 155-159.
20. Simó JM, Castellano I, Ferré N, Joven J, Camps J: Evaluation of a homogeneous assay for high density lipoprotein cholesterol: Limitations in patients with cardiovascular, renal and hepatic disorders. Clin Chem 1998; 44: 1233-1241.
21. Levine DM, Sloan BJ, Donner JE, Lorenz JD, Heinzerling RH: Automated measurement of lipoprotein(a) by immunoturbidimetric analysis. Int J Clin Lab Res 1992; 22: 173-178.
22. Gaubatz JW, Ghanem KI, Guevara J, Nava ML, Patsch W, Morrisett JD: Polymorphic forms of human apolipoprotein(a): Inheritance and relationship of their molecular weights to plasma levels of lipoprotein(a). J Lipid Res 1990; 31: 603-613.
23. Martin S, Ladona MG, Pedro-Botet J, Covas MI, Rubies-Prat J: Differential expression of double-band apolipoprotein(a) phenotypes in healthy Spanish subjects detected by SDS-agarose immunoblotting. Clin Chim Acta 1998; 277: 191-205.
24. Hoover-Plow JL, Miles LA, Fless GM, Scanu AM, Plow EF: Comparison of the lysine binding functions of lipoprotein(a) and plasminogen. Biochemistry 1993; 32: 13681-13687.
25. Kraft HG, Sandholzer C, Menzel HJ, Utermann G: Apolipoprotein(a) alleles determine lipoprotein(a) particle density and concentration in plasma. Arterioscler Thromb 1992;

12: 302-306.

26. Lindén T, Taddei-Peters W, Wilhelmsen L, et al: Serum lipids, lipoprotein(a) and apo(a) isoforms in patients with established coronary artery disease and their relation to disease and prognosis after coronary by-pass surgery. Atherosclerosis 1998; 137: 175-186.
27. Klausen IC, Sjol A, Hansen PS, et al: Apolipoprotein(a) isoforms and coronary heart disease in men - A nested case-control study. Atherosclerosis 1997; 132: 77-84.
28. Gazzaruso C, Geroldi D, Garzaniti A, et al: Apolipoprotein(a) phenotypes as genetic markers of coronary atherosclerosis severity. Int J Cardiol 1998; 64: 277-284.
29. Marcovina SM, Albers JJ, Wijsman E, Zhang Z, Chapman NH, Kennedy H: Differences in Lp[a] concentrations and apo[a] polymorphs between black and white Americans. J Lipid Res 1996; 37: 2569-2585.
30. Rotimi CN, Cooper RS, Marcovina SM, McGee D, Owoaje E, Ladipo M: Serum distribution of lipoprotein(a) in African Americans and Nigerians: Potential evidence for a genotype-environmental effect. Genet Epidemiol 1997; 14: 157-168.
31. Srinivasan SR, Dahlén GH, Jarpa RA, Webber LS, Berenson GS: Racial (black-white) differences in serum lipoprotein (a) distribution and its relation to parental myocardial infarction in children: Bogalusa Heart Study. Circulation 1991; 84: 160-167.
32. Hughes SD, Lou XJ, Ighani S, et al: Lipoprotein(a) vascular accumulation in mice - In vivo analysis of the role of lysine binding sites using recombinant adenovirus. J Clin Invest 1997; 100: 1493-1500.
33. Karmansky I, Shnaider H, Palant A, Gruener N: Lysine-binding species of lipoprotein(a) in coronary artery disease. Eur J Clin Invest 1994; 24: 360-366.
34. Hoover-Plow J, Khaitan A, Fless GM: Phospholipase A_2 modification enhances

lipoprotein(a) binding to the subendothelial matrix. Thromb Haemost 1998; 79: 640-648.

35. Kronenberg F, Kronenberg MF, Kiechl S, et al: Role of lipoprotein(a) and apolipoprotein(a) phenotype in atherogenesis - Prospective results from the Bruneck study. Circulation 1999; 100: 1154-1160.
36. Hervio L, Girard-globa A, Durlach V, Angles-Cano E: The antifibrinolytic effect of lipoprotein(a) in heterozygous subjects is modulated by the relative concentration of each of the apolipoprotein(a) isoforms and their affinity for fibrin . Eur J Clin Invest 1996; 26: 411-417.
37. Hervio L, Chapman M, Thillet J, Loyau S, Anglés-Cano E: Does apolipoprotein(a) heterogeneity influence lipoprotein(a) effects on fibrinolysis? Blood 1993; 82: 392-397.
38. Boerwinkle E, Leffert CC, Lin J, Lackner C, Chiesa G, Hobbs HH: Apolipoprotein(a) gene accounts for greater than 90% of the variation in plasma lipoprotein(a) concentrations. J Clin Invest 1992; 90: 52-60.
39. Gazzaruso C, Garzaniti A, Buscaglia P, et al: Association betrween apolipoprotein(a) phenotypes and coronary heart disease at a young age. J Am Coll Cardiol 1999; 33: 157-163.
40. Geroldi D, Bellotti V, Buscaglia P, et al: Characterization of apo(a) polymorphism by a modified immunoblotting technique in an Italian population sample. Clinica Chimica Acta 1993; 221: 159-169.
41. Rainwater DL, Ludwig MJ, Haffner SM, VandeBerg JL: Lipid and lipoprotein factors associated with variation in Lp(a) density. Arterioscler Thromb Vasc Biol 1995; 15: 313-319.
42. Valenti K, Aveynier E, Laporte F, Hadjian AJ: Evaluation of the genotyping and

phenotypind approaches in the investigation of apolipoprotein(a) size polymorphism. Clin Chim Acta 1997; 263: 249-260.

43. Prins J, Van der Hoek YY, Biesheuvel TH, Leus FR, van Rijn HJM, Kastelein JJP: The func□tional and clinical significance of the Met-->Thr substitution in kringle IV type 10 of apolipoprotein(a). Thromb Res 1998; 90: 125-130.
44. Rejante MR, Llinás M: Solution structure of the Œ-aminohexanoic acid complex of human plasminogen kringle 1. Eur J Biochem 1994; 221: 939-949.
45. Ramesh V, Petros AM, Llinás M, Tulinsky A, Park CH: Proton magnetic resonance study of lysine-binding to the kringle 4 domain of human plasminogen. J Mol Biol 1987; 198: 481-498.
46. Rejante MR, Byeon I-JL, Llinás M: Ligand specificity of human plasminogen kringle 4. Biochemistry 1991; 30: 11081-11092.
47. Xue S, Green MA, LoGrasso PV, et al: Comparison of the effects of Apo(a) kringle IV-10 and plasminogen kringles on the interactions of lipoprotein(a) with regulatory molecules. Thromb Haemost 1999; 81: 428-435.

Table 1
Lp(a) plasma concentration, apo(a) size, and LBS function.

	Mean	Median	Standard deviation	Range
Plasma Lipoprotein(a) (mg/L)				
Myocardial infarction	334	297	299	< 49-1346
Age-matched controls	176*	146	160	< 49-596

apo(a) size (Kringle number)				
Largest isoform				
Myocardial infarction	25.24	25.00	4.42	11-34
Age-matched controls	26.12	25.00	5.10	14-39
Smallest isoform				
Myocardial infarction	18.61	18.00	4.48	8-30
Age-matched controls	20.41†	20.00	4.77	11-34
LBS activity (%)[a]				
Myocardial infarction	52.8	57.0	24.7	0-103
Age-matched controls	50.3	53.0	24.6	0-98

*$p < 0.0005$ with respect to patients, and †$p = 0.008$ with respect to patients. LBS activity is the percent of the reference Lp(a). Largest and smallest isoforms refer to the largest or smallest isoform determined for each subject with 2 isoforms.

Table 2.
Association of Lp(a) Concentration, Smallest kringle number, and LBS activity with CAD.

Variable	Odds Ratio (95% confidence Ratio)	p value
1)Lp(a) concentration	1.18 (1.09-1.27)	< 0.001
Smallest kringle	0.92 (0.86-0.98)	0.009
LBS Activity	1.02 (0.96-1.08)	0.49
2) Small kringle number (< 22)		
Low LBS	0.58 (0.27-1.28)	0.18
High LBS	10.10 (2.1-48.8)	0.004
3) High Lp(a) (>300mg/L		
Low LBS	2.55 (0.97-6.7)	0.06
High LBS	4.40 (1.7-11.34)	0.002

Models considered one risk factor at a time. In models 2 and 3, a subset was evaluated, either smaller kringles (model 2) or high Lp(a) (model 3).

Figure Legends

Figure 1. Frequency distribution of plasma Lp(a) concentration in patients with myocardial infarction (Panel A, n = 95), and age-matched controls (Panel B, n = 95).

Figure 2. Frequency distribution of apo(a) alleles in patients with myocardial infarction (Panel A), and age-matched controls (Panel B). Black bars represent the smallest isoforms and white bars the largest isoforms determined for each subject with 2 isoforms.

Figure 3. Lp(a) Concentration of Various Size Isoforms (mean ± SEM). Lp(a) concentration is plotted versus the smallest kringle determined for each subject with 2 isoforms. The number of subjects per group for the various kringle sizes are: patients -- ≤ 15, n = 28, 16-18, n = 23, 19-22, n = 21, 23-25, n = 17, > 26, n = 6; and age-matched controls -- ≤ 15, n = 15, 16-18, n = 25, 19-22, n = 25, 23- 25, n = 14, > 26, n = 16. Gray bars are values for patients and black bars for the controls.

Figure 4. Scatterplot of LBS activity values versus Lp(a) concentration for patients (Panel A •) and controls (Panel B □).

Figure 5. Scatterplot of the Lp(a) LBS activity versus the number of KIV repeats of the phenotypes of single banded subjects), controls -- n = 54 (Panel A) and patients -- n = 39 (Panel B**.** Correlation coefficients of LBS activity for CAD patient phenotype was statistically significant ($p<0.0005$).